New Drugs against Trypanosomatid Parasites: Rediscovery of Fexinidazole

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Summary

Neglected tropical diseases (NTDs) are a group of communicable diseases mostly affecting people in developing countries. These diseases are responsible for a major part of the global morbidity, mortality and poverty. There is no doubt that the well-being of people in the developing world can only be improved if the NTDs are controlled. An important tool for disease control is the drug treatment. The few available drugs are unsatisfactory because of the limited efficacy, adverse effects and the high price. Chagas disease, leishmaniasis and human African trypanosomiasis belong to this group of NTDs. They are caused by infections with protozoa of the family *Trypanosomatidae*. For these three diseases new drugs are urgently needed.

By definition there is no commercial market for drugs against NTDs. Drug research and development (R&D) for NTDs is mainly driven by the public sector, the so-called product development partnerships (PDPs). Drug R&D is a very long (10-15 years), risky and therefore expensive process. Three different series of compounds (agrochemicals, marketed drugs and nitro-heterocyclic compounds) were tested for their antiparasitic effects, with the aim to identify new lead compounds or even clinical candidates against leishmaniasis, sleeping sickness, and Chagas disease.

Agrochemicals are used worldwide on a large scale in food production. They undergo a rigorous toxicological testing prior to launch. Over 600 compounds were screened for their antiparasitic activity. Agrochemicals are not optimized for use in mammals, yet a significant number of molecules were found with good and selective in vitro activity. Some of them showed also efficacy in the corresponding rodent model. These results indicate that agrochemicals can provide very interesting starting structures for drug research against parasitic diseases.

Drugs or drug-like compounds are an ideal starting point for antiparasitic drug discovery, because very often pharmacokinetic and toxicological data are available. A number of drugs, including antibiotics, antivirals, antifungals, and anti-psychotics were assayed for antiparasitic activity. Some of the drugs tested showed selective antiparasitic activity. These compounds can be regarded as new lead structures and should be further investigated.

Nitroheterocycles belong to a well- known class of compounds with the stigma of being mutagenic or genotoxic. Over 700 compounds, mainly nitroimidazoles, have been systematically tested for their antiparasitic activity, and their pharmacokinetics and mutagenicity was investigated. A number of effective, non-mutagenic and non- genotoxic compounds was identified. So fexinidazole was rediscovered, a drug that had been in clinical development already in the 70's as a broad-spectrum antimicrobial drug. Fexinidazole is rapidly metabolized to fexinidazole-sulfoxide and -sulfone. The parent compound and the two principle metabolites showed in vitro trypanocidal activity against all (sensitive and resistant) tested *T. brucei* strains (IC_{50} of 0.2 - 0.9 μg / ml). Fexinidazole cured the first stage mouse model with a 4-day oral treatment of 100 mg/kg/day and the 2nd stage mouse model with a 5-day oral treatment of 200 mg/kg/day. The two metabolites are mainly responsible for the good efficacy in animal models. Both reach very high concentrations in blood and brain tissue. Fexinidazole has successfully completed preclinical development and Phase I clinical trials and is currently in a clinical phase II / III study.

With the approach of phenotypic screening of compounds that have been developed for other purposes, new leads for drug R&D against Chagas' disease, leishmaniasis and human African trypanosomiasis were identified.

Fexinidazole is the first drug candidate in clinical Phase II / III trials since decades. It would be the first oral drug for the treatment of stage 1 and 2 of human African sleeping sickness. If fexinidazole overcomes all obstacles, this would be a major breakthrough in the fight against African sleeping sickness. With a well tolerated, orally active drug like Fexinidazole the elimination of sleeping sickness seems finally tangible.

Zusammenfassung

Vernachlässigte Tropenkrankheiten („Neglected Tropical Diseases“ NTDs) sind eine Gruppe von übertragbaren Krankheiten, welche vor allem die Bevölkerung der Entwicklungsländer betreffen. Diese Krankheiten verursachen einen großen Teil der weltweiten Morbidität, Mortalität und Armut. Es besteht kein Zweifel, dass das Wohlergehen der Menschen in Entwicklungsländern nur verbessert werden kann, wenn diese Krankheiten unter Kontrolle gebracht werden. Ein wichtiges Instrument dafür ist die medikamentöse Behandlung. Die wenigen verfügbaren Medikamente sind unbefriedigend, aufgrund ihrer beschränkten Wirksamkeit, Nebenwirkungen und des hohen Preises. Chagas-Krankheit, Leishmaniose und die menschliche afrikanische Schlafkrankheit gehören zu dieser Gruppe von NTDs. Sie werden durch eine Infektion mit Protozoen aus der Familie Trypanosomatidae verursacht. Für diese drei Krankheiten werden dringend neue Medikamente benötigt.

Es gibt keinen kommerziell interessanten Markt für Medikamente gegen vernachlässigten Krankheiten. Die Medikamentenforschung wird hauptsächlich von der öffentlichen Hand, sogenannten Produktentwicklung Partnerschaften (PDPs), angetrieben. Die Medikamentenforschung und -entwicklung ist ein sehr langwieriger (10-15 Jahre), riskanter und daher teurer Prozess. Drei verschiedene Substanzserien (Agrochemikalien, vermarktete Medikamente und nitroheterozyklische Verbindungen) wurden auf ihre antiparasitäre Wirkung untersucht, mit dem Ziel, neue Leitstrukturen oder sogar klinischen Kandidaten gegen Leishmaniose, Schlafkrankheit und Chagas-Krankheit zu identifizieren.

Agrochemikalien werden weltweit in großem Maßstab in der Nahrungsmittelproduktion eingesetzt. Sie durchlaufen vor der Markteinführung eine strenge toxikologische Prüfung. Über 600 Verbindungen wurden auf ihre antiparasitische Aktivität getestet. Agrochemikalien sind nicht für den Einsatz in Säugetieren optimiert, dennoch wurde eine beträchtliche Anzahl von Molekülen mit hoher und selektiver in vitro-Aktivität gefunden. Einige davon waren auch im entsprechenden Nagetier-Modell wirksam. Diese Ergebnisse zeigen, dass Agrochemikalien sehr interessante Ausgangsstrukturen für die Medikamenten-forschung gegen parasitäre Erkrankungen liefern können.

Bekannte Wirkstoffe oder wirkstoffähnliche Verbindungen sind ein idealer Ausgangspunkt für die antiparasitäre Medikamentenforschung, weil sehr oft

pharmakokinetische und toxikologische Daten bereits zur Verfügung stehen. Eine Reihe von Arzneimittel, einschließlich Antibiotika, antivirale Wirkstoffe, Antimykotika und Antipsychotika wurde auf die antiparasitische Wirkung untersucht. Einige der getesteten Arzneimittel zeigten eine selektive antiparasitische Aktivität. Diese Verbindungen können als neue Leitstrukturen betrachten werden und sollten weiter untersucht werden.

Nitroheterozyklische Substanzen gehören zu einer bekannten Klasse von Verbindungen mit dem Makel, erbgutverändernd oder genotoxisch zu sein. Über 700 Verbindungen, hauptsächlich Nitroimidazole, wurden systematisch auf antiparasitäre Aktivität untersucht und ihre Pharmakokinetik und Mutagenität geprüft. Eine Anzahl wirksamer, weder mutagener noch genotoxischer Verbindungen konnte identifiziert werden. So wurde Fexinidazol wiederentdeckt, ein Wirkstoff der bereits in den 70er Jahren als Breitspektrum-Antibiotikum in der klinischen Entwicklung war. Fexinidazol wird schnell zu Fexinidazol-sulfoxid und -sulfon metabolisiert. Die Stammverbindung und die zwei Metaboliten sind gegen alle (sensitive und resistente) getesteten *T. brucei* Stämme aktiv (IC_{50} 0.2 – 0.9 mg/ml). Fexinidazol heilt das Mausmodell des ersten Krankheitsstadiums bei einer oralen 4-tägigen Behandlung mit 100 mg/kg/Tag und das 2. Stadium-Mausmodell bei einer oralen 5-tägigen Behandlung mit 200mg/kg/Tag. Verantwortlich für die gute Wirksamkeit in den Tiermodellen sind die beiden Metaboliten, welche sehr hohe Blut- und Gehirnkonzentrationen erreichen. Fexinidazol durchlief erfolgreich die präklinische Entwicklung und klinische Phase I und ist derzeit in einer klinischen Phase II / III-Studie.

Mit dem Ansatz des phänotypischen Screenings von Verbindungen, die für andere Zwecke entwickelt worden waren, konnten neue Leitstrukturen für die Medikamenten-forschung gegen drei parasitischen Erkrankungen identifiziert werden.

Fexinidazol ist der erste Wirkstoff in klinischen Phase II / III-Studien seit Jahrzehnten. Es wäre das erste orale Medikament zur Behandlung des ersten und zweiten Stadiums der menschlichen afrikanischen Schlafkrankheit. Sollte Fexinidazol alle Hindernisse überwinden, wäre dies ein großer Durchbruch im Kampf gegen die menschliche Afrikanische Schlafkrankheit und könnte einen essentiellen Beitrag zur Eliminierung der Krankheit leisten.

CHAPTER 1

General introduction

Neglected tropical diseases (NTDs) such as human African trypanosomiasis (HAT), Chagas' disease and Leishmaniasis affect the poorest people. NTDs are responsible for substantial global morbidity, mortality, and economic adversity [1]. 1-2 billion people are at risk and hundred thousands of people die by an NTD every year. NTDs have a huge social and economic impact due to loss of education potential and reduced productivity. The human toll is measured in disability-adjusted life year (DALY). The estimate of DALYs caused by NTDs is 20 million [1].

For successful combating these neglected diseases, all aspects must be considered such as treatment, diagnosis, transmission and distribution. Transmission and distribution of NTDs could be reduced and controlled by specific vector control tools, e.g. mosquito nets, fly traps, insecticide spraying. Accurate diagnostic tools are required in order to detect the disease early, to treat as early as possible and further transmission can be prevented. As example there are highly sensitive serological tests available for *T. b. gambiense* HAT, but the parasitological confirmatory tests are insufficient and needs to be improved. The diagnosis for *T. b. rhodesiense* HAT still relies on microscopy; there is no serological field test [2]. In general for NTDs there is a lack of effective medications. All recent used drugs have their drawbacks, often hospitalization is necessary, or the treatment is logistically very complicated or takes a long time. Most medications have significant side effects. There is an urgent need for new drugs that are safe to use, easy to administer and inexpensive.

Human African trypanosomiasis (HAT)

Human African trypanosomiasis (HAT) also known as African sleeping sickness, is caused by two protozoan parasites, *Trypanosoma brucei gambiense* and *T .b. rhodesiense* [3]. *T. b. rhodesiense* is prevalent in East Africa and causes an acute infection; it lasts only few months until death [4]. *T. b. gambiense* is prevalent in central and West Africa causing a chronic infection and takes up to 4 years until death [5]. Trypanosomes elude the adaptive

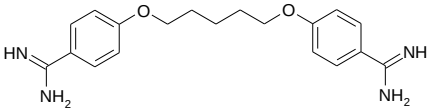
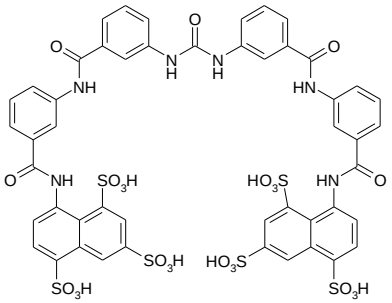
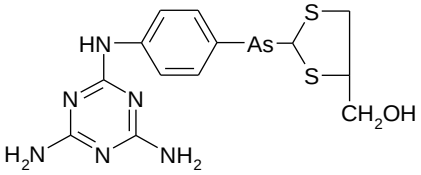
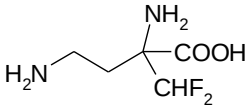
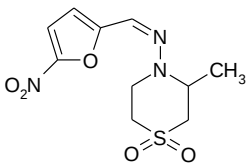
immune response by continuous variation of the surface glycoproteins [6]. Antigenic variation is the main reason for the lack of success of developing a vaccine.

HAT is transmitted by tsetse flies (Glossina sp). The disease course is divided into two stages. In the first stage, the trypanosomes are localized mainly in the hemolymphatic system, and causing rather unspecific symptoms such as headache, fever and lymphadenopathy [3]. In the second stage of the disease, the parasites have overcome the blood-brain barrier and have invaded the brain tissues and cerebrospinal fluid (CSF) [7, 8]. The trypanosomes in the brain provoke neurological dysfunctions [9, 10]. The neuropathological changes include also the disruptions of sleep/wake patterns, which has led to the common name of 'sleeping sickness' being used for the disease [11].
Primary diagnosis is done serologically by use of the card agglutination test for trypanosomiasis (CATT) [12], detecting antibodies produced against the variant surface glycoproteins (VSGs). CATT is very sensitive but may produce false positives. Therefore the microscopical identification of the parasites in lymph or blood is required. The stage of the disease is determined by lumbar puncture, necessary because stage specific drugs are used. The presence of parasites or white blood cells ($\geq$ 5 cell per microliter) in the CSF are indicative of stage 2 of the disease [13,14].

HAT occurs only in sub-Saharan Africa (14° North and 29° South) and is correlated with the distribution of the vector. According to the World Health Organization (WHO), the disease is endemic in 36 countries [15,16]. But HAT is a significant public health problem in only 20 countries [17]. Over 95% of all reported cases are from the countries in West and Central Africa, the distribution area of *T. b. gambiense* [16,18]. Since the discovery of trypanosomes as the causative agent of sleeping sickness by Bruce, three major epidemics occurred [19]. The last major epidemic was at the end of the 20th century with an estimated 300'000 cases [16].

Human African trypanosomiasis is transmitted by tsetse flies and the disease threatens millions of people in 36 countries in sub-Saharan Africa. Due to reinforced surveillance and vector control the number of reported cases decreased in the last 10 years from approximately 30'000 to <10'000 cases [18].

Table 1. Available chemotherapy for the treatment of acute and chronic stage of human African trypanosomiasis, problems and main adverse effects.

First stage of human African trypanosomiasis		
Drug	Route of application & dosage	Problems & main adverse effects
Pentamidine (1940) 	Intramuscular; 4 mg/kg/day for 7-10 days	Only efficacious for T.b.gambiense; hypoglycaemia, injection site pain, diarrhea, nausea, vomiting
Suramin (1920s) 	Intravenous; 20mg/kg once per week over 5 weeks (maximum dose 1g per injection)	For T.b. rhodesiense; hypersensitivity reactions, haematuria, albuminuria, cylinduria, peripheral neuropathy. Due to the risk of a severe anaphylactic reaction, a test dose of 4-5 mg/kg/ the first day is recommended.
Second stage of human African trypanosomiasis		
Melarsoprol (1949) 	Intravenous; *T.b.gambiense:* 2.2mg/kg/day for 10 days *T.b.rhodesiense*: 3 series of 3.6 mg/kg for 3 days, spaced by intervals of 7 days.	10 painful daily injections Increasing number of treatment failures (up to 30% in some regions) ~5% treatment-related mortality due to encephalopathic syndromes, skin reactions, neuropathies, gastrointestinal upset
Eflornithine (1981) 	Intravenous infusion 100 mg/kg at 6 h intervals for 14 days	Only efficacious for T.b.gambiense; administration difficult, diarrhoea, nausea, vomiting, convulsions, anaemia, leucopenia, thrombocytopenia;
Nifurtimox (1970s) 	Oral 5mg/kg three times per day for 14 days	Oral drug developed for Chagas disease, not registered for HAT; sometimes used after melarsoprol relapse; probably ~70% efficacy; anorexia and neurological alterations
Nifurtimox-eflornithine combination (2009)	Eflornithine: 200 mg/kg intravenous infusion 2 times per day for 7 days Nifurtimox: 5 mg/kg 3 times per day oral for 10 days	Only efficacious for T.b.gambiense, Simplified stage 2 treatment Included in WHO's List of Essential Medicines (EML) in May 2009

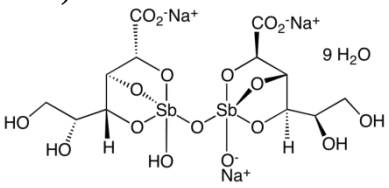
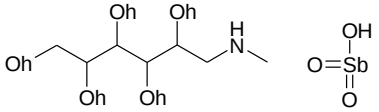
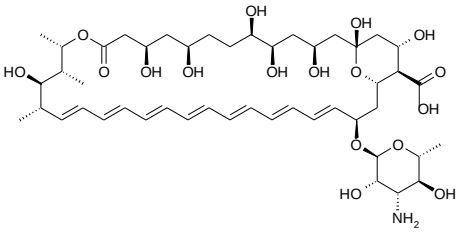
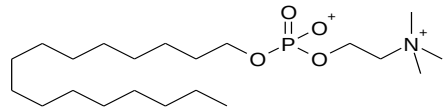
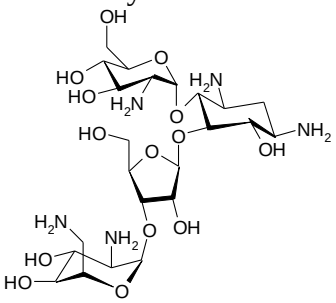
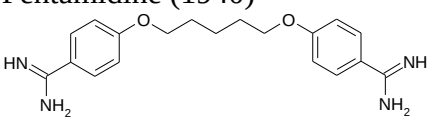
Chemotherapy depends on a limited number of available drugs [3,20,21]. All current used drugs have their drawbacks such as toxicity, problems of efficacy, poor oral bioavailability (Tab1). For the treatment of the first stage of the disease two drugs are used, pentamidine
for T.b.gambiense and suramin for T.b.rhodesiense infections. Melarsoprol was for a long time the first-line drug to treat T.b.gambiense second stage infection and is the only option for T.b.rhodesiense second stage disease. Today, eflornithine (DFMO) is used for second stage gambiense disease. Since few years the nifurtimox-eflornithine combination therapy (NECT) is recommended by WHO as first-line treatment [22]. The introduction of NECT is the only progress in the chemotherapy of HAT in the last 25 years.

Leishmaniasis

Leishmaniasis is caused by more than 20 species of the kinetoplastid protozoan parasites belonging to the genus Leishmania. The disease is prevalent worldwide in southern Europe and in the subtropic and tropic belt. Leishmaniasis is classified in three major clinical forms, visceral (VL), cutaneous (CL) and mucocutaneous leishmaniasis (MCL) [23]. The forms differ in immunopathologies, in degree of morbidity and mortality.

Leishmania parasites are transmitted by the bite of an infected phlebotomine sandfly, about 30 sandfly species can transmit the disease [24]. Promastigote forms of the parasite are ingested by macrophages and transform into amastigotes [25]. The amastigotes multiply and survive in the phagolysosomes of macrophages until the cell eventually bursts, then new phagocytic cells are infected. There are two main forms of leishmaniasis with a broad range of severe clinical manifestations. Cutaneous leishmaniasis (CL) is the most common form causing skin lesions and skin ulcers on exposed areas as face, arms and legs. After healing often remains scarring and serious disability. Chronic skin lesions similar to those of lepromatous leprosy are the characteristics of diffuse cutaneous leishmaniasis. Visceral leishmaniasis (VL) also known as kala azar is another form of the disease. It is caused by an infection of the lymphatic system and leads to fever, weight loss, swelling of liver and spleen and anaemia. This most severe form of the disease is fatal if untreated. A third disease form is the mucocutaneous leishmaniasis (MCL) which affects the naso-oropharyngeal mucosa.

Table 2. Available chemotherapy for the treatment of Leishmaniasis, problems and main adverse effects.

Leishmaniasis		
Drug	Route of application & dosage	Problems & main adverse effects
Pentavalent Antimonials: Sodium stibogluconate (Pentostam, SSG)  and meglumine antimoniate (Glucantime) 	Intravenous and intramuscular 20 mg/kg daily for 20-30 days (depends on geographic area)	For VL and CL. Organo-metal complexes on polymeric forms. Length of treatment Painful injection Resistance in India Cardiac toxicity, pancreatitis, nephrotoxicity hepatotoxicity
Amphotericin B  Liposomal amphotericin B	Intravenous 1 mg/kg every other day for up to 30 days (15mg/kg total dose) Intravenous Unilamellar liposome	For VL, CL and MCL. Polyene antibiotic. First line drug for VL in areas of India where antimonial resistence occurs. Need for slow iv infusion; Dose-limiting, nephrotoxicity, hypokalaemia, fever, chills, hypotension Most effective formulation for VL, also used for complex forms (PKDL, MCL)
Miltefosine 	Oral 2.5mg/kg/day for 28 days	First oral drug for VL. Hexadecylphosphocholine Anorexia, nausea, vomiting, diarrhoea, potentially teratogenic
Paromomycin 	Intramuscularly (VL), topical (CL) 15mg/kg/day for 28 days CL topical one daily for 20 days	For VL and CL aminoglycoside antibiotic as sulfate salt, Mild pain at the injection site, Reversible ototoxicity Topical formulation (12%) with methyl benzylmethonium chloride. Topical formulation with gentamicin and surfactants in Phase III trial.
Pentamidine (1940) 	Intramuscular; 4mg/kg/alternate days or 3 times/week for 15-30 dosage (IV or IM)	Only for specific forms of CL in South America; hypoglycaemia, injection site pain, diarrhea, nausea, vomiting, hypotension

Leishmaniasis is transmitted in 98 countries of the tropical and subtropical belt but also in Southern Europe [24]. 350 million people life at risk and 12 million people worldwide are affected by the disease, with an estimated 1.5-2 million new cases per year [24].

Leishmaniasis is a poverty related disease but it is also linked to environmental changes [26]. Deforestation, urbanization, and migration of non-immune people to endemic areas are manmade risk factors [23].

It is widely acknowledged that the reported case figures represent an underestimate of the true burden. For VL 0.2 to 0.4 million cases per year are estimated, but only 60,000 cases are reported. 90% of reported VL cases occur in Bangladesh, Brazil, India, Nepal and Sudan [27]. VL interacts with HIV infection; it is one of the major threats to control of the disease. A co-infection with HIV increases the risk of developing active VL over 100 times [28].

210000 CL cases per year are reported, but it is estimated that there are 0.7 to 1.2 Mio cutaneous Leishmaniasis cases per year [27]. CL occurs mainly in the Middle East (Afghanistan, Iran, Saudi Arabia and Syria) and Latin America (Brazil and Peru), about 90% of cases are found in those countries. MCL occur mainly in Latin America; Bolivia, Brazil and Peru are most affected.

The current situation of treatment options for leishmaniasis are similar to the other neglected protozoan diseases. Only a limited number of drugs are available and they show severe side effects and lack of efficacy [29] (Tab.2). Each drug has its drawback, such as difficulty in administration, length of treatment, toxicity, cost, and increasing parasitic resistance. Pentavalent antimonials, meglumine antimoniate and sodium stibogluconate were introduced in the 1940s and are still the first-line treatment in most part of the world. New drugs and new formulation of old drugs led to some improvement in the treatment of VL [30]. These new treatments include liposomal amphotericin B, paromomycin (both antibiotics) and the oral anticancer drug miltefosine. For the treatment of CL the same drugs are used as for VL. But the treatments are all unsatisfactory due to high failure rates and toxicity. Species variation,15 Leishmania species can cause CL, and pharmacokinetics are the major problems in the development of new drugs [31].

Chagas disease

The hemoflagellate parasite *Trypanosoma cruzi* is the causative agent of Chagas disease also called American trypanosomiasis [32]. The disease is found mainly in Latin America and was discovered 1909 by the Brazilian physician Carlos Chagas.

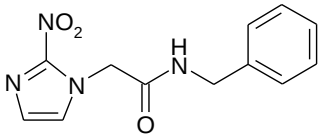
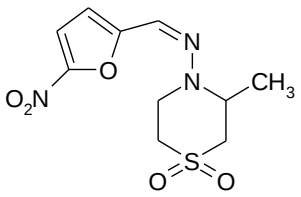
T.cruzi parasites are mainly transmitted by the infected feces of blood-sucking triatomine bugs [32]. The night active bugs feed on human blood and defecate close to the bite. The parasite can enter the body when a person instinctively lubricates the feces into the bite, the mouth, or the eyes. Chagas disease cans also be transmitted by other routes [33,34,35,36]. such as: i) food or drink contaminated with T.cruzi, ii) blood transfusion and organ transplants if the donor is infected, iii) congenital, iv) laboratory accidents. The initial acute phase lasts few weeks or months with parasites circulating in the blood. In the acute phase symptoms are absent or mild but can include skin lesion, swelling of the eyelid (Romaña's sign) fever, enlarged lymph nodes, headache and pain [37]. The chronic phase of the disease is asymptomatic and the parasites hide mainly in the heart and digestive muscle. Most people are unaware of their infection and many of them do not develop any disease related symptoms. Up to 30% of the infected people will develop problems as cardiac disorders (heart rhythm abnormalities, dilated heart), enlargement of the esophagus or colon [38,39]. The progressive destruction of the heart muscle can lead to sudden death. HIV infection or any immunosuppression may reactivate the chronic disease; the consequences are parasites in the blood and a severe disease [49].

The acute stage of Chagas disease can be diagnosed microscopically by blood smear examination. Congenital infection is best identified with microhaematocrit, due to the small amount of blood needed. At least two different serological tests are needed for the diagnosis of the chronic disease [41]. The direct detection in the blood is not possible because of the very low parasitemia.

Chagas disease is endemic in 21 countries of Central and South America [42]. Due to the population mobility and less frequently due to blood transfusion, organ donation and congenital transmission, Chagas disease is increasingly detected in North America, Europe and some Western Pacific countries. It is estimated that 8 million people are infected and that in 2008 over 10'000 people died from the disease [32]. People living under poor

housing conditions in rural areas of Latin America, are most at risk to acquire the disease. Prevention and control of the disease in Latin America is mainly focused on vector control. Triatomine bugs typically live in the cracks of poorly-constructed homes in rural or suburban areas. Therefore, improved housing and spraying insecticides inside houses are the most effective ways to combat the disease [43].

Table 3. Available chemotherapy for the treatment of Chagas disease, problems and main adverse effects.

Chagas disease		
Drug	Route of application & dosage	Problems & main adverse effects
Benznidazole (1972) 	Oral; 5-7 mg/kg in two divided doses daily for 60 days. Children (up to 12 years): 10 mg/kg in two divided doses daily for 60 days.	- limited effectiveness (60 – 80%) in the acute phase of the disease - regional variations in efficacy due to naturally resistant T.cruzi strains low effectiveness (10 – 20%) in the chronic phase of the disease - long period of treatment, and dose-dependent toxicity -no paediatric strengths -contraindicated during pregnancy - need for monitoring under specialized medical supervision - rash and gastrointestinal symptoms such as nausea. Rarely, peripheral neuropathy leukopenia, agranulocytosis
Nifurtimox (1967) 	Oral Adults: 8-10 mg/kg in three divided daily doses for 90 days. Children: 15-20 mg/kg in four divided daily doses for 90 days.	Safety in pregnancy has not been established high number of side effects: digestive disturbances (epigastric and abdominal pain, nausea and vomiting, anorexia, weight loss)

There are only two drug available (Tab. 3), benznidazole and nifurtimox, for chemotherapy of Chagas disease [44]. Both medicines can cure infected people. Nifurtimox and benznidazole show the highest efficacy in early infection but low effectiveness in the chronic stage of the disease. Both drugs show a number of side effects, a dose dependent toxicity and a long duration of treatment is necessary to successfully combat an infection. Another problem for the efficacy of the drugs is the genetical heterogeneity of T.cruzi. The

parasite strains were grouped into six phylogenetic lineages with different drug sensitivities [45].

Drug discovery and development

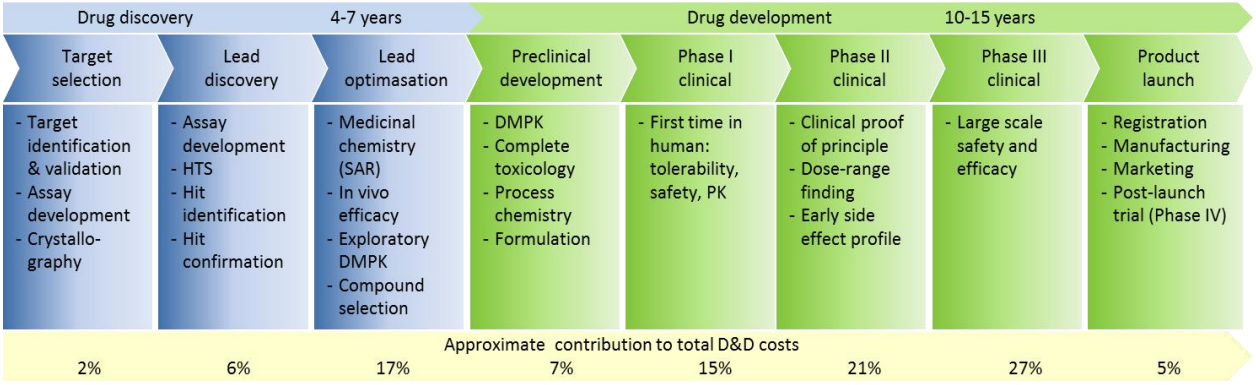
Drug discovery and development is an expensive and lengthy process taking approximately 10-15 years. The costs for preclinical development are estimated to be up to 500 Mio USD and for the full development to bring a new drug on the market 500-1800 Mio USD [46].

Drug discovery and development is a complex process which starts from basic research (target identification, validation) and processes to lead identification, preclinical and clinical development until registration and marketing and ends with the access of the drug [47,48] (Figure 1). All aspects are important and have their own complexity and difficulties. The risk of failure is very high. Due to the length and cost of the drug developing process, the risk of failure should be minimized during this process. In an optimal drug development process the attrition rate is high at the beginning and decreases with duration [49].

Antiparasitic drug discovery and development

There is no doubt, for neglected tropical disease (NTD) new drugs are urgently needed. The current drugs have their drawbacks, they are old, expensive, not easy to administer and often toxic. New candidates have to fulfill some pre-defined criteria, so called target product profile (TPP). Drug discovery is an iterative and integrated process; a TPP can guide selection of candidate molecules [50].

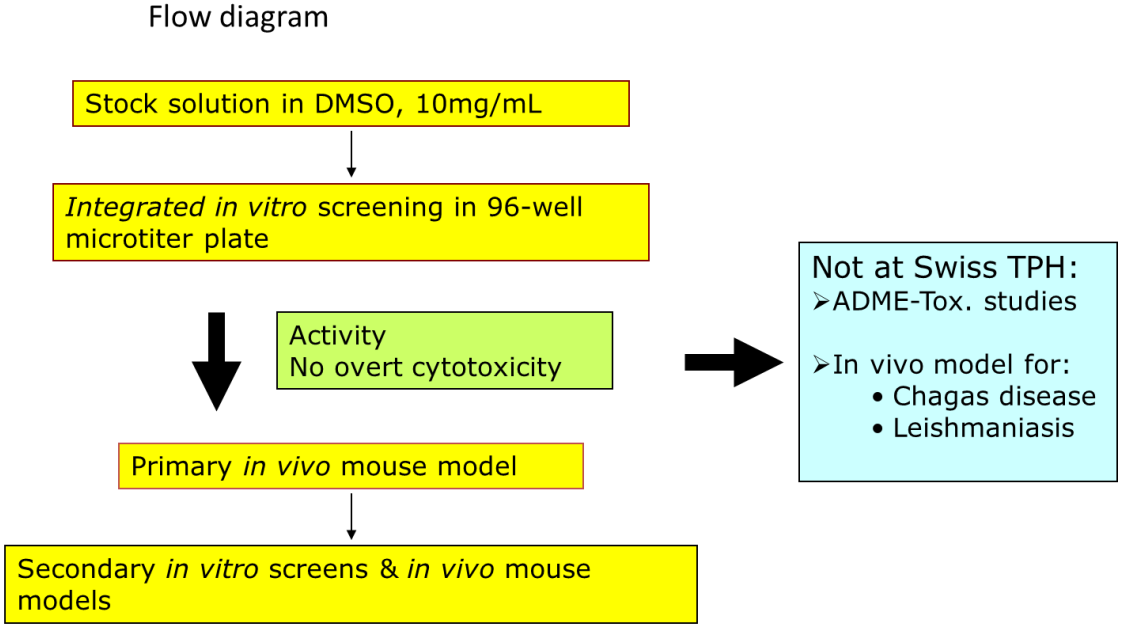
Figure 1. Drug discovery and development process



There are several strategies to develop new drugs against NTDs. De novo drug discovery and drug development is a very rational approach but it is a lengthy and expensive process. Frearson et al [51] introduced the "traffic light" system for target assessment as a prioritization and management tool. The goal of the target based drug discovery is to produce a specific acting new medicine. This process as mentioned above is very long and high risky. Drugs that act on a specific enzyme have the advantage that the mechanism of action is known. However, such drugs may have the disadvantage that resistance can easily result from mutation. The knowledge of the mode of action of a drug can be helpful in the drug discovery process but it is not mandatory.

There are other drug discovery strategies. The in vitro screening against whole parasites is a very valuable strategy for anti-parasitic drug discovery [52]. Large compound libraries should be screened against living parasites and whenever possible using the human infectious form. This strategy of phenotypic screening was also used for establishing the" malaria box" [53,54,55,56].

Figure 2 Flow diagram of drug screening



Another good approach for drug discovery is to follow the advice of the Nobel prize winner Sir James Black 'the most fruitful basis for the discovery of a new drug is to start with an old drug' [57]. Using the piggyback approach has its merit [58]. It is a low risk and low cost approach by looking for new indications of existing human, veterinarian drugs and

agrochemicals. This opportunistic approach includes whole libraries of compounds coming from any drug discovery projects including cancer research.

We follow the strategy of phenotypic screening since our screening center was established in the 90's. Additionaly we use the approach of an integrated in vitro drug screening (Fig. 2). This means the compounds are tested whenever possible against 4 protozoan parasites, *T.b.rhodesiense, T.cruzi*, *L. donovani*, *P.falciparum,* and for cytotoxicity assessment against a mammalian cell line.

Goal

The first goal of this thesis is the use of phenotypic screening to re-investigate the old compound class of nitroimidazoles in order to identify a clinical drug candidate for the treatment of human African trypanosomiasis. A second aim is the identification of nitroimidazoles as preclinical candidates to treat Chagas disease and Leishmaniasis. A third goal is the use of the piggy-back approach to evaluate a library of agrochemicals and a series of approved drugs in order to identify preclinical/clinical candidates against the trypanosomatid parasites.

Objectives

The study's specific objectives were:

- To evaluate the in vitro efficacy of a collection of nitroimidazoles against *T.b.rhodesiense, T.cruzi. L. donovani*.
- To evaluate the most in vitro active nitroimidazole in both, the first and second stage, HAT rodent model
- To characterize the in vitro efficacy of the most active nitroimidazoles
- To evaluate the in vitro and in vivo efficacy of a collection of agrochemical
- To evaluate in vitro a collection of approved drugs

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CHAPTER 2

Fexinidazole - A New Oral Nitroimidazole Drug Candidate Entering Clinical Development for the Treatment of Sleeping Sickness

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Abstract

Background: Human African trypanosomiasis (HAT), also known as sleeping sickness, is a fatal parasitic disease caused by trypanosomes. Current treatment options for HAT are scarce, toxic, no longer effective, or very difficult to administer, in particular for the advanced, fatal stage of the disease (stage 2). New safe, effective and easy-to-use treatments are urgently needed. Here it is shown that fexinidazole, a 2-substituted 5-nitroimidazole rediscovered by the Drugs for Neglected Diseases *initiative* (DND*i*) after extensive compound mining efforts of more than 700 new and existing nitroheterocycles, could be a short-course, safe and effective oral treatment curing both acute and chronic HAT that could be implemented at the primary health care level. In order to complete the regulatory requirements for demonstrating efficacy and safety before initiating human trials, the anti-parasitic properties and the pharmacokinetic, metabolic and toxicological profile of fexinidazole have been assessed.

Methods and Findings: Standard *in vitro* and *in vivo* anti-parasitic activity assays were conducted to assess drug efficacy in experimental models for HAT. In parallel, a full range of preclinical pharmacology and safety studies, as required by international regulatory guidelines before initiating human studies, have been conducted.

Fexinidazole is moderately active *in vitro* against African trypanosomes (IC_{50} against laboratory strains and recent clinical isolates ranged between 0.16 and 0.93 μg/mL) and oral administration of fexinidazole at doses of 100 mg/kg/day for 4 days or 200 mg/kg/day for 5 days cured mice with acute and chronic infection respectively, the latter being a model for the advanced and fatal form of the disease when parasites have disseminated into the brain. In laboratory animals, fexinidazole is well absorbed after oral administration and readily distributes throughout the body, including the brain. The absolute bioavailability of oral fexinidazole was 41% in mice, 30% in rats, and 10% in dogs. Furthermore, fexinidazole is rapidly metabolised *in vivo* to at least two biologically active metabolites (a sulfoxide and a sulfone derivative) that likely account for a significant portion of the therapeutic effect. Key pharmacokinetic parameter after oral absorption in mice for fexinidazole and its sulfoxide and sulfone metabolites are a C_{max} of 500, 14171 and 13651 ng/mL respectively, and an AUC_{0-24} of 424, 45031 and 96286 h.ng/mL respectively. Essentially similar PK profiles were observed in rats and dogs. Toxicology studies

(including safety pharmacology and 4-weeks repeated-dose toxicokinetics in rat and dog) have shown that fexinidazole is well tolerated. The No Observed Adverse Effect Levels in the 4-weeks repeated dose toxicity studies in rats and dogs was was 200 mg/kg/day in both species, with no issues of concern identified for doses up to 800 mg/kg/day. While fexinidazole, like many nitroheterocycles, is mutagenic in the Ames test due to bacterial specific metabolism, it is not genotoxic to mammalian cells *in vitro* or *in vivo* as assessed in an *in vitro* micronucleus test on human lymphocytes, an *in vivo* mouse bone marrow micronucleus test, and an *ex vivo* unscheduled DNA synthesis test in rats.

Conclusions: The results of the preclinical pharmacological and safety studies indicate that fexinidazole is a safe and effective oral drug candidate with no untoward effects that would preclude evaluation in man. The drug has entered first in-human studies in September, 2009. Fexinidazole is the first new clinical drug candidate with the potential for treating advanced-stage sleeping sickness in thirty years.

Introduction

A major challenge for new drug development is the identification of pharmacologically active compounds with a favourable activity and toxicity profile that can be turned into new drug candidates. The contemporary approach to identifying such compounds is high-throughput screening of large and chemically diverse compound libraries to identify novel pharmacophores, followed by lead optimisation [1,2]. Sometimes, this screening effort is narrowed down by using more targeted libraries that are thought to be enriched in compounds with a desired type of activity (e.g. kinase inhibitors [3]). However, promising candidates can also be found by revisiting the wealth of past drug discovery research, during which promising lines of research were sometimes not pursued for commercial or other strategic reasons.

In this paper, we report the successful result of a proactive compound mining approach into a well-known class of anti-infectives, the nitroimidazoles, to rediscover fexinidazole, a long forgotten anti-parasitic drug candidate. Fexinidazole turned out to be an excellent candidate to cure human African trypanosomiasis (HAT), including the advanced and fatal stage of the disease.

An estimated sixty million people in 36 sub-Saharan African countries are at risk for HAT, especially poor and neglected populations living in remote rural areas [4,5]. While the number of reported HAT cases has decreased in recent years due to intensified control activities, 50,000 to 70,000 people are estimated to be infected [6. In west and central Africa, *Trypanosoma brucei gambiense* causes a chronic form of sleeping sickness, whereas in eastern and southern Africa *T. b. rhodesiense* causes an acute form of the disease [7,8]. Both forms of HAT occur in two stages: stage 1 (early, hemolymphatic) is characterized by non-specific clinical symptoms such as malaise, headache, fever, and peripheral oedema, whereas stage 2 (late, meningoencephalic) is characterized by neurological symptoms including behavioural changes, severe sleeping disturbances, and convulsions, which, if left untreated, lead to coma and death [9.10].

Available treatments for HAT [8] (Table 1) are few, old, and limited due to toxicity, diminishing efficacy in several geographical regions [11,12], and complexity of use [13]. Treatment is stage-specific, with the more toxic and difficult-to-use treatments being used for stage 2 HAT.

NECT, a combination treatment of a simplified course of intravenous eflornithine and oral nifurtimox, has been the only advance in the past 25 years [14,15], and has been recently accepted into the WHO's Essential Medicines List as treatment for stage 2 HAT [16]. Despite being a clear improvement with reduced toxicity and treatment duration, the requirement for intravenous administration is still a limitation.

Table 1. Available treatment options for HAT.

Indication	**Drug**	**Associated Problems**
Stage 1	Pentamidine (1940)	7-10 daily intramuscular injections; only efficacious for stage 1
	Suramin (1920's)	Used primarily for stage 1 *T. b. rhodesiense* HAT
Stage 2	Melarsoprol (1949)	Ten painful daily intravenous injections; highly toxic, with ~5 % treatment-related mortality. Increasing number of treatment failures (up to 30% in some regions); used for stage 2 HAT
	Eflornithine (1981)	Administration difficult – 4 slow intravenous infusions per day for 14 days; increasingly used as 1st line for *T. b. gambiense* stage 2 HAT
	NECT (2009)	Simplified regimen combining 7 days eflornithine (two infusions/day) and 10 days oral nifurtimox; expected to replace eflornithine monotherapy and melarsoprol for stage 2 *T. b. gambiense* HAT

It is estimated that less than 20% of currently infected people have access to treatment or are under any HAT surveillance, due to a combination of lack of effective and field-adapted diagnostics and treatments, combined with extreme poverty and remoteness of the affected populations, including in conflict zones [4,17]. To change the dynamics of HAT control and access more patients while improving their case-management, a safe, effective, affordable, and easy-to-use (short course, preferably oral) treatment is urgently needed. Nitroimidazoles are a well-known class of pharmacologically active compounds, among which several have shown good activity against trypanosomes [18] . The best-known anti-trypanosomal drug candidate in this class was megazol [19,20]; its development was abandoned because of toxicity, in particular mutagenicity [21,22], a known possibility in this chemical family [23,24]. However, other members of this family including metronidazole [25], are widely used as antibiotics, indicating that it is possible to select compounds with an acceptable activity/toxicity profile in this class.

A systematic review and profiling of more than 700 nitroheterocyclic compounds (mostly nitroimidazoles) from diverse sources was undertaken and included an assessment of anti-parasitic activity and mutagenic potential using state-of-the-art scientific methods. From these efforts, fexinidazole, a 2-substituted 5-nitroimidazole, was identified as a promising drug candidate for the treatment of HAT. Fexinidazole (1-methyl-2-((p-(methylthio)phenoxy)methyl)-5-nitroimidazole, CAS registry number 59729-37-2) had been in preclinical development in the 1970s and early 1980s as a broad-spectrum antimicrobial agent by Hoechst AG (now sanofi-aventis), selected within a broader series because of its wider range of action, lower toxicity and comparative ease of chemical synthesis [26,27]. In 1983, the in vivo activity of fexinidazole against African trypanosomes was also documented [28]. However fexinidazole's further development was not pursued at the time.

This paper describes the trypanocidal efficacy and preclinical profile of fexinidazole as a novel clinical drug candidate for HAT, devoid of genotoxic risks for patients. The results show fexinidazole's potential to become a safe, efficacious, affordable, short-course (less than 14 days), oral treatment with a suitable shelf life in tropical conditions. Ideally the treatment will be safe and effective in both stages 1 and 2 HAT, allowing for significantly simplified diagnosis, treatment and patient-management and ultimately a better control of the disease.

Methods

Ethics Statement

All work was conducted in accredited laboratories and according to international guidelines. Specific references to the relevant authorities are provided below as appropriate. Where not stated details of specific license holders can be obtained from the appropriate laboratories if required.

Drug preparation

Fexinidazole and metabolites were prepared for *in vitro* studies as a stock solution in DMSO further diluted with water or 0.5% methylcellulose in water to appropriate concentration required for the assay.

For *in vivo* studies fexinidazole was prepared as an optimized suspension comprising 5% w/v Tween 80/0.5% w/v Methocel in water, unless stated otherwise.

Anti-trypanosomal activity studies

***In vitro* trypanocidal and cytotoxicity assays [29,30,31]:** Parasites were cultured with or without the test article at a concentration of $3x10^4$/mL in Minimal Essential Medium, according to Baltz et al. [30] with the following modifications: 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5 mM hypoxanthine and 15% heat inactivated horse serum at 37°C in 5% CO_2 in 96-well microtitre plates for 72 h. 10 μL of Alamar Blue was added for the final 3 h to determine viability. The assay was assessed by reading the fluorescence in each well at an excitation wavelength of 536 nm and at an emission wavelength of 588 nm. The IC_{50} values were calculated from the sigmoidal inhibition curves. For cytotoxicity, $4×10^4$/mL L-6 rat skeletal myoblast cells were used, and incubations and assessments carried out as above.

***In vivo* trypanocidal assays [32,33]:** Female NMRI mice were infected via intraperitoneal injection with bloodstream forms of *T. b. rhodesiense* STIB900 or *T. b. gambiense* 130R and treated with test drugs daily for 4 consecutive days, starting on day 3 or 7 post infection, respectively, or with bloodstream forms of *T. b brucei* GVR35 and treated daily from day 21 after infection for 5 days. Parasitemia was evaluated by tail blood examination and surviving, aparasitaemic mice at 60, 90, or 180 days, respectively, were considered cured.

The mouse assays were conducted in accordance to relevant national and international guidelines. The studies were approved by the local veterinary office (Kantonales Veterinäramt Basel) under licence No. 739.

Pharmacokinetic (PK) studies

A range of pharmacokinetic studies have been performed in different species (mouse, rat, dog) in the context of this paper, either as pharmacokinetic studies to establish the PK profile of fexinidazole and its metabolites, or as part of other studies (safety pharmacology and toxicity) to demonstrate the systemic exposure of fexinidazole and its metabolites in the conditions of that particular study.

Plasma sampling: Blood samples were taken via into heparinised collection tubes and, following centrifugation, plasma was removed and stored frozen until required for assay.

Brain sampling in mice: Following sacrifice, the cranium of mice was opened and the brain removed. Excess blood was washed from the brain with distilled water and any excess fluid blotted on absorbent paper. The brain was then snap frozen using liquid nitrogen and placed into a suitably labelled container and stored at approximately -70°C, pending analysis. Brains were homogenized and extracted samples were prepared appropriately for analysis via liquid chromatography-mass spectrometry (LC-MS).

Plasma and brain pharmacokinetic (PK) analytical assessment: All PK evaluations were carried out using validated high performance liquid chromatography (HPLC) and LC-MS assays as per standard operating procedures of the laboratories involved.

The majority of PK studies were carried out by Accelera, Nerviano Medical Sciences, Italy following internal Standard Operating Procedures as non-GLP regulated studies and all procedures for housing and handling of animals were in strict compliance with EEC and Italian Guidelines for Laboratory Animal Welfare.

One mouse PK and brain sampling study was carried out by BioDynamics Ltd., Rushden, UK according to internal Standard Operating Procedures as a non-GLP regulated study and animals were maintained as required by the "Code of practice for the housing and care of animals used in scientific procedures" (Home Office, London, 1989).

ADME (Absorption, Distribution, Metabolism, Excretion) studies

***In vitro* hepatocyte metabolism [34]:** Fexinidazole was incubated with cryopreserved hepatocytes from male CD-1 mice, male Sprague Dawley rats, male beagle dogs, male cynomolgus monkeys, and from pooled white or African-American human donor samples, at concentrations of 1 μM and 10 μM. 1 μM samples were used for the cross-species intrinsic clearance determination, whereas 10 μM samples were used for the cross-species metabolite profile determination. The incubated samples were analyzed by LC-MS after extraction into acetonitrile.

***In vitro* CYP-450 metabolism [35]:** Duplicate samples of human recombinant CYP enzyme isotypes CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5 (10-30 pmol/mL) with NADPH as cofactor were incubated with fexinidazole,

fexinidazole sulfoxide, or fexinidazole sulfone at a concentration of 1.0 μM for up to 60 min (pH 7.4, 37°C). Detection of the compounds was via HPLC-MS.

***In vitro* transcellular permeability:** Caco-2 [36] or MDR1-MDCK (Madin Darby canine kidney cells transfected with the human multidrug resistance gene) [37] cell monolayers were grown to confluence on collagen-coated, microporous, polycarbonate membranes in 12-well microtitre plates. The buffer for the donor chamber was Hanks balanced salt solution with 10 mM HEPES and 15 mM glucose at pH 7.4. The buffer in the receiver chamber contained the same with the addition of 1% bovine serum albumin (BSA). The test article was added at 5 μM in the assay buffer. Cells were dosed on the apical side or baso-lateral side and incubated at 37°C with 5% CO_2 in a humidified incubator. After 2 h, duplicate aliquots were taken from the donor and receiver chambers and assayed via LC/MS. The apparent permeability and recovery were calculated using standard methods.

***In vivo* metabolism and distribution:** Whole-body autoradiography in rats was conducted using [^{14}C]-radiolabelled fexinidazole. The site of the [^{14}C] label in the fexinidazole molecule on the bridging carbon atom ensured that the radioactivity would remain associated with both the sulfoxide and sulfone metabolites. The radiolabelled compound was administered at the dose of 800 mg/kg (approximately 3.7 MBq/kg, 100 μCi/kg) by gastric gavage to eight male albino Sprague Dawley rats. Two animals were sacrificed at each time point 2, 8, 24 or 48 h after administration and the quantitative radioactivity distribution in the organs and tissues was evaluated using quantitative whole-body autoradiography.

Excretion balance in rats was carried out using a single oral dose of radiolabelled fexinidazole administered at the dose of 800 mg/kg (approximately 3.7 MBq/kg, 100 μCi/kg) by gastric gavage to three male albino Sprague Dawley rats.

The animal studies above were carried out by Accelera, Nerviano Medical Sciences, Italy following internal Standard Operating Procedures as non-GLP regulated studies and all procedures for housing and handling of animals were in strict compliance with EEC and Italian Guidelines for Laboratory Animal Welfare.

Safety pharmacology profiling [38,39]

In vitro **cardiac safety [40]:** HEK 293 (human embryonic kidney) cells stably expressing hERG (human ether-a-go-go-related gene) were treated with fexinidazole, fexinidazole sulfoxide, or fexinidazole sulfone at 1, 5 or 30 µM, and the hERG peak tail current was measured.

Dog cardiovascular safety assessment [40] : Cardiovascular safety parameters were assessed in beagle dogs. Four adult (>1 year old) beagle dogs were given oral doses of fexinidazole at 100, 300 and 1000 mg/kg in an escalating dose design. During the experimental phase animals were monitored via a Closed Circuit TeleVision CCTV system and physically inspected 24 h after dosing. On the day of dosing, a predefined set of telemetry signals from each animal were collected continuously from at least 1 h before dosing to at least 24 h after treatment.

In vivo **neurobehavioural safety profiling:** Neurobehavioural safety was carried out using the Irwin's test of general behaviour and body temperature in male rats [41,39]. Twenty-four male rats (Crl:CD(SD)BR, aged about 7 weeks on the day of dosing were divided into four experimental groups of 6 animals per group. Each group of animals was treated orally with fexinidazole at doses of 100, 300, or 1000 mg/kg, or vehicle. General behaviour was assessed before treatment and 2 h and 24 h after vehicle or drug administration. A defined set of behavioural observations using an arbitrary intensity scale of 0 (no behavioural change) to 8 was made before treatment and 2 h and 24 h after treatment. Factors present in normal animals (e.g. alertness, mobility, etc.) were scored as 4; potentiation or depression of these factors was indicated as higher or lower integers, respectively. Factors absent in normal animals were scored from 0 (normal) to 8.

In vivo **respiratory safety profiling:** Respiratory parameters were assessed in the rat [42]. Groups of eight male rats (Crl:CD(SD)BR, aged about 8 weeks on the day of dosing were administered doses of 100, 300, or 1000 mg/kg of fexinidazole by oral gavage. A pre-determined standard set of respiratory parameters was acquired continuously from individual animals housed in plethysmographic chambers. Basal values were calculated as the mean of values from 30 to 10 min before treatment. After treatment, values were extracted every 30 min up to 4 h. Body weights were recorded on the day of treatment for calculation of dose volumes and for calculation of tidal and minute volumes per kg of body weight.

All safety pharmacology studies in animals were carried out by Accelera, Nerviano Medical Sciences, Italy following internal Standard Operating Procedures and applicable ICH guidelines (ICH S7A, S7B), were GLP regulated and were conducted in compliance with the DECRETO LEGISLATIVO 2 Marzo 2007, No. 50 and OECD Principles of GLP (January 1998) ENV/MC/CHEM (98) 17.

Repeated –Dose Study / Toxicokinetics [43,44]

In the rat repeat-dose toxicokinetic study, fexinidazole was administered orally by gavage once a day for 28 consecutive days to ten or fifteen Crl:CD (SD)IGS BR rats/sex/group at doses of 50, 200, or 800 mg/kg/day. A control group received the vehicle alone (5% Tween 80/0.5% methocel). Ten animals/sex/group were sacrificed at the end of the treatment period on day 29 or 30 of study. The remaining 5 animals/sex/group in the control and high-dose groups were sacrificed on day 43 at the end of a 2-weeks observation period. Systemic exposure to fexinidazole and its sulfoxide and sulfone metabolites was evaluated in three additional animals/sex/group. Samples were taken at predose, and 30 min, 1, 2, 4, 8 and 24 h after dosing on days 1, 14, and 28, and in addition at 48 and 72 h after treatment on day 28.

In the dog repeat-dose toxicokinetic study, fexinidazole was given orally by gavage once a day for 28 days to five (control and high dose) or three (low and mid dose) beagle dogs /sex/dose at the doses of 0 (control group), 50, 200, or 800 mg/kg/day. The control group received the vehicle alone (same as above). Systemic exposure to fexinidazole and of its sulfoxide and sulfone metabolites was evaluated on days 1, 14 and 28 in the same animals used for the toxicological study. Samples were taken at predose, and 30 min, 1, 2, 4, 8 and 24 h after dosing on days 1, 14, and 28, and in addition at 48 and 72 h after treatment on day 28.

For both species, the standard package of toxicological analyses was carried out.

Both rat and dog studies were carried out by Accelera, Nerviano Medical Sciences, Italy according to their internal Standard Operating Procedures, were GLP regulated and were conducted in compliance with the DECRETO LEGISLATIVO 2 Marzo 2007, No. 50 and OECD Principles of GLP (January 1998) ENV/MC/CHEM (98) 17 and the ICH regulatory guidelines for repeated dose toxicokinetics studies (ICH M3 and S3A)

Developmental and reproductive toxicology [45]

Preliminary studies in rat and rabbit have been performed to have an early idea of reproductive and developmental toxicity risks, and to determine dose levels to be used in further pivotal studies.

In the rat study, fexinidazole was administered orally by gavage once a day from day 6 to day 17 of gestation or from day 6 of gestation to day 7 of lactation to 10 mated rat Crl:CD (SD)IGS BR rats/sex/group at doses of 50, 200, or 800 mg/kg/day. A control group received the vehicle alone (5% Tween 80/0.5% methocel).

In the rabbit study, fexinidazole was administered orally by gavage once a day from day 6 to day 20 of gestation to 6 inseminated New Zealand White Rabbit KBL females at the dose of 20,40 and 80 mg/kg/day. A control group received the vehicle alone (5% Tween 80/0.5% methocel).

Both rat and rabbit studies were carried out by Accelera, Nerviano Medical Sciences, Italy according to their internal Standard Operating Procedures, were GLP regulated and were conducted in compliance with the DECRETO LEGISLATIVO 2 Marzo 2007, No. 50 and OECD Principles of GLP (January 1998) ENV/MC/CHEM (98) 17 and the appropriate ICH regulatory guidelines for reproductive toxicology (ICH S5A).

Genotoxicity assessments [46]

In vitro **Ames test:** To evaluate bacterial mutagenicity, a full Ames test was carried out on fexinidazole and fexinidazole sulfone using the five strains of *Salmonella typhimurium* recommended by the relevant ICH regulatory guideline, namely TA1535, TA1537, TA98, TA100, and TA102, as well as the corresponding nitroreductase-deficient strains [47,48]. Standard bacterial plate incorporation assays were carried out, essentially as described by Maron and Ames [49]. Tests on fexinidazole were carried out with and without rat liver post-mitochondrial fraction plus co-factors (S9 mix) to provide a mammalian metabolic activation system. The S9 fraction was prepared from Spague-Dawley rats pretreated with the mixed cytochrome P 450 enzyme inducer Aroclor 1254. Tests on fexinidazole sulfone were carried out only in the absence of rat liver S9 (as this is a metabolite). After incubation at 37°C for three days, plates were scored for mutant colonies using a Colony Counter plate reader.

***In vitro* micronucleus test on human lymphocytes [50]:** Duplicate human lymphocyte cultures were prepared from the pooled blood of two donors in two independent experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were done both in the absence and presence of metabolic activation (S9) from Aroclor-1254-induced animals. The highest concentration of fexinidazole was 220 μg/mL. Cells were treated with the drug either 24 h or 48 h after mitogen stimulation by phytohaemagglutinin (PHA). The test concentrations for micronucleus analysis were selected by evaluating the effect of fexinidazole on the replication index (RI). In each experiment, micronuclei were analysed at three concentrations. Similar tests were carried out on fexinidazole sulfone. As per the Ames test, this was carried out in the absence of rat liver S9 only.

***In vivo* mouse bone marrow micronucleus test [51]:** Groups of six young adult male Crl:CD-1 (ICR) mice were treated with fexinidazole at two oral doses of 0, 500, 1000, or 2000 mg/kg given 24 h apart, and bone marrows were harvested 24 h after the second dose. Slides of bone marrow cells were prepared, Giemsa stained, and 2000 polychromatic erythrocytes per animal were scored for micronuclei.

***Ex vivo* unscheduled DNA synthesis (UDS) in rats [52]:** Groups of young male Sprague Dawley rats received fexinidazole at doses of 500, 1000, or 2000 mg/kg orally, and the livers were sampled either 2-4 h or 12-14 h after administration. Hepatocyte suspensions were prepared and incubated in the presence of titrated thymidine. Slides of fixed hepatocytes were coated in photographic emulsion and stored for 14 days at 4°C in the dark. The silver grains above the nuclei or cytoplasm (background) were counted, providing a measure of DNA uptake during DNA repair. The difference between these two counts indicated the extent of DNA repair (nuclear net grain count).

All genotoxicological studies in animals were carried out by Covance Laboratories Ltd, Harrowgate, England following internal Standard Operating Procedures, the applicacble ICH guidelines (ICH S2) and in compliance with the UK GLP Regulations 1999, Statutory Instrument No. 3106 as amended by the GLP (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on GLP (January 1998) ENV/MC/CHEM (98) 17. Animals were maintained as required by the "Code of practice for the housing and care of animals used in scientific procedures" (Home Office, London, 1989).

Redox potential measurement: One-electron reduction potentials were determined by pulse radiolysis following an established procedure [53].

Results

Anti-parasitic activity

The anti-parasitic activity of fexinidazole was assessed in experimental models of HAT. *In vitro* fexinidazole and its two main metabolites showed trypanocidal activity against the STIB900 laboratory strain of *T. b. rhodesiense* with very steep dose-response relations when assessed after 72 h of culture (Figure 1). With an IC_{50} of 0.48-0.82 μg/mL fexinidazole's *in vitro* potency is weaker than that of the reference drug melarsoprol (IC_{50}=0.003 μg/mL) and other trypanocidal drugs (Table 2) or the abandoned drug candidate megazol (IC_{50}=0.02 μg/mL), although the two drugs currently used as first line to treat stage 2 HAT have a similarly modest *in vitro* potency (eflornithine: 0.9 μg/mL ; nifurtimox: 0.4 μg/mL). Importantly, in contrast to melarsoprol and other drugs, fexinidazole has little or no non-specific cytotoxicity. Fexinidazole has a comparable IC_{50} of 0.16-0.36 μg/mL against a laboratory *T. b. gambiense* strain (STIB930), and against 6 recent *T. b. gambiense* clinical isolates (IC_{50} values from 0.30 to 0.93 μg/mL) (data not shown).

In vivo, fexinidazole is effective in curing both *T. b. rhodesiense* and *T. b. gambiense* acute models of infection, at an oral dose of 100 mg/kg/day (or 50 mg/kg twice a day) for 4 days (Table 3A). Most significantly, in a *T. b. brucei* GVR35 infected mouse model of stage 2 HAT with brain infection involvement, fexinidazole given orally showed a dose-related increase in efficacy, with a dose of 200 mg/kg/day for 5 days being highly effective (Table 3B). In two other independent experiments, 100% cure was obtained in groups of 5 mice receiving an oral dose of 100 mg/kg, twice per day for 5 days (in these experiments, five daily intraperitoneal injections of 15 mg/kg melarsoprol also cured 100%). Of the drugs currently in clinical use (Table 1), only melarsoprol is effective in this experimental stage 2 HAT model.

Figure 1. Effect of fexinidazole and its two main metabolites on T. b. rhodesiense (STIB 900).Parasite viability was measured in vitro after 72-h drug exposure. Fexinidazole - open circles (n=11). Fexinidazole sulfoxide - open squares (n=4). Fexinidazole sulfone - open diamonds (n=4).

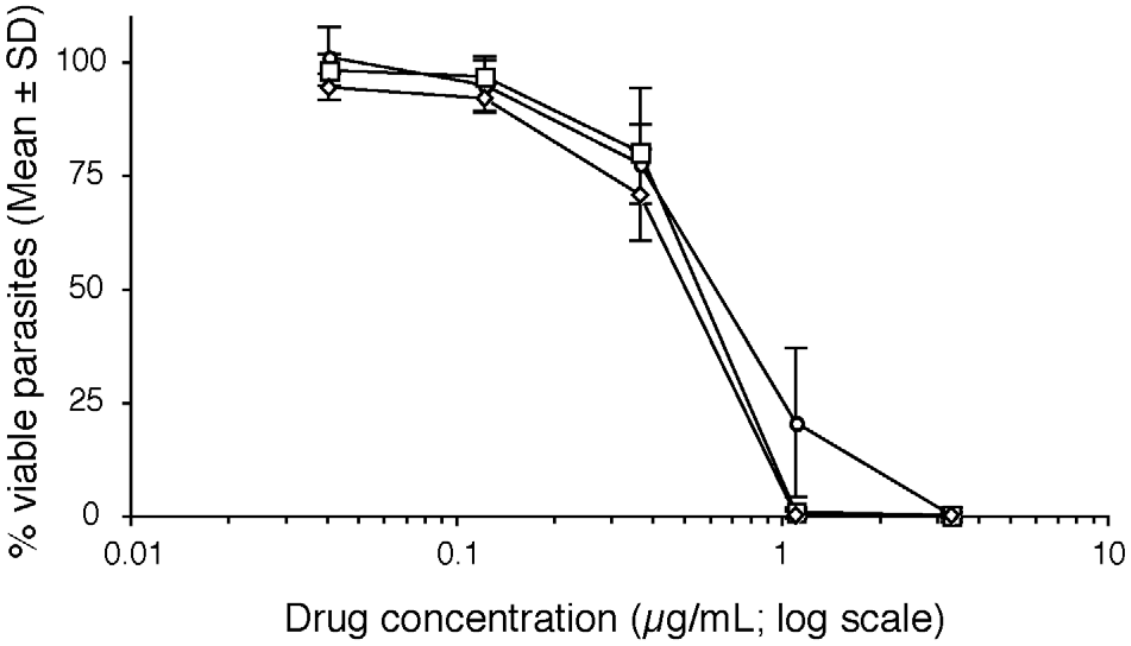


Table 2. *In vitro* anti-parasitic activity of fexinidazole, its metabolites, and reference compounds.

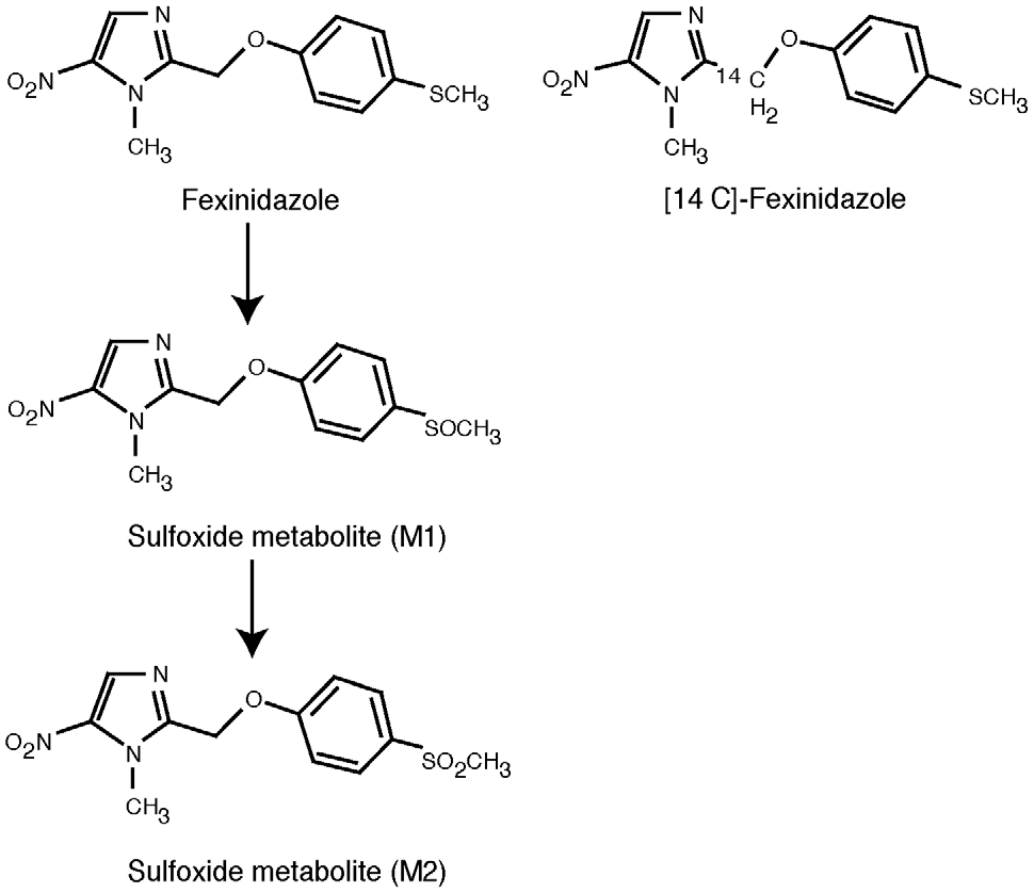
Compound	***T. b. rhodesiense*** (STIB 900)	***T. b. gambiense*** (STIB 930)	**Cytotoxicity** L-6 rat myoblast cells
	IC_{50} in µg/mL (µM)*		
Fexinidazole	0.48-1.12 (1.71-4.00)	0.16–0.36 (0.58–1.29)	>90 (>322)
Fexinidazole sulfoxide	0.41-0.49 (1.33-1.65)	0.18–0.36 (0.61–1.22)	> 90 (>305)
Fexinidazole sulfone	0.35-0.40 (1.14-1.30)	0.16–0.39 (0.48–1.25)	> 90 (>289)
Reference molecules			
Melarsoprol	0.002–0.004 (0.004-0.009)	0.0015-0.003 (0.004-0.006)	1.3 (3.3)
Megazol	0.02 (0.10)	Not available	57 (254)
Eflornithine	0.90 (3.80)	0.40 (1.67)	12 (51)
Nifurtimox	0.41 (1.44)	0.31 (1.08)	25 (87)
Pentamidine	0.003 (0.009)	0.002 (0.01)	3 (9)
Suramin	0.062 (0.046)	Not available	>90 (>70)

* IC_{50}: concentration of drug required to kill 50% of the parasites or skeletal myoblast cells.

ADME and Pharmacokinetics (PK)

Fexinidazole is rapidly metabolised in vivo, with the main metabolites being the sulfoxide and sulfone derivatives (Figure 2) [26]. This principle metabolic conversion was confirmed in vitro using rat S9 fractions and hepatocytes from the mouse, rat, dog, monkey and human (results not shown). In a comparative hepatocyte metabolism assay, fexinidazole was rapidly metabolised by all species, with in vitro intrinsic clearance rates highest in monkey (6500 mL/min/kg) > dog (5000 mL/min/kg) > mouse (4300 mL/min/kg) > rat (2900 mL/min/kg) > human (125 mL/min/kg). No meaningful differences were observed

Figure 2. Chemical structure of fexinidazole and its main metabolites [26], including 14C-labeled fexinidazole indicating which carbon atom was labelled.



when comparing the in vitro metabolism of fexinidazole by hepatocytes from African-American or Caucasian donors. The main metabolic reactions were oxidation to the

sulfoxide and sulfone derivatives. This metabolic pathway was also confirmed to be the major route of metabolism in vivo in mice, rats and dogs (see below). As shown above, both metabolites have in vitro anti-trypanosomal activity similar to the parent compound (IC50 in μg/ml: 0.41-0.49 for the sulfoxide and 0.35-0.40 for the sulfone versus 0.48-1.12 for the parent compound) (Table 2).

The potential hepatic oxidative pathways involved in fexinidazole metabolism were assessed by testing the clearance of the fexinidazole and its two primary metabolites by a range of cytochrome P450 (CYP450) enzymes. The data show that fexinidazole is extensively metabolised by a range of CYP450 enzymes, including 1A2, 2B6, 2C19, 3A4, and 3A5 and, to a lesser extent, 2D6. The 2C8 and 2C9 enzymes were inactive.

Table 3. In vivo efficacy of fexinidazole experimental infection models for acute and chronic HAT.

	Compound	**Dose (mg/kg)**	**Route***	**Cured/infected**	**Mean relapse time (days)**
A	*T. b. rhodesiense* STIB900 (acute infection)				
	No treatment	..	..	0/4	7
	Fexinidazole	25 × 4 days	po	0/4	12
	Fexinidazole	50 × 4 days	po	1/4	>27
	Fexinidazole	100 × 4 days	po	4/4	>60
	T. b. gambiense 130R (acute infection)				
	No treatment	..	..	0/4	10
	Fexinidazole	100 × 4 days	po	3/3	>90
	Melarsoprol	4 × 4 days	ip	4/4	>90
B	*T. b. brucei* GVR35 (chronic infection)				
	Diminazene^{+}	40 × 1 day	ip	0/4	48.6
	Fexinidazole	50 × 5 days	po	0/8	41.3
	Fexinidazole	100 × 5 days	po	2/8	>82.1
	Fexinidazole	200 × 5 days	po	7/8	>163.8
	Melarsoprol	10 × 5 days	ip	2/8	>96.6
	Melarsoprol^{++}	15 × 5 days	ip	4/5	>180

*ip: intraperitoneal; po: per os. Fexinidazole was formulated as a suspension in 5% Tween80/95% methyl-cellulose (0.5% w/v in water) and administered via gastric gavage. $^{+}$Diminazine diaceturate is used as a control as it is able to eliminate bloodstream parasitaemia but is not effective after CNS infection is established. Single dose on day 21 after infection. $^{++}$Data included from a separate experiment for illustration only.

Interestingly, none of the enzymes tested metabolised either the sulfoxide or the sulfone to any significant degree (their metabolic pathways remain to be established). These data are in agreement with *in vivo* data showing the long systemic half-lives of the sulfoxide and sulfone metabolites in animal studies (see below). Since fexinidazole is metabolised extensively by multiple CYP450 isoforms, its metabolism is unlikely to be significantly affected by other drugs.

The oral absorption potential of fexinidazole was assessedin the well-known Caco-2 cell model for intestinal epithelial permeability [36, Dataset S4]. In this assay, fexinidazole showed high absorption potential (apparent permeability P_{app}= 57.2 10^{-6} cm/s and no significant efflux). Intestinal permeability of fexinidazole is therefore not expected to be a limiting factor for absorption in humans.

The PK profile of fexinidazole was assessed in single-dose and multiple-dose studies in mice, rats, and beagle dogs. The absolute bioavailability of oral fexinidazole was 41% in mice, 30% in rats, and 10% in dogs. In all species tested, fexinidazole was rapidly and extensively metabolised to the sulfoxide and subsequently sulfone derivatives. Key pharmacokinetic parameter after oral absorption in mice for fexinidazole and its sulfoxide and sulfone metabolites are shown in Table 4 [Dataset S5]. Essentially similar PK profiles were observed in rats and dogs [Dataset S6, S7], even if the exact values varied among species (see also below).

Table 4. Mouse pharmacokinetics of fexinidazole and its metabolites in plasma and brain after oral administration.

	C_{max} (ng/mL)	**T_{max} (h)**	**$T_{1/2}$ (h)**	**AUC_{0-24} (h.ng/mL)**
Fexinidazole	500	0.25	0.8	424
Sulfoxide metabolite	14171	0.5	1.0	45031
Sulfone metabolite	13651	4.0	1.7	96286

Fexinidazole was formulated as a suspension in 1% DMSO/99% methyl-cellulose (1% w/v in water) and administered to female NMRI mice (n = 3) via gastric gavage at a concentration of 25 mg/kg.
Cmax: maximum plasma concentration. Tmax: time of maximum plasma concentration. T1/2: terminal elimination half life. AUC0–24: area under curve from time of dosing to the last measurable concentration.

The ability to cross the blood-brain barrier is crucial for drugs intended to treat stage 2 HAT. The ability of fexinidazole to do so was initially assessed *in vitro* in a MDR1-MDCK model [37, Dataset S8]. Fexinidazole showed high predicted brain permeation (apparent

permeability P_{app}= 60.6 10^{-6} cm/s and no significant efflux). In mice, the presence of fexinidazole and both metabolites in the brain was confirmed after oral dosing (Table 5 Dataset S5), and is consistent with the data showing efficacy in the murine model of chronic HAT (Table 3B).

The PK profile of fexinidazole was further characterised in mice which were administered the same treatment schedule that was curative in the chronic disease model (Table 3B). The plasma profile in mice of fexinidazole and its sulfoxide and sulfone metabolites after 5 days of fexinidazole treatment at the effective dose (200 mg/kg/day) is illustrated in Figure 3. The data show that a high and prolonged systemic bioavailability of biologically active compounds is achieved a few hours after drug administration, seemingly without drug accumulation and associated potential toxicity [Dataset S9].

Table 5. Presence of fexinidazole and metabolites in the brain after oral administration of fexinidazole to mice.

Time point (min)	Fexinidazole (ng/g)	Sulfoxide (ng/g)	Sulfone (ng/g)
15	1136 ± 54.1	ND	ND
30	800 ± 92.6	3315 ±1611	469 ± 222
60	763 ± 90.7	4873 ± 2335	1183 ± 322

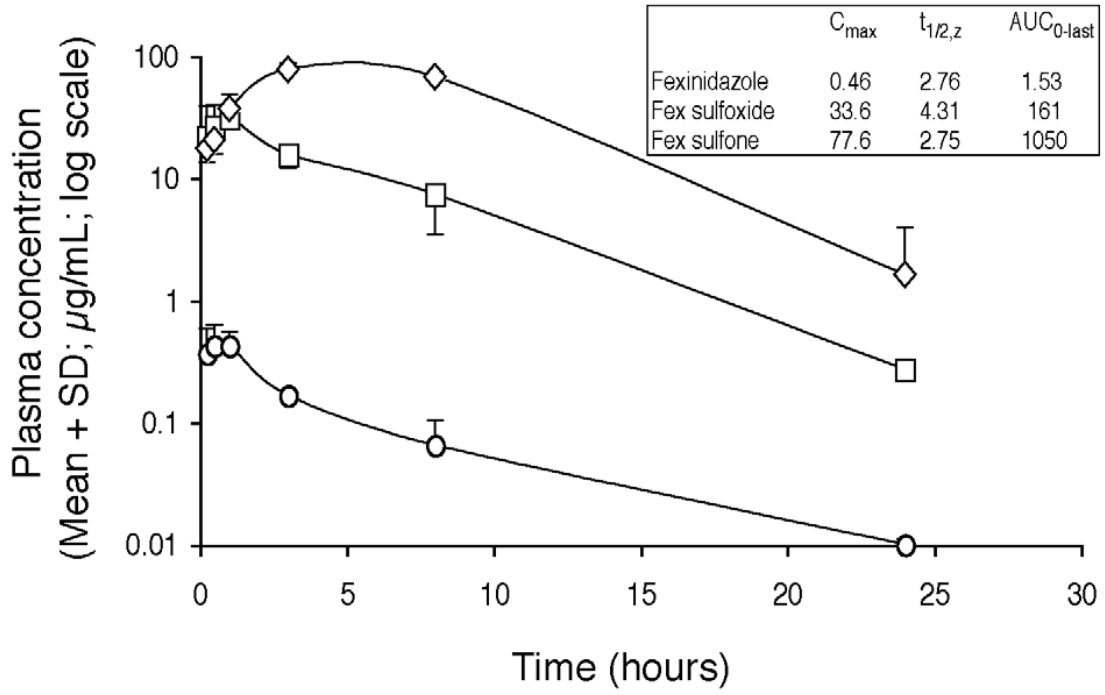
Data are expressed as mean 6 SD (n = 3). ND: not determined. Fexinidazole was formulated as a suspension in 1% DMSO/99% methyl-cellulose (1% w/v in water) and administered to female NMRI mice via gastric gavage at a concentration of 25 mg/kg.

The PK profile of fexinidazole was further characterised in mice which were administered the same treatment schedule that was curative in the chronic disease model (Table 3B). The plasma profile in mice of fexinidazole and its sulfoxide and sulfone metabolites after 5 days of fexinidazole treatment at the effective dose (200 mg/kg/day) is illustrated in Figure 3. The data show that a high and prolonged systemic bioavailability of biologically active compounds is achieved a few hours after drug administration, seemingly without drug accumulation and associated potential toxicity [Dataset S9].

This pattern of parent and metabolite plasma profiles without significant drug accumulation is further illustrated in Tables 6 and 7, which show plasma PK parameters in Sprague-Dawley rats and beagle dogs after 1 and 14 days of daily oral dosing with fexinidazole (data taken from the 28-day toxicokinetics studies, see below).In both species, it is interesting to note that there is no apparent accumulation in the plasma of either parent drug or metabolites, irrespective of dose, at least during the treatment period of 1–14 days. In the

dog, and to some extent in the rat, the only difference seen between the data from day 1 versus day 14 is that the T_{max} for the sulfone metabolite occurs some hours earlier on day 14 compared to day 1, although the overall amount of the metabolite in plasma is similar on both days.

Figure 3. Plasma concentrations of fexinidazole and its two main metabolites after 5 days of oral administration. 200 mg/kg fexinidazole was administered to mice (n = 3). Fexinidazole - open circles. Fexinidazole sulfoxide - open squares. Fexinidazole sulfone – open diamonds.



Whole-body autoradiography in rats using [^{14}C]-radiolabelled fexinidazole (see Figure 2 for labelling site) showed that the parent drug and/or its metabolites are broadly distributed to all organs and tissues (the assay did not distinguish between fexinidazole and its metabolites), with peak concentrations in most tissues 2 h after oral dosing. After 48 h, most radioactivity was eliminated from the body and no tissue specific accumulation was noted [Datasheet S10]. Furthermore, radioactivity was detected at all times in the brain, with a brain-to-blood concentration ratio of 0.4-0.6.

Excretion balance studies in rats showed that 30% and 59% of fexinidazole-related material was excreted via urine and faeces, respectively, within 96 h [Dataset S11]. Elimination of

the radioactivity after oral dosing was rapid, with 84% eliminated within 48 h. About 1.4% of the dose was recovered from the carcass with an overall recovery of the total radioactivity of approximately 93%.

Table 6. Rat plasma pharmacokinetic parameters for fexinidazole and its metabolites after oral administration of fexinidazole.

Dose (mg/kg/day) (no. animals)	Sample day	C_{max} * (µg/mL)	t_{max} (h)	$AUC_{0\text{-}t(last)}$ (µg·h/mL)
Fexinidazole				
50 (6)	1	0.09 ± 0.07	1.42 ± 1.28	0.47 ± 0.34
	14	0.18 ± 0.14	1.92 ± 1.20	0.83 ± 0.54
200 (6)	1	0.38 ± 0.28	1.92 ± 1.20	2.16 ± 1.34
	14	0.52 ± 0.30	2.08 ± 1.11	3.02 ± 1.47
800 (6)	1	1.48 ± 0.71	1.58 ± 0.66	12.8 ± 4.10
	14	1.02 ± 0.87	3.17 ± 2.84	9.29 ± 5.20
Fexinidazole sulfoxide				
50 (6)	1	2.70 ± 1.40	2.50 ± 1.22	15.4 ± 9.28
	14	5.23 ± 3.28	2.17 ± 0.98	29.8 ± 19.4
200 (6)	1	11.4 ± 2.17	2.33 ± 0.82	85.9 ± 14.1
	14	15.8 ± 3.17	3.33 ± 1.03	118 ± 38.9
800 (6)	1	31.7 ± 3.74	4.67 ± 1.63	410 ± 101
	14	25.0 ± 8.99	2.67 ± 1.03	277 ± 160
Fexinidazole sulfone				
50 (6)	1	2.92 ± 2.26	7.33 ± 1.63	36.1 ± 28.6
	14	6.38 ± 2.30	6.00 ± 2.19	89.6 ± 34.1
200 (6)	1	9.29 ± 1.75	8.00 ± 0.00	126 ± 17.5
	14	20.2 ± 2.42	7.33 ± 1.63	287 ± 36.5
800 (6)	1	42.6 ± 13.1	10.7 ± 6.53	574 ± 256
	14	40.5 ± 13.0	6.67 ± 2.07	543 ± 252

Data are expressed as mean 6 SD. Cmax: maximum plasma drug concentration achieved. Tmax: time to reach Cmax. AUC0-t(last): area under the plasma concentration time curve from initial to final data point

Safety pharmacology

In regulatory safety pharmacology assessments, *in vitro* exposure of hERG-transfected HEK 293 cells to fexinidazole sulfone, but not fexinidazole or the sulfoxide, showed a statistically significant decrease of 33% on hERG peak tail current at the highest of the 3 doses tested (30 µM, 9.34 µg/mL: no effect at 1 or 5 µM) [Dataset S12]. However, assessment of cardiovascular parameters in beagle dogs after single oral doses up to 1000 mg/kg showed no meaningful effects on blood pressure, heart rate, and ECG intervals,

including the Q-T interval. Similarly, no meaningful effects were observed after single oral doses in rats of up to 1000 mg/kg on general behaviour and body temperature (modified Irwin's test) or on respiratory parameters [Datasets S14, S15].

Repeated dose toxicity and reproductive toxicity

Because fexinidazole treatment for HAT is expected to be a single regimen of 14 days or less, 28-day regulatory toxicokinetic studies were carried out in rats and dogs.

Once daily oral fexinidazole doses of 50, 200 and 800 mg/kg/day were well tolerated in rats at all doses tested [Dataset S16]. Only a minimal-to-slight decrease in food consumption and in the expected body weight increases (due to normal growth) was observed at 200 and 800 mg/kg, in male animals only. Minimal-to-moderate changes were observed in the liver of all fexinidazole-treated animals (increased liver weight and/or hypertrophy of the centrilobular hepatocytes). However there was no increase in liver enzymes including AST and ALT, and all other clinical pathology parameters were also normal. Taken together with the observation that these changes were restricted to the dosing period, these were considered of adaptive origin (metabolism) and not indicative for liver toxicity. The No Observed Adverse Event Level (NOAEL) in rat was therefore 200 mg/kg/day.

In Beagle dogs, daily oral fexinidazole doses of 50, 200 and 800 mg/kg/day were also well tolerated [Dataset S17]. Slight-to-moderate body weight loss and reduction in food intake were observed at 800 mg/kg/day during treatment. A minimal-to-slight decrease in the number of lymphocytes was seen at the highest dose. The No Observed Adverse Event Level (NOAEL) in dog was also set at 200 mg/kg/day.

In both rat and dog studies, plasma levels of fexinidazole and both metabolites were measured and showed that fexinidazole was adequately absorbed, resulting in a significant and prolonged exposure of especially fexinidazole sulfoxide and sulfone (data up to 14 days of treatment in rats and dogs is shown in Table 6 and 7).

Preliminary studies on the potential effects of fexinidazole on embryo-foetal and early postnatal development were carried out in pregnant rats and no adverse effects on embryos/foetuses, parturition, and neonates were identified in dams. Further standard development and reproductive toxicology (DART) studies are currently ongoing to confirm and extend the preliminary results.

Table 7. Dog plasma pharmacokinetic parameters for fexinidazole and its metabolites after oral administration of fexinidazole.

Dose (mg/kg/day) (no. animals)	Sample day	C_{max} * (µg/mL)	t_{max} * (h)	$AUC_{0\text{-}t(last)}$* (µg·h/mL)
Fexinidazole				
50 (6)	1	0.04 ± 0.01	0.75 ± 0.61	0.19 ± 0.13
	14	0.03 ± 0.01	1.08 ± 0.74	0.15 ± 0.13
200 (6)	1	0.07 ± 0.03	1.00 ± 0.55	0.44 ± 0.09
	14	0.08 ± 0.01	1.50 ± 0.77	0.45 ± 0.14
800 (10)	1	0.14 ± 0.07	1.15 ± 0.47	0.84 ± 0.32
	14	0.14 ± 0.05	1.20 ± 0.42	1.05 ± 0.30
Fexinidazole sulfoxide				
50 (6)	1	3.76 ± 0.97	1.17 ± 0.41	19.6 ± 5.68
	14	2.99 ± 0.98	1.33 ± 0.52	13.6 ± 5.99
200 (6)	1	8.12 ± 2.66	1.25 ± 0.61	51.4 ± 14.1
	14	8.99 ± 2.83	2.00 ± 0.00	56.7 ± 16.6
800 (10)	1	14.5 ± 3.95	1.55 ± 0.60	112 ± 31.8
	14	13.6 ± 4.09	1.60 ± 0.97	129 ± 39.8
Fexinidazole sulfone				
50 (6)	1	8.58 ± 2.15	7.33 ± 1.63	146 ± 38.3
	14	7.78 ± 2.89	5.33 ± 2.07	126 ± 59.1
200 (6)	1	17.6 ± 2.46	12.7 ± 8.91	348 ± 63.9
	14	21.8 ± 3.86	5.33 ± 2.07	384 ± 49.9
800 (10)	1	36.1 ± 7.94	14.4 ± 8.26	660 ± 164
	14	36.6 ± 6.24	6.80 ± 1.93	653 ± 118

Data are mean±SD.
*C_{max}: maximum plasma drug concentration achieved. T_{max}: time to reach C_{max}.
$AUC_{0\text{-}t(last)}$: area under the plasma concentration time curve from initial to final data point

Genotoxicity

Fexinidazole and its primary metabolites are nitroimidazoles and, like many other nitroheterocyclic compounds, are potentially mutagenic [48]. To evaluate bacterial mutagenicity, a standard full Ames test was carried out on five strains of *Salmonella typhimurium*, with and without rat liver microsomes [Dataset S18]. Fexinidazole elicited both frameshift and base substitution mutations. However, this activity was significantly reduced or abolished when nitroreductase-deficient *Salmonella* strains were used for the assay (representative example shown in Figure 4). Rat liver microsomes metabolise fexinidazole efficiently to the sulfoxide metabolite under these experimental conditions (data not shown), so mutagenicity of this metabolite is covered by the above data. A

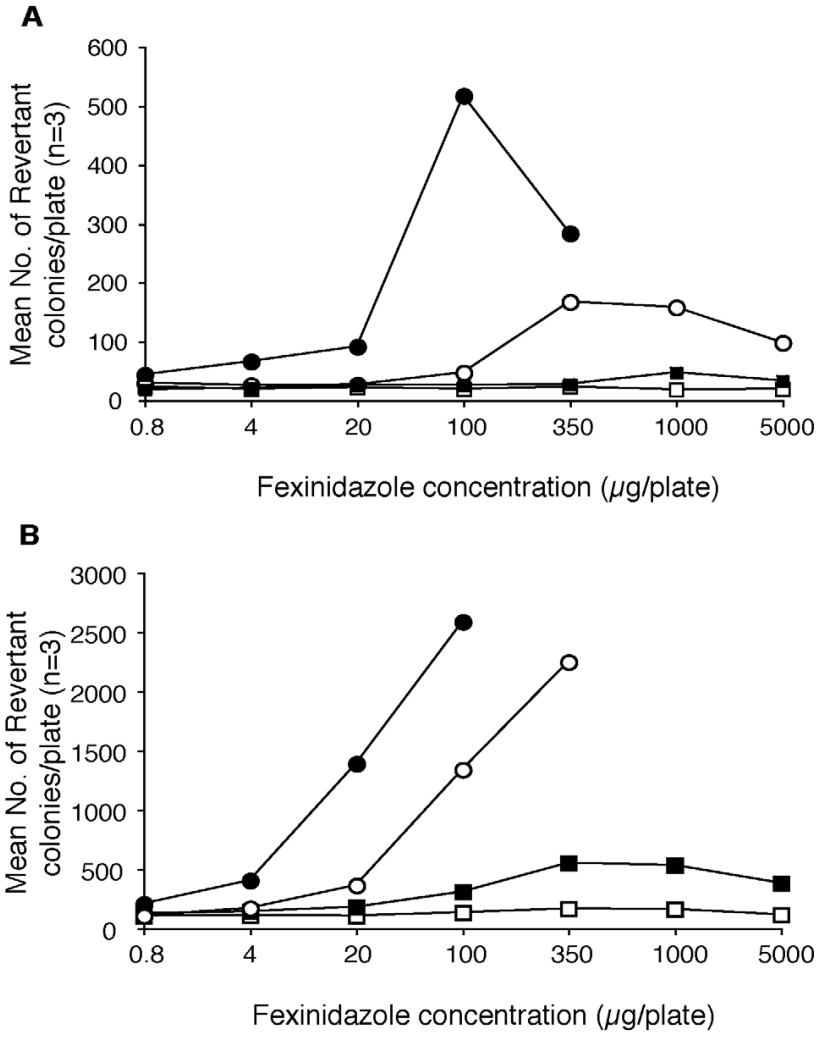
separate Ames test of the sulfone metabolite gave similar results to fexinidazole (data not shown). These data suggest that the observed mutagenic activity is due to bacterial activation of fexinidazole and its metabolites by nitroreductases, and is not an inherent property of the compounds. A detailed analysis of fexinidazole's genotoxic potential on mammalian systems was undertaken subsequently. First, genotoxicity in mammalian cells was evaluated in an in vitro micronucleus test using human peripheral lymphocytes [Dataset S19]. Fexinidazole did not induce the formation of micronuclei, and thus no clastogenic damage, either in the presence or absence of rat liver microsomal enzymes (Table 8A). A separate in vitro micronucleus assay of the sulfone metabolite was also negative (data not shown). An *in vivo* bone-marrow micronucleus test in mice administered high oral doses of fexinidazole (up to 2 g/kg) confirmed the lack of clastogenicity (Table 8B Dataset S20), while plasma analysis of these mice confirmed the exposure to fexinidazole and its two major metabolites (data not shown). Finally, an *ex vivo* rat liver unscheduled DNA synthesis study (Table 8C) confirmed the lack of mammalian genotoxic activity for fexinidazole and its metabolites [Dataset S21]. Taken together, these data support the conclusion that fexinidazole does not pose a genotoxic risk to patients.
No direct studies have been done on the mode of action of fexinidazole. However, fexinidazole might act as a prodrug like other 5-nitroimidazoles that are toxic to the parasites only after bioreductive activation [54]. From studies of trypanosomes resistant to the action of nitroimidazoles, it appears that these parasites have bacterial-like nitroreductases, which can activate nitroimidazole drugs into reactive intermediates that in turn cause cellular damage [55]. Fexinidazole and the sulfoxide and sulfone metabolites were shown to have a low single electron redox potentials being -511 mV, -493 mV, and -488 mV, respectively. In the same study, the single electron redox potential of metronidazole was -516 mV, and of megazole was -422 mV.

Discussion

This paper provides data showing that fexinidazole, a 2-substituted 5-nitroimidazole identified among a series of existing but long forgotten compounds, is a promising drug candidate for HAT. A full set of preclinical studies have been conducted in accordance with the regulatory requirements for pharmaceuticals for human use, and fexinidazole has now

successfully entered phase I clinical trials. Fexinidazole is the first new drug candidate for 30 years that is in clinical development for the advanced and fatal stage of the disease (stage 2). In addition, being an oral drug with the potential to be effective against both stage 1 and stage 2 HAT, it could become the much needed breakthrough for HAT control by drastically simplifying case management.

Figure 4. Mutagenic activity of fexinidazole in the Ames test. Salmonella typhymurium strains TA98 (A) and TA100 (B) and their nitroreductase-deficient variants TA98NR and TA100NR were used, in the presence and absence of metabolic activation (+/2 S9). A: Solid circles: TA98 +S9; Open circles: TA98 -S9; Solid squares: TA98NR +S9; Open squares: TA98NR -S9; Negative control: Mean number of revertants per plate were TA98 (2S9): 21; TA98 (+S9): 34; TA98NR (2S9): 29; TA98 (+S9): 18. B: Solid circles: TA100 +S9; Open circles: TA100 2S9; Solid squares: TA100NR +S9; Open squares: TA100NR 2S9; Negative control: Mean number of revertants per plate were TA100 (2S9): 104; TA100 (+S9): 116; TA100NR (2S9): 90; TA100NR (+S9): 111.



Fexinidazole has been shown to be selectively trypanocidal *in vitro* on *T. b. rhodesiense* and *T. b. gambiense* parasites, both on established laboratory strains and recent clinical isolates. Whilst *in vitro* potency is modest, with IC_{50} values between 0.1 and 0.8 µg/mL, a short course (4 or 5 days) of oral fexinidazole treatment is curative in experimental mouse models of acute and chronic (stage 2) HAT at doses of 100-200 mg/kg/day. This would correspond to a daily human equivalent dose (HED) for adults of 16 mg/kg calculated based on body surface area [56]; however, more detailed mouse pharmacodynamics studies are required together with human PK data to be able to propose an effective therapeutic dose, including durationThe experimental curative capacity of fexinidazole is significant as amongst the currently used drugs in the clinic, only the highly toxic drug melarsoprol is curative in the chronic mouse model which involves an established brain infection that mimics stage 2 HAT. The observation that a single high dose of fexinidazole was also partially curative in the acute model (data not shown) underscores the potential for a short course treatment which will be critical to achieve an easy-to-use treatment for remote and rural areas. While the predictive value of these murine models in terms of the potential for curing stage 2 patients is not fully established (only melarsoprol cures both), the demonstration that a drug candidate can clear systemic trypanosome infections in both the acute and chronic model, as well as clearing the brain infection (no relapse in the chronic model), is widely considered as the critical feature for a stage 2 HAT drug candidate.

It has been argued by some that obtaining data from other animal models (rat, monkey) before moving into clinical development is desirable. However, the urgency to find new drugs for HAT combined with the lack of clinical candidates in the pipeline warrants a bolder strategy. Moreover, as fexinidazole's in vivo efficacy is likely to depend on the combined exposure profile of the parent drug and its two major metabolites, and knowing that metabolism can vary between species, it is uncertain what can be learned from additional animal disease models. Clearly, the critical studies ahead to determine the curative potential of fexinidazole in humans will be the human safety and PK studies in phase I, and subsequently a proof-of-concept phase II study in patients.

Upon oral administration, fexinidazole is well absorbed and rapidly metabolised into the sulfoxide and sulfone derivatives, both of which have similar *in vitro* trypanocidal activity to the parent compound. The excellent *in vivo* activity of fexinidazole when administered

Table 8. Mutagenicity assessments of fexinidazole on mammalian cells.

A. *In vitro* micronucleus assay on human lymphocytes

Micronucleated binucleate cells (%)*				
	24-h PHA		**48-h PHA**	
Fexinidazole dose (μg/mL)	**- S9**	**+S9**	**-S9**	**+S9**
0	0.75	0.40	1.1	0.90
20	0.50	0.50	1.1	0.60
40	0.40	0.55	0.60	1.20
80	0.70	0.75	0.90	0.40
**4-nitroquinoline N-oxide, 5.0 μg/mL	10.80		8.95	
**Cyclophosphamide, 6.25 mg/mL		3.60		12.85

B. *In vivo* bone marrow micronucleus assay

Fexinidazole dose (mg/kg)	**Micronucleated PCE/1000**	**PCE/NCE**
0	1.0	0.75
500	1.4	0.82
1000	1.1	0.75
2000	1.8	0.89
**Cyclophosphamide, 4.0 mg/kg,	19.3	0.89

C *Ex vivo* unscheduled DNA synthesis assay on rat liver cells

Fexinidazole dose (mg/kg)	**Net nuclear grain count (2–4 h)**	**Net nuclear grain count (12–14 h)**
0	0.2	0.6
500	0.0	0.4
1000	-0.1	0.5
2000	-0.4	0.2
**dimethylnitrosamine, 10 mg/kg	8.9	
**2-acetamidofluorene, 75 mg/kg		9.8

*Relative Replication Index (RI: relative number of nuclei compared to controls) expressed as a %.
**positive control.
PCE: poly-chromatic erythrocyte. NCE: normo-chromatic erythrocyte. PHA: phytohemagglutinin.

orally is likely to be due to the cumulative exposure to not one but three active compounds which distribute throughout the body with different but overlapping kinetics, thus ensuring

effective exposure in both the systemic circulation and the brain. In mice, rats and dogs, the half-life of fexinidazole after oral treatment ranges from 1-3 h, whilst the half-life of the sulfoxide ranges from 2-7 h and that of the sulfone can be up to 24 h after dosing. As the *in vitro* intrinsic clearance rate by human hepatocytes was lower than of all other species tested, it can be expected that the half lives in humans will be even longer which further supports fexinidazole's potential for a once per day short-duration treatment schedule. On the other hand, a non-linear dose-related absorption and consequent exposure was observed in both rats and dogs (not done in mice). It will thus be important to carefully analyse the dose-related pPK of fexinidazole and both metabolites after oral dosing in humans to better predict the dose-response relationship.

While fexinidazole and the sulfoxide are metabolised by multiple liver microsomal enzymes, suggesting a low risk for drug-drug interactions, the metabolic route of the sulfone remains to be established. No accumulation of either fexinidazole or the primary metabolites was found in rats and almost all drug-related material was eliminated from the body within 48 h of oral dosing, excreted mainly through faeces (59%) and urine (30%). The distribution of fexinidazole and metabolites to the brain was confirmed in mice and rats, and, considering the lipophilicity of the molecules ($logD_{pH\ 7.4}$ 2.83 [fexinidazole], 0.74 [sulfone], 0.52 [sulfoxide]), there is no reason to assume that the brain penetration potential, critical for the efficacy in stage 2 HAT, would be different in humans.

A full regulatory toxicology package has been conducted, including safety pharmacology (respiratory, cardiovascular, and general behaviour) and 4-weeks repeated-dose toxicokinetics studies in rat and dog. Overall, fexinidazole was well tolerated, with no specific issues of concern or target organs for toxicity identified. Fexinidazole is positive in the classical *in vitro* Ames test, but this effect is highly dependent on the presence of bacterial nitroreductases. A carefully designed set of *in vitro* and *in vivo* assays to detect possible signals of mammalian genotoxicity remained negative.

While a clearly positive Ames test result has long been considered a no-go for drug development (except for terminal diseases) as it would indicate a possible risk for (human) carcinogenicity, bacterial mutagenicity is not necessarily a relevant indication for mammalian genotoxicity, when bacterial specific metabolism is involved, especially with certain compound classes such as nitroimidazoles [57]. In fact several examples exist of nitroaromatic drug candidates currently in development for diseases requiring a much

longer treatment than HAT, for instance epilepsy and tuberculosis, in which either the positive Ames test was not considered decisional to indicate a hazard to patients or no bacterial mutagenicity was detected [58,59,60]. Instead, a carefully-designed series of *in vitro* and *in vivo* mammalian genotoxicity assays can be used to rule out the different possible mechanisms of mutagenicity that would indicate a risk for genotoxic-related carcinogenicity. The observation by us and others that it is possible to select non-mammalian mutagenic compounds within the nitroheterocycles family reopens the potential for the further use of this family of compounds with well-known anti-infective properties.

It is important to emphasize that the observed positive Ames results in nitroreductase-containing tester strains in no way point to a residual risk for carcinogenicity not captured by the detailed *in vitro* and *in vivo* mammalian genotoxicity studies as performed with fexinidazole. In contrast to what is often assumed, the *in vitro* micronucleus test measuring chromosome damage is no less sensitive as a screening test than the Ames test, even if it involves larger scale genetic damage than bacterial point mutations [61]. Although there are a few documented examples of genotoxic carcinogens that can induce chromosome damage but not bacterial point mutations (e.g. arsenic), there are our knowledge, no examples of genotoxic carcinogens that induce bacterial point mutation but not chromosome damage.

It has also been argued that gut flora contains bacterial nitroreductases, which could convert nitroaromatics into mutagenic species, much like what is observed in the *in vitro* Ames test and thus still present a genotoxic risk *in vivo*. A recent study of AMP397, a nitroaromatic compound previously in clinical development for epilepsy has attempted to address the issue of potential generation of gut-bacteria derived mutagens [58]. This compound has a similar profile to fexinidazole, with positive Ames test results in standard strains and lack of activity in nitroreductase-deficient bacterial strains and in mammalian cell assays. Suter *et al*. carried out a mutagenicity study of AMP397 in vivo in the transgenic MutaMouse model using five daily doses at the maximum tolerated dose and sampling at 3, 7 and 31 days after treatment. No evidence of mutagenicity was seen in the colon or liver. Likewise, a comet assay (measuring DNA strand breakage) did not detect any genetic damage in the jejunum or liver of treated rats after dosing the animals at a dose six times higher than that possible in the mouse study. A radioactive DNA binding study also failed to show any DNA binding in rat liver. Thus, if a mutagenic metabolite was formed by intestinal

bacteria, it is unable to exert any genotoxic activity in adjacent intestinal tissue. As the genetic toxicology profile of fexinidazole is the same as AMP397 and the mechanism behind the bacterial specific mutation seen is the same, there is no reason to expect a different assessment regarding gut flora activation.

The mechanism of action of fexinidazole is not yet elucidated, but likely involves bioreductive activation. Fexinidazole and the sulfoxide and sulfone metabolites were shown to have a low single electron redox potentials (ranging between -511 and -488 mV). The nitroreductive enzymes present in mammalian cells can only reduce compounds with relatively high redox potentials under aerobic conditions. In contrast, bacterial nitroreductases such as those in the Salmonella assay can act at much lower redox potentials than equivalent mammalian systems. This gives a plausible explanation for the positive results in the standard Ames test and the reduced or abolished activity in nitroreductase-deficient strains. In line with these observations, it is of interest to note that the single electron redox potential of metronidazole was -516 mV, while megazole's is significantly higher at -422 mV.

The rediscovery of fexinidazole as a drug candidate also shows the success of the compound mining approach, during which a careful investigation of existing compounds within a family of known pharmacologically active compounds using state-of-the-art science, has yielded a new drug candidate for clinical development in a relatively short time. Starting the experimental work within this limited set of existing compounds in 2005 (around 700 compounds tested, mainly parasitology and genotoxicity assays), a preclinical candidate could be selected early 2007, and clinical trials initiated in the second half of 2009. Compared to drug discovery "from scratch", this represents a significant shortcut. It also shows that it is worthwhile to dig into past research efforts to find those potential drug candidates which are lingering in drawers or on shelves. In particular in the context of non-profit drug development such as for neglected diseases where the existence of patent is not considered a prerequisite for development, this compound mining strategy may be worthwhile to pursue more vigorously.

Based on the data presented in this paper fexinidazole has entered clinical development, and a phase I trial is currently ongoing to establish its PK and tolerability in healthy volunteers from African origin (in a combined single ascending dose and multiple

ascending dose study) [62]. If well tolerated, fexinidazole is expected to progress to phase II trials in patients with stage 2 HAT by the end of 2010.

If fexinidazole successfully completes clinical development, it will represent a real breakthrough for the control of HAT in rural Africa for several reasons. Fexinidazole would be the first oral drug for stage 2 HAT, well tolerated and effective upon a short course treatment. Compared to the current options of either 10 days of daily intravenous melarsoprol with its dreadful toxicity and waning efficacy, the very complicated eflornithine monotherapy (56 infusions over 14 days), or even the recent improvement NECT (a combination therapy of 10 days oral nifurtimox and 7 days of 12 hourly eflornithine infusions) this would be ground-breaking. Moreover, based on its simple chemistry and short synthesis, fexinidazole is expected to be relatively cheap (certainly not more than US$ 50 per treatment, likely significantly less). Furthermore, stability data to date show that the fexinidazole drug substance is very stable, which is a good starting point for the development of a stable solid dosage formulation for use in tropical climates. Finally and most significantly, it could be the first treatment to be used for both stage 1 and stage 2 HAT, thereby overturning the long-standing but complicated diagnosis and treatment paradigm which includes systematic lumbar punctures of every diagnosed patient to determine which stage of the disease they are in before deciding which treatment to prescribe (to avoid exposing a stage 1 patient to the risks and burden of the stage 2 treatments).

A safe, effective, cheap and easy to use treatment for both stage 1 and 2 HAT, ideally in combination with an easy field-diagnostic, would make HAT control a realistic option for the future. In contrast to the current diagnosis and treatments options which are largely dependent on vertical HAT control approaches, this safe, effective, easy to use stage 1+2 treatment could be integrated into more horizontal approaches which are more likely to reach the extremely poor and remote populations most affected by HAT. Clearly, there are many hurdles to overcome before fexinidazole can reach this target, but it surely is the most promising candidate in many years. A concerted effort to progress fexinidazole efficiently through clinical development and registration is warranted.

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Author Contributions

Conceived and designed the experiments: ET BBT DT MK RB GM MAB BP. Performed the experiments: BBT MK RB MAB. Analyzed the data: DT MK RB GM. Contributed reagents/materials/analysis tools: BBT. Wrote the paper: ET BBT MAB.

Competing Interest

DT, GM and MAB are paid consultants to DNDi. All other authors declare that they have no competing interests.

Supporting Information

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CHAPTER 3

Anti-trypanosomal activity of Fexinidazole – A New Oral Nitroimidazole Drug Candidate for the Treatment of Sleeping Sickness

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Abstract

Fexinidazole is a 5-nitroimidazole drug currently in clinical development for the treatment of human sleeping sickness (human African trypanosomiasis (HAT)) caused by infection with species of the protozoan parasite *Trypanosoma brucei*. The compound and its two principal metabolites the sulfoxide and sulfone have been assessed for their ability to kill a range of *T. brucei* parasite strains *in vitro* and to cure both acute and chronic HAT disease models in the mouse. The parent molecule and both metabolites have shown trypanocidal activity *in vitro* in the 0.7 – 3.3 μM range against all parasite strains tested. *In vivo* fexinidazole is orally effective in curing both acute and chronic disease in the mouse at doses of 100 mg/kg/day for 4 days and 200 mg/kg/day for five days respectively. Pharmacokinetic data indicate that it is likely that the sulfoxide and sulfone metabolites provide most if not all of the *in vivo* killing activity (33). Fexinidazole and its metabolites require up to 48 hours exposure in order to induce maximal trypanocidal efficacy *in vitro*. The parent drug and its metabolites show no *in vitro* cross reactivity in terms of trypanocidal activity with either themselves or other known trypanocidal drugs in use in man. The *in vitro* and *in vivo* anti-trypanosomal activity of fexinidazole and its two principal metabolites provides evidence that the compound has the potential to be an effective oral treatment for both the *T. b. gambiense* and *T. b. rhodesiense* forms of human sleeping sickness and both stages of the disease.

Introduction

Human African trypanosomiasis (HAT), also know as sleeping sickness, is caused by two subspecies of the protozoan parasite *Trypanosoma brucei* and is transmitted through the bite of infected tsetse flies. In west and central Africa *T. b. gambiense* is responsible for the chronic form of the disease whereas *T. b. rhodesiense* is responsible for a more acute form of the disease endemic in eastern Africa. Poor and neglected populations living in remote rural areas of sub-Saharan Africa are at risk for HAT and in 2006, it was estimated that 50 - 70,000 individuals were infected (35). In recent years the reported HAT cases have decreased to approximately 10,000 (29, 36) with over 95% of the reported cases due to *T. b. gambiense* infection.

There are four drugs currently registered for use against sleeping sickness. Pentamidine and suramin are used against the hemolymphatic stage (stage 1) of the disease whilst melarsoprol and eflornithine (DFMO) are used against stage 2 of the disease when the parasites have invaded the central nervous system (CNS). The disease is fatal if left untreated. The drugs currently in use are unsatisfactory due to cost, toxicity, poor oral bioavailability, long treatment and lack of efficacy. Melarsoprol treatment is highly toxic and up to 5% of the second stage patients treated with melarsoprol die of a reactive encephalopathy. Eflornithine treatment requires four daily intravenous infusions over fourteen days meaning that this therapy is expensive and logistically difficult in rural clinics. The only advance in the last twenty-five years has been the introduction of the eflornithine-nifurtimox combination therapy (NECT) (26). Despite the reduced toxicity and treatment duration of NECT when compared to melarsoprol or eflornithine, the requirements for seven days of intravenous administration is still a limitation.

The aim of the present study was to characterize the anti-trypanosomal activity of the 5-nitroimidazole drug candidate fexinidazole and its two principal metabolites fexinidazole sulfoxide and fexinidazole sulfone using phenotypic *in vitro* and *in vivo* screening. Fexinidazole is targeted for the treatment of HAT, currently in phase I clinical studies and had been in preclinical development as a broad spectrum antimicrobial agent during the 1970's when the *in vivo* efficacy in the *T. b. brucei* strain GVR35 mouse CNS model of HAT was first demonstrated (14).

Some of the data presented here have previously been published in summary form (33).

Materials and Methods

Materials. Fexinidazole (1-methyl-2-((p-(methylthio)phenoxy)methyl)-5-nitroimidazole) manufactured under GMP conditions (Axyntis), its sulfoxide and sulfone derivatives (1-Methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitro imidazole and 1-Methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitro imidazole) at laboratory grade (Axyntis) and nifurtimox (Bayer) were provided by DNDi, pentamidine isethionate and diminazene aceturate were purchased from Sigma-Aldrich; melarsoprol (Aventis) was provided by the WHO. The chemical structures of the experimental drug fexinidazole and the two metabolites fexinidazole sulfoxide and fexinidazole sulfone have been previously published (33).

All other reagents were of standard laboratory grade and purchased from commercial suppliers.

Preparation of compounds. For *in vitro* studies compounds were dissolved in 100% DMSO and finally diluted in culture medium prior to assay. The maximum DMSO concentration in the *in vitro* assays was 1%.

For *in vivo* studies, the compounds were dissolved in DMSO and further diluted with distilled water to a final DMSO concentration of 10%, unless stated otherwise. In some studies fexinidazole was prepared in an optimized suspension medium for oral administration comprising 5% w/v Tween 80/0.5% w/v Methocel in water which has previously been shown to maximize absorption of the drug (33).

Parasites and cell culture conditions. (i) *T. b. rhodesiense*: The STIB900 strain is a derivative of the STIB704 strain isolated from a patient in Ifakara, Tanzania, in 1982 (5). STIB900mel and STIB900pent are melarsoprol and pentamidine resistant lines, respectively which were generated by growing STIB900 in increasing sub-curative drug concentrations (3).

(ii) *T. b. gambiense*: The STIB930 strain is a derivate of the TH1/78E(031) strain isolated from a patient in Côte d'Ivoire in 1978 (9). The DAL 898R strain was also isolated from a patient in Côte d'Ivoire in 1985 (5).

T. b. gambiense strains 40R, 45R, 130R, 349Pi and 349R were all isolated from patients in the Democratic Republic of Congo in 2003-2004 (24). The K03048 strain was isolated from a patient in South Sudan in 2003 (20).

(iii) *T. b. brucei*: The strains used include BS221, a derivative of the S427 strain isolated in Uganda in 1960 (7); AT1KO, a P2 transporter knockout of the BS221 strain (21); STIB950mdr strain which is a derivative of the CP 2469 strain isolated in 1985 from a cow in Hakaka, Soakow District, Somalia (15). The GVR35 strain was isolated from a wildebeest in the Serengeti in 1966 (primary isolate S10) (13).

T. b. rhodesiense and *T. b. brucei* parasites were cultured at 37°C under a humidified 5% CO_2 atmosphere in Minimum Essential Medium (MEM) with Earle's salts, supplemented according to the protocol of Baltz et al. (2) with the following modifications: 0.2mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5mM hypoxanthine, and 15% heat-inactivated horse serum as supplement. *T. b. gambiense* strains were grown in HMI-9 medium (11) supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 5% human serum. To ensure maintenance of a log growth phase, parasites were sub cultured into fresh medium at appropriate dilutions every 2 to 3 days.

***In vitro* growth inhibition assays.** The compounds were tested in a serial drug dilution assay in order to determine the IC_{50} values (concentration of drug causing 50% growth inhibition) by using the Alamar Blue assay (27).

Serial drug dilutions were prepared in 96-well microtitre plates containing appropriate culture medium as described above for each parasite strain, and wells were inoculated with either 2,000 bloodstream forms for *T. b. rhodesiense* or *T. b. brucei* assay or 10,000 trypanosomes for *T. b. gambiense* assay. Cultures were incubated for 70 h at 37°C under a humidified 5% CO_2 atmosphere. After this time ten microliters of resazurin (12.5 mg resazurin [Sigma] dissolved in 100 ml phosphate buffered saline) was added to each well. The plates were incubated for an additional 2 to 4 h for *T. b. rhodesiense* and *T. b. brucei* and an additional 6-8 h for *T. b. gambiense* isolates. The plates were read in a Spectramax Gemini XS microplate fluorescence scanner (Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression (12) from the sigmoidal dose inhibition curves using SoftmaxPro software.

***In vitro* dynamic assays.** *T.b.rhodesiense* (STIB900) was seeded in clear 96-well V-bottom plates at a density of 10,000 parasites per well in 100 µl medium and incubated for 1, 6, and 24 h with serially diluted test compounds. One plate was prepared for each time point. At the designated time point a plate was spun at 650 rcf (relative centrifugal force) for 5 min to

sediment the parasites. The supernatant was removed and 100 µl of warmed MEM media was added to each well to resuspend the parasites. The wash process was repeated four more times. After the washing procedure the parasites were resuspended in 100 µl media and transferred into new culture plates and further incubated. After a total of 70 h incubation resazurin was added and the trypanocidal activity (IC_{50} and IC_{90} values) determined as described for the *in vitro* growth inhibition assays.

***In vitro* combination assays.** Drug combination studies were performed as previously described (10). Initially, the IC_{50} values of the test drugs alone were determined. Subsequently, drug solutions were diluted with culture medium to initial concentrations of 10 times the predetermined IC_{50} value. The solutions were combined in ratios of 1:3, 1:1, and 3:1. Single and combination drug solutions were then introduced into 96-well plates and the parasites cultured as described above. The IC_{50} values of the drugs alone and in combination were determined as described above. For data interpretation, the IC_{50} values of the drugs in combination were expressed as fractions of the IC_{50} values of the drugs alone. These data were expressed as fractional inhibitory concentrations (FIC) for drug A and drug B, respectively.

Isobolograms were constructed by plotting the FIC of drug A against that of drug B for each of the three drug ratios, with concave curves indicating synergism, straight lines indicating addition and convex curves indicating antagonism. To obtain numeric values for the interactions, results were expressed as the sum FICs (ΣFICs) of the FIC-A and FIC-B. Cutoff ranges were determined by mixing the same drug at various ratios and accounting for experimental variation. Changes in FIC values indicate the nature of the interactions as follows: $\Sigma FIC<0.5$ is synergism; ΣFIC 0.5 to 4.0 is indifferent, $\Sigma FIC>4$ is antagonism (8,23). Mean ΣFICs were used to classify the overall nature of the interaction.

***In vivo* experiments**. Adult female NMRI mice (Harlan Laboratories, The Netherlands) weighing between 20 and 25 g at the beginning of the study were housed under standard conditions with food pellets and water *ad libitum*. All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland.

***T. b. rhodesiense* (STIB900) acute mouse model.** The STIB900 acute mouse model mimics the first stage of the disease. Experiments were performed as previously described (32), with minor modifications. Female NMRI mice were infected intraperitoneally (ip)

with 10^4 *T. b. rhodesiense* (STIB900) bloodstream forms. Experimental groups of four mice were treated ip or orally (per os [po]) with compounds on four consecutive days from day 3 to 6 post infection. A control group was infected but remained untreated. The tail blood of all mice was checked for parasitemia up to 60 days post infection. Surviving and aparasitemic mice at day 60 were considered cured and were euthanized. The day of relapse of the animals was recorded (including the cured mice, as >60) and data expressed as the mean day of relapse (MRD).

***T. b. brucei* (GVR35) CNS mouse model.** The GVR35 mouse CNS model mimics the second stage of the disease. Five female NMRI mice per experimental group were inoculated ip with 2 x 10^4 *T. b. brucei* (GVR35) bloodstream forms. Treatment (i.p. or p.o.) with compound was given on five consecutive days from days 21 to 25 post infection. Some experimental groups were treated twice daily with a time interval of 7-8 h. In all experiments with fexinidazole a control group was treated on day 21 with a single intraperitoneal dose of diminazene aceturate at 40 mg/kg, which is sub-curative since it clears the trypanosomes only in the hemolymphatic system and not in the CNS, leading to a subsequent reappearance of trypanosomes in the blood (13). Parasitemia was monitored twice per week in the first five weeks after treatment followed by once a week up to 180 days post infection. Surviving and aparasitemic mice at day 180 were considered cured and were euthanized. The day of relapse of the animals was recorded (including the cured mice, as >180) to calculate the MRD.

Results

In vitro activity of fexinidazole and its primary metabolites against African trypanosomes

Fexinidazole and its sulfoxide and sulfone metabolites and the reference drugs melarsoprol, pentamidine, eflornithine, nifurtimox and the veterinary compound diminazene aceturate have been assessed for *in vitro* efficacy against *T. brucei* subspecies isolates (Table 1). Fexinidazole showed *in vitro* trypanocidal activity against all tested *T. brucei* subspecies and strains in the range of 0.7 – 3.3 μM (0.2 to 0.9 μg/ml). The fexinidazole sulfoxide and sulfone metabolites were slightly more potent but within the same order of magnitude as the parent compound. Fexinidazole and its sulfoxide and sulfone metabolites showed

comparable activity to eflornithine and nifurtimox but were considerably less potent than the three other drugs tested.

In vivo efficacy of fexinidazole in an experimental model of acute infection with African trypanosomes

Fexinidazole showed dose related efficacy in the *T. b. rhodesiense* (STIB900) acute mouse model at intra-peritoneal (ip) doses of 20 – 50 mg/kg/day and oral (per os (po)) doses of 25 – 100 mg/kg/day given on four consecutive days with 100 mg/kg/day po being 100% curative (Table 2). In a separate experiment the two fexinidazole metabolites were less potent than fexinidazole when administered ip or orally in the acute model of infection. Fexinidazole sulfoxide cured one out of four infected mice at a dose of 50 mg/kg/day ip and two mice at 100 mg/kg/day po. Fexinidazole sulfone was not effective at 50 mg/kg/day ip and cured one mouse at a dose of 100 mg/kg/day.

In vivo efficacy of fexinidazole in an experimental model for chronic infection with African trypanosomes, involving brain infection

Fexinidazole was shown to be effective in the GVR35 mouse model which mimics the advanced and fatal stage of the disease when parasites have disseminated into the brain (Table 3). At ip doses of 50 mg/kg given twice per day (bid) or po doses of 100 mg/kg also given twice per day for 5 consecutive days, all mice were cured; at single doses of 200 mg/kg/day po for five consecutive days 7 out of 8 mice were cured and at single doses of 100 mg/kg/day po 3 out of 5 mice (DMSO/water vehicle), and 2 out of 8 mice (Tween/Methocel vehicle) were cured, respectively. In another experiment using the same vehicle fexinidazole was compared to nifurtimox at the dose range of 50 - 200 mg/kg/day po given for five days. While fexinidazole resulted in partial cure at 100 mg/kg/day (2/8 mice cured) and almost complete cure at 200 mg/kg/day (7/8) (data from Ref. 33), nifurtimox had no curative effect at any dose tested. Significant levels of fexinidazole and the sulfoxide and sulfone metabolites can be detected in mice treated using the same protocol and assessed for plasma drug levels after day 5 (33). The plasma levels of both fexinidazole sulfoxide and fexinidazole sulfone following five days of once per day oral treatment with fexinidazole were found to be in the same range as that shown to kill all

TABLE 1: *In vitro* trypanocidal activity against different *T. brucei* subspecies. IC_{50} values (μM) are the mean ± standard deviation (SD) from 3-5 cultures.

Parasite	**Strain**	**Fexinidazole** MW** 279.3	**Fex-sulfone** MW 295.3	**Fex-sulfoxide** MW 311.3	**Melarsoprol** MW 398.3	**Pentamidine** MW 592.7	**Eflornithine /DMFO** MW 236.7	**Nifurtimox** MW 287.3	**Diminazene** MW 515.5
T.b. rhodesiense	STIB900 wt	2.17 ± 0.29 5.56 ± 1.9*	1.44 ± 0.22 3.2 ± 0.15*	1.64 ± 0.36 3.2 ± 0.44*	0.011 ± 0.003	0.002 ± 0.0003	8.58 ± 2.7	1.09 ± 0.33	0.009 ± 0.002
	STIB900 mel	2.66 ± 0.57	1.26 ± 0.51	1.16 ± 0.29	0.092 ± 0.028	0.095 ± 0.035	nd	nd	0.019 ± 0.002
	STIB900 pent	2.71 ± 0.87	1.16 ± 0.39	1.48 ± 0.75	0.043 ± 0.022	0.058 ± 0.019	nd	nd	0.011 ± 0.004
T.b. brucei	BS221 wildtype	2.38 ± 0.88	1.63 ± 0.92	1.49 ± 0.61	0.013 ± 0.004	0.002 ± 0.0003	nd	nd	0.005 ± 0.001
	BS221 AT1KO	1.33 ± 0.21	0.56 ± 0.04	0.85 ± 0.32	0.034 ± 0.003	0.008 ± 0.002	nd	nd	0.060 ± 0.016
	STIB950 mdr	2.44 ± 0.99	0.99 ± 0.34	1.21 ± 0.14	0.038 ± 0.011	0.002 ± 0.0002	nd	nd	0.062 ± 0.05
T.b. gambiense	STIB930	1.84 ± 1.13	0.91 ± 0.27	0.94 ± 0.39	0.012 ± 0.005	0.016 ± 0.001	2.85 ± 0.98	2.24 ± 0.66	0.021 ± 0.009
	DAL 898R	1.01 ± 0.36	0.76 ± 0.30	1.03 ± 0.13	0.009 ± 0.002	0.002 ± 0.0002	nd	nd	0.014 ± 0.001
	K3048	0.95 ± 0.19	nd	nd	0.032 ± 0.012	0.084 ± 0.015	7.63 ± 2.5	0.99 ± 0.12	0.076 ± 0.03
	45R	2.47 ± 1.59	0.95 ± 0.47	1.24 ± 0.60	0.033 ± 0.011	0.069 ± 0.044	9.98 ± 2.4	1.06 ± 0.38	0.074 ± 0.033
	40R	2.61 ± 1.03	0.67 ± 0.35	0.95 ± 0.33	0.032 ± 0.006	0.088 ± 0.024	11.4 ± 5.8	1.46 ± 0.20	0.12 ± 0.02
	349Pi	1.07 ± 0.14	nd	nd	0.043 ± 0.011	0.066 ± 0.012	16.7 ± 3.6	0.78 ± 0.19	0.043 ± 0.025
	349R	3.31 ± 0.88	nd	nd	0.033 ± 0.015	0.095 ± 0.012	22.8 ± 13.9	2.73 ± 0.66	0.064± 0.031
	130R	2.37 ± 1.14	nd	nd	0.055 ± 0.023	0.074 ± 0.011	9.4 ± 2.19	1.34 ± 0.17	0.051 ± 0.013

* IC_{90} values ± standard deviation in mM
** MW: molecular weight

parasites in vitro indicating that these compounds probably provided the bulk of the trypanocidal activity of the administered parent compound.

***In vitro* dynamic results**

In order to better understand the *in vitro* trypanocidal activity of fexinidazole, and the sulfoxide and sulfone metabolites pulse incubation experiments were performed and IC_{50} and IC_{90} values determined following compound wash out at various time points after exposure. The results are shown in Figure 1. A 48- h period of exposure to the compounds is required to produce similar activities as in the standard 72-h assay indicating that maximum killing effectiveness requires up to 48 hours exposure to the drugs.

TABLE 2: *In vivo* anti-trypanosomal activity in the STIB900 acute mouse model

Compound	Dose mg/kg	Route	Cured/ Infected	Mean day of relapse
Control	-	-	0/12	8.75*
Fexinidazole	4x 20	ip	0/4	11± 2
Fexinidazole	4x 50	ip	4/4	>60
Fexinidazole**	4x 25	po	0/4	12± 2
Fexinidazole**	4x 50	po	1/4	>27
Fexinidazole**	4x 100	po	4/4	>60
Fexinidazole sulfoxide	4x 50	ip	1/4	>24.5
Fexinidazole sulfoxide	4x 100	po	2/4	>38.25
Fexinidazole sulfone	4x 50	ip	0/4	11± 2
Fexinidazole sulfone	4x 100	po	1/4	>31.5
Melarsoprol	4x 4	ip	4/4	>60

* Mean survival days post infection of untreated control animals, the value given is the average of three experiments
10% DMSO was used as vehicle
** Data published E.Torreele PLoS Negl Trop Dis. 4(12): e923 (Ref 33)

***In vitro* drug combination results**

Although NECT is currently the only available drug combination therapy to treat HAT the development of resistance to existing therapies is making the potential use of combination therapies increasingly relevant. Data on the *in vitro* interaction of possible

combinations has been proposed to support such development options (30). Fexinidazole and the biologically

TABLE 3: *In vivo* anti-trypanosomal activity in the GVR25 chronic disease mouse model.

Compound	Dose mg/kg	Route	Vehicle	Cured/ Infected	Mean day of relapse
Fexinidazole	5 x 50	ip	DMSO/water	1/5	73.8
Fexinidazole	5 x 50 bid	ip	DMSO/water	5/5	>180
Fexinidazole	5 x 100	po	DMSO/water	3/5	>127
Fexinidazole	5 x 100 bid	po	DMSO/water	11/15	$>156.5^{+}$
Fexinidazole*	5 x 50	po	MethocelTween **	0/8	41.3± 9
Fexinidazole*	5 x 100	po	MethocelTween	2/8	>82.1
Fexinidazole*	5 x 200	po	MethocelTween	7/8	>163.8
Nifurtimox	5 x 50	po	MethocelTween	0/8	31.0± 2
Nifurtimox	5 x 100	po	MethocelTween	0/8	31.0± 2
Nifurtimox	5 x 200	po	MethocelTween	0/8	37.4± 5
Diminazene	1 x 40	ip	DMSO/water	0/24	$49.8\pm 6^{++}$
Eflornithine	10 x 2%***	po	Water	0/4	$76.3\pm 8^{+++}$
Melarsoprol	5 x 5	ip	Propyleneglycol/H2O	0/5	$57.6\pm 14^{++++}$
Melarsoprol	5 x 10	ip	Propyleneglycol/H2O	1/5	$>103.4^{++++}$
Melarsoprol	5 x 15	ip	Propyleneglycol/H2O	4/5	$>180^{++++}$

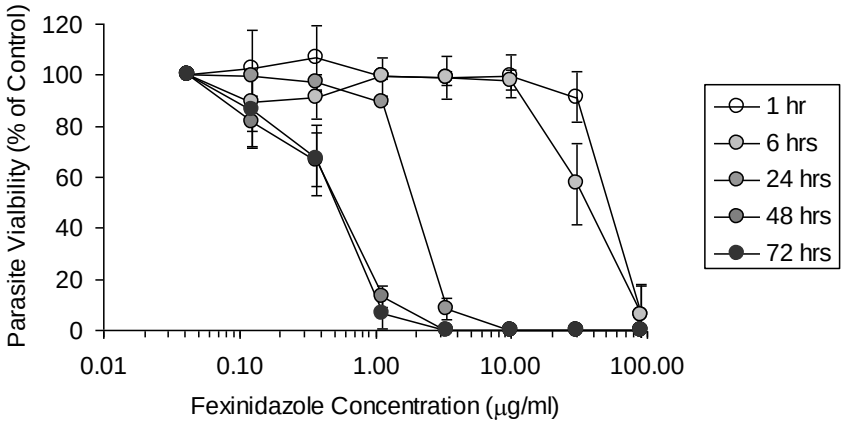
* Data published E.Torreele PLoS Negl Trop Dis. 4(12): e923 (Ref. 33).
** An optimized suspension medium for oral administration comprising 5% w/v Tween 80/0.5% w/v Methocel in water to maximize absorption. These data have been previously published and are reproduced here for comparative purposes (33).
*** A 2% solution of eflornithine provided in drinking water for 10 days.
$^{+}$ Mean result from 3 separate experiments (n = 15)
$^{++}$ Mean result from 5 separate experiments (n = 24)
$^{+++}$ Data from 1 experiment (n = 4)
$^{++++}$ Representative data from 1 experiment (n =5/group)

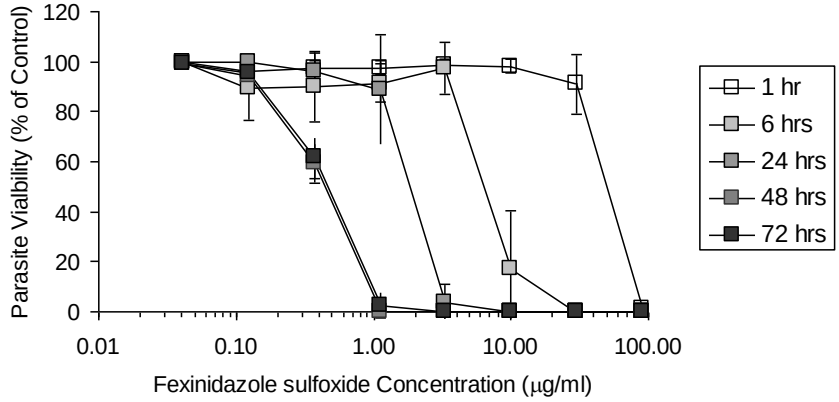
active sulfoxide and sulfone metabolites have been assessed in combination with several drugs currently available to patients. All drug combination studies were performed at three different ratios (1:3, 1:1 and 3:1) using the fixed-ratio isobologram method (10) and the data analysed using the IC50 results. Results of all drug interaction studies are shown in Table 5. Fexinidazole combined with its sulfoxide and sulfone metabolites as

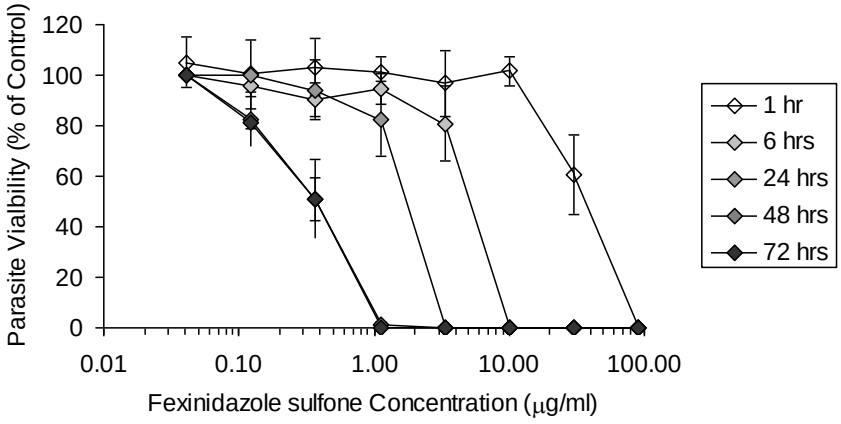
well as the combination of sulfoxide and sulfone all showed indifferent effects. The combinations of fexinidazole or either of its metabolites with melarsoprol, eflornithine or pentamidine also resulted in an indifferent effect. These data indicate that there are no cross-reactivities between these compounds which would preclude their use in, albeit unlikely, combination therapies.

FIG. 1. (A) Growth inhibition curves after compound washout at specified times and viability assessment at 72 h. (B) IC50 and IC90 values calculated from the compound washout procedure. Values are the means and standard deviations for 4 experiments (*n* =4).

A







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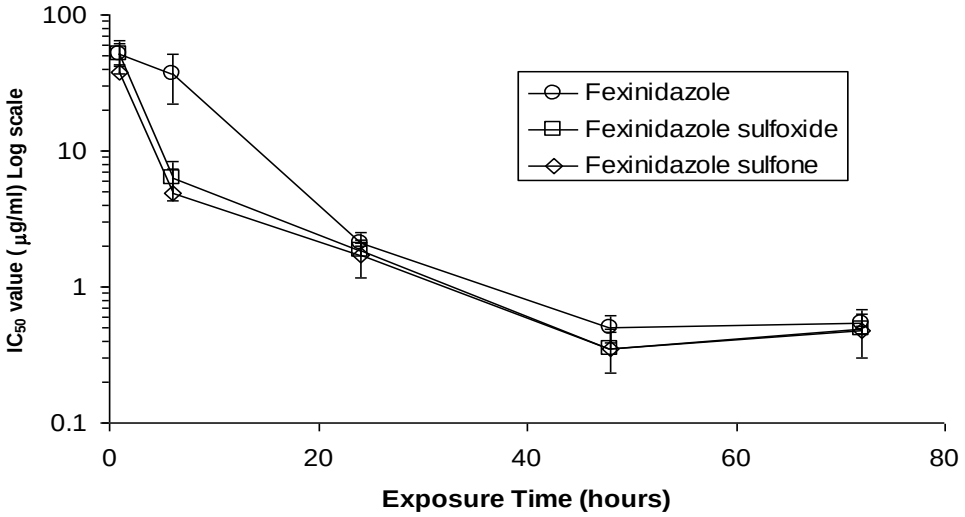

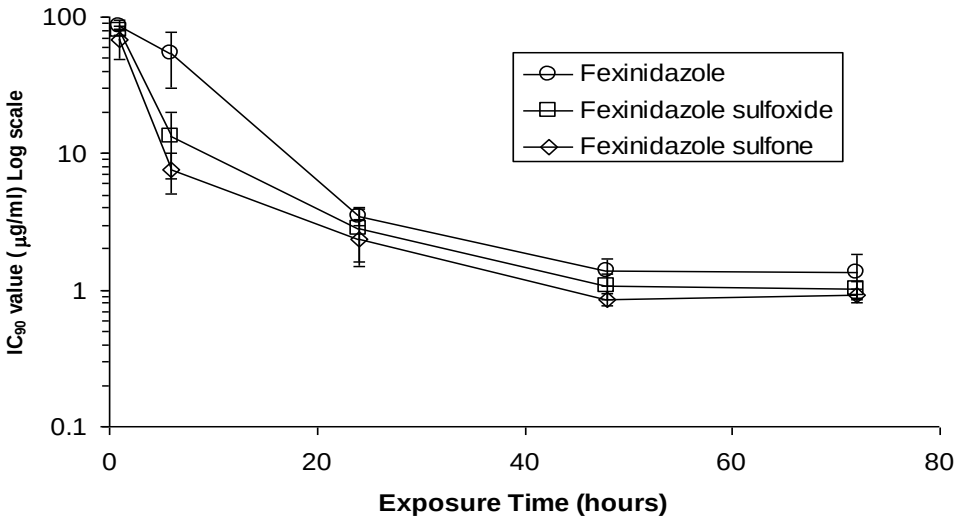

Discussion

Only four drugs are registered for HAT treatment. Pentamidine and suramin are used against the early stage of the disease whilst treatment of the second stage depends on melarsoprol, eflornithine and the recently introduced combination therapy nifurtimox-eflornithine (NECT). Melarsoprol is an arsenical compound and is highly toxic with severe adverse effects (18). In addition there have been alarming reports of treatment failures with both melarsoprol and eflornithine, until recently the only available drugs for second stage treatment (1) and it is hoped that the broad implementation of the NECT regimen may avert the further development of eflornithine resistance. New safe and effective drugs with simplified dosing regimens are urgently needed. Ideally, such new treatments would be effective in both acute and chronic disease stages. Such new treatment options would largely simplify disease management and, importantly, avoid

the painful lumbar puncture procedure currently required for distinguishing between disease stages.

Fexinidazole has recently been identified as a promising new drug candidate for treatment of HAT (33) and data presented here provide *in vitro* and *in vivo* profiling of the anti-trypanosomal efficacy of fexinidazole and its two primary metabolites, the sulfoxide and sulfone.

Fexinidazole and the sulfoxide and sulfone metabolites were tested *in vitro* alongside reference drugs against a panel of African trypanosomes of the *T. brucei spp*. (Table 1) which included sensitive and resistant wild type, laboratory-induced melarsoprol and pentamidine resistant and P2-transporter knockout strains as well as new field isolates. The data showed that there is no evidence of innate resistance to fexinidazole or the two metabolites within any of the strains tested as all IC_{50} values were in a similar range and varied by less than a factor of four. The new *T. b. gambiense* strains showed reduced IC_{50} values for pentamidine but this is unlikely to indicate resistance in the field given the higher blood levels and long terminal half-life of the drug found in patients after standard treatment (4).

Fexinidazole showed *in vivo* efficacy in both the acute mouse model and, more importantly, the chronic mouse model with established brain infection. In the STIB900 acute mouse model fexinidazole demonstrated 100% efficacy at an ip dose of 50 mg/kg/day and an oral dose of 100 mg/kg/day both given for 4 days (Table 2). Whilst a dose of 50 mg/kg/day ip fexinidazole was fully effective, the sulfoxide only partially cured with the same dose and route of administration and the sulfone was ineffective. After oral administration at a dose of 100 mg/kg/day both the sulfoxide and sulfone metabolites were only partially effective whereas fexinidazole cured 100% of the animals. Although no pharmacokinetic data are currently available to formally demonstrate oral absorption of the sulfoxide or sulfone metabolites in mice it may be that neither are as readily absorbed as fexinidazole via the oral route. However, it is apparent that, even using the ip route of administration which should maximize the systemic bioavailability of the compounds, neither metabolite was as effective as the parent fexinidazole in this acute model of disease. In addition it is unlikely that protein binding could account for the lack of effectiveness of the metabolites when given orally as, whilst fexinidazole is highly protein bound in plasma (93% in miice; 95% in man) neither metabolite is highly protein bound, at least in human plasma (26% and 42% respectively for the sulfoxide and sulfone metabolites) (Data on file at DNDi). Overall

these data support the view that the use of fexinidazole itself, acting as a biologically active pro-drug, whilst rapidly metabolized to the sulfoxide and sulfone metabolites in all animals tested (33), is likely to be the more useful compound for oral treatment compared to either of the two metabolites given alone.

In 1983, Jennings and Urquhart reported that fexinidazole, given in combination with suramin, cured a *T. brucei* CNS infection in mice (14). We have tested fexinidazole as monotherapy in the GVR35 mouse model of stage 2 HAT involving brain infection using two different vehicle formulations (Table 3). Using the optimized methocel/Tween vehicle, fexinidazole showed a dose related increase in efficacy and cured 7 out of 8 infected mice at a single oral daily dose of 200 mg/kg/day for 5 days. In comparison, nifurtimox was ineffective in the GVR35 mouse model up to a dose of 200 mg/kg/day for 5 days. It is of interest to note that the presumed trough levels of the two metabolites after 24 h are reported to be around 1 μg/ml (33) which would allow for a daily dosing schedule to be maintained with systemic drug levels near to those required to kill the parasite *in vitro*. Clearly, in this model, the drug levels in the CNS are of key importance and, whilst no data are available from the experiments presented, published data indicate that, in mice, brain levels of fexinidazole, the sulfoxide and the sulfone metabolites are approximately 0.8, 5 and 1 μg/ml respectively 60 minutes post oral dosing with fexinidazole (33). Further experiments are underway to more fully assess the brain levels of the compounds in mice at different times. Whilst the most effective oral dose of 200 mg/kg may seem high fexinidazole is well tolerated in laboratory animals at significantly higher doses (33) and, although no data are available in mice regarding a no toxic effect level an LD_{50} of >10,000 mg/kg has been reported (DNDi data on file).

It is important to note that, of the drugs currently in clinical use, only melarsoprol has been shown to be effective in this experimental stage 2 HAT model.

Pulse incubation of *T. b. rhodesiense* with fexinidazole and the sulfoxide and sulfone metabolites shows that a 48 hours period of exposure is required to produce irreversible effects on trypanosomal survival for all three compounds (Fig 1). This result has implications for *in vivo* efficacy as it suggests that plasma or CSF concentrations may need to be maintained at or above optimal trypanocidal concentrations for >48 h to achieve elimination of all parasites. As discussed above it is apparent, at least in mice, that, whilst plasma levels of fexinidazole may not be maintained at a sufficient killing concentration, both the sulfoxide and sulfone metabolites are present in plasma and in

brain, at concentrations sufficient to kill all parasites. In addition the data indicate that a five day dosing schedule would ensure sufficient trough levels of these metabolites at 24 h to maintain effective killing concentrations, in plasma. Concentrations in brain reach several μg/ml one hour after oral application (33), information on the persistence of fexinidazole and its metabolites is not available. It can be assumed that the metabolites and mainly the sulfone are responsible for the trypanocidal effect in the brain. The CSF is often used as surrogate for the brain since it is accessible without the need to kill the animal (6). Thus these data provide support to the observations in both mouse models that oral treatment with fexinidazole for 4 days (acute model) or 5 days (CNS model) can achieve cure. This time-dose relationship has been previously described for diamidines such as diminazene aceturate which are able to kill trypanosomes after a short exposure time of 15 min at 1 μg/ml (16), whilst other trypanocidal agents (e.g. trybizine hydrochloride) with an *in vitro* potency similar to or greater than diminazene aceturate require a much longer exposure time of >8 h at 10 μg/ml to lead to death of the parasites (17).

Fexinidazole and the sulfoxide and sulfone metabolites have similar *in vitro* trypanocidal activity (Tables 1, 2 and Ref, 33). The *in vivo* activity of fexinidazole is likely to be due to the concerted action of the three molecules. The *in vitro* combination studies performed support this hypothesis. All combinations of fexinidazole and its metabolites were investigated using the fixed-ratio isobologram method (10). The IC_{50} values for fexinidazole, the sulfoxide and the sulfone in combination did not differ from those of each drug alone, resulting in indifferent mean ΣFICs values between 1 and 1.4 for the combinations.

In several foci, melarsoprol treatment failures have reached 30% of those treated (19, 22, 28, 31) and treatment failures of up to 16% with eflornithine have been recently reported (1, 25). A strategy to prevent the development of resistance is the use of drugs in combination and the introduction of nifurtimox-eflornithine combination therapy (NECT) is an important development in the treatment of *T. b. gambiense* infections (26). The rationale behind combination treatments in general is that the likelihood of developing resistance to a single drug is relatively high, but much lower with a drug combination (34). Although *in vitro* cross-resistance studies have yet to be fully validated as predictive of human drug resistance the recently published study on cross resistance of fexinidazole and its sulfoxide and sulfone metabolites in a nifurtimox-resistant *T. b. brucei* strain supports the approach of utilizing chemically unrelated drug

combinations (30). The same authors also showed that resistance against fexinidazole could easily be generated in the laboratory thus underlining the potential need for a combination partner for fexinidazole. In the present study fexinidazole and the sulfoxide and sulfone metabolites were tested *in vitro* in combination with three existing drugs - pentamidine, melarsoprol and eflornithine. All combinations resulted in indifferent mean ΣFICs values. This observation supports the proposition that fexinidazole could be a candidate for combination with existing drugs that are currently acceptable treatments such as pentamidine, eflornithine and NECT or, more likely, with other new drug candidates that may become available in the future.

In conclusion the data presented in this paper demonstrate that fexinidazole and the sulfoxide and sulfone metabolites rapidly formed *in vivo* are effective at killing the parasites responsible for human African trypanosomiasis. Fexinidazole is effective in both acute and chronic mouse models of HAT at doses and dosing regimens which are expected to be practicable for human treatment. Time-dose studies indicate that effective drug levels need to be maintained for at least 48 hours and interaction data show that there is no cross-inhibition between fexinidazole and the sulfoxide or sulfone metabolites or other, chemically unrelated, treatment modalities. Overall these data provide evidence that fexinidazole has the potential to be an effective oral treatment for both *T. b. gambiense* and *T. b. rhodesiense* forms of human sleeping sickness and both stages of the disease.

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CHAPTER 4

1-Aryl-4-nitro-1*H*-imidazoles, a new promising series for the treatment of Human African Trypanosomiasis

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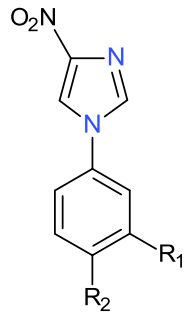
Abstract

Nitroimidazoles are a well-known class of antibacterial and antiprotozoal drugs but in spite of the widespread clinical and veterinary use of these drugs, this family has been stigmatized in part due to associated genotoxicity problems. Here we report the synthesis, the anti-trypanosomal activity and a structureeactivity relationship (SAR) study of a series of about fifty 1-aryl-4-nitro-1*H*-imidazoles, with an emphasis on selected in vivo active molecules. Compounds 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole are curative in mouse models of both acute and chronic African trypanosomiasis when given orally at doses of 25-50 mg/kg for 4 days for the acute infection, and 50-100 mg/kg (bid) for 5 days in the chronic model. While both compounds are bacterial mutagens, activity is lost in strains lacking bacterial specific nitro-reductases. Mammalian nitroreductases do not reduce nitroaromatic compounds with low redox potentials with same avidity as their bacterial counterparts and these compounds were shown to be devoid of genotoxicity in mammalian cells. Both compounds are promising leads for the treatment of human African trypanosomiasis (HAT or sleeping sickness), including the fatal stage 2 of the disease, for which new treatments are urgently needed.

Graphical Abstract



16: R_1 = H; R_2 = OCF_3
31: R_1, R_2= Cl

This article describes the synthesis, antitrypanosomal activity, and non genotoxic profile of a promising new series of 1-aryl-4-nitro-1*H*-imidazoles with potential for the treatment of human African trypanosomiasis (HAT or sleeping sickness), with an

emphasis on the lead compounds 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole (**16**) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (**31**).

Introduction

Classified among the most neglected diseases, human African trypanosomiasis (HAT), also known as sleeping sickness and caused by the two pathogenic parasite subspecies *Trypanosoma brucei rhodesiense* and *T. b. gambiense,* is a major health problem in sub-Saharan Africa [1,2]. Treatment of HAT is difficult [3], especially in its advanced fatal stage when the parasites have crossed the blood-brain barrier (BBB) for which only two drugs are currently registered and neither is adequate. Melarsoprol, an old arsenical drug in use for the treatment of HAT since 1949, is toxic (5-10% associated risk of mortality due to drug induced encephalopathy) and requires painful intravenous injections. Eflornithine, originally developed as an anticancer drug and registered for the treatment of HAT in 1981, has a narrow therapeutic window and requires 14 days of 6-hourly slow infusions, which is an impractical regimen for many patients in the countries where the disease is prevalent. Recently, a simplified co-administration of oral Nifurtimox and intravenous Eflornithine (NECT) has proven good safety and efficacy and provides an improved first line treatment for stage 2 HAT, although as above it remains a severe challenge to implement in remote and rural settings where HAT is endemic [4,5]. With a renewed interest in neglected diseases, a new drug candidate has also emerged in recent years: Fexinidazole is today in phase I clinical development with potential for the advanced stage of HAT [6,7]. Despite this progress, and taking into account the inevitable attrition rates in drug development, there is still a long way to go before an improved treatment could become available for patients.

Nitroimidazoles are a well-known family of antibacterial and antiprozoal drugs [8], including antitrypanosomal drugs or compounds with known antitrypanosomal activity [9,10,11]. Metronidazole, the first drug to be introduced for this purpose and probably also the best-known drug in this class, has been in use for more than 50 years [12]. Other well-known examples include Tinidazole [13] closely related to Metronidazole and the antitrypanosomal drug Benznidazole indicated for the treatment of Chagas disease [14,15]. In spite of extensive use, this class of compounds has often been stigmatized for reasons of perceived genotoxic risks associated with the nitroaromatic group [16]. A well-known case here is the antitrypanosomal drug

candidate Megazol [10,11] which was abandoned because of clear mammalian cell genotoxicity [17,18]. In recent years however, several new nitroimidazole drug candidates have emerged, for instance PA-824 [19] and OPC-67683 [20], both in clinical development for the treatment of tuberculosis, and Fexinidazole for HAT [6]. In each of these cases, a detailed analysis of the genotoxic properties of the compounds concluded that they did not pose a genotoxic risk to humans.

One electron reduction potentials of nitroimidazoles as well as other nitroazoles mainly depend on the position of the nitro group on the azole ring and for nitroimidazoles generally increase in the following order 4-NO_2<5-NO_2<2-NO_2 [21,22] . It is believed that generally genotoxicity problems increase in a similar order. Examples of non-genotoxic anti-infective 4-nitroimidazoles have been described [19,20], but while 1-alkyl derivatives have been largely explored, few 1-aryl-4-nitro-1*H*-imidazoles have been studied due to serious synthetic limitations. We have developed a general method which allowed the synthesis of several 1-aryl-4-nitro-1*H*-imidazoles from a very simple coupling reaction between 1,4-dinitro-1*H*-imidazole and the corresponding anilines [23]. Several compounds in this series have shown anti-tuberculosis activity [24,25]. Here we report the antitrypanosomal activity and a structure-activity relationship (SAR) study, with an emphasis on selected *in vivo* active molecules, with evidence for lack of mammalian cell genotoxicity. Several 1-aryl-4-nitro-1*H*-pyrazoles [26] were also prepared but proved inactive and are not included in the present paper.

Results and discussion

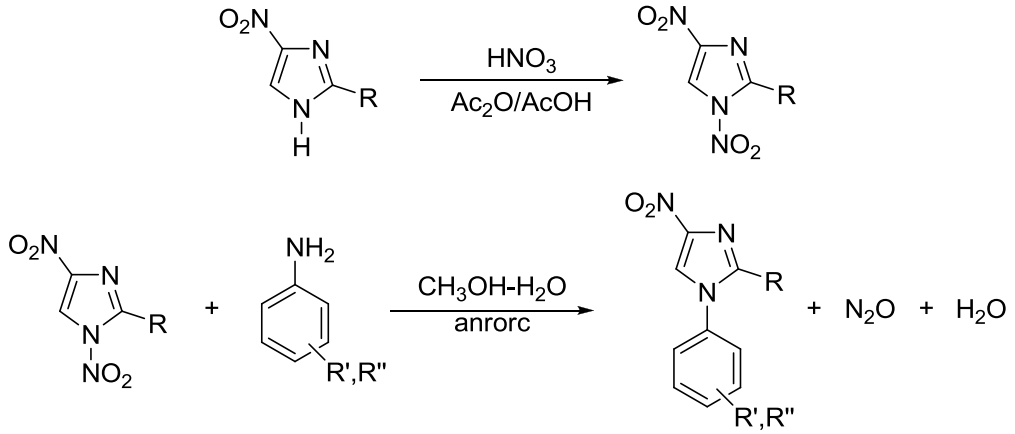
Chemistry

This work has further validated and demonstrated the wide applicability of the coupling reaction between 1,4-dinitro-1*H*-imidazoles and anilines for the synthesis of 1-aryl-4-nitro-1*H*-imidazoles: the forty-three 1-aryl-4-nitro-1*H*-imidazoles and six 1-aryl-2-methyl-4-nitro-1*H*-imidazoles of the present study were obtained by this method in relatively good yields (40-90%), with substituents on the benzene ring ranging from strong electron-withdrawing (e.g. NO_2, CO_2R) to electron-donating groups (e.g. Me, MeO) (Scheme 1). For a detailed description of the method, see [23,25]. The mechanism of the reaction involves a degenerated ring transformation reaction also called *anrorc* reaction *(**a**ddition of **n**ucleophile, **r**ing **o**pening, **r**ing closure)*; on the

anrorc reaction, see [27] and references cited herein. The reaction is generally performed at room temperature though a higher temperature is often required for completion of the reaction. Water and gaseous nitrogen(I) oxide are the single easy to separate by-products; the so called 'atom economy' of this reaction [28], describing the conversion efficiency of a chemical process in terms of all atoms involved, exceeds 80%. Alternative approaches exist, nevertheless none of them competes with the present *anrorc* coupling reaction between 1,4-dinitro-1*H*-imidazoles and anilines. Nitration of 1-phenyl-1*H*-imidazole is not selective, compounds from nitration of both aromatic rings are usually obtained [29], and to our knowledge, selective reduction of the resulting dinitro- or trinitro-compounds has not been reported yet. 1-Arylation of 4(5)-nitro-1*H*-imidazole anions with 1-fluoro(or chloro)-2(or/and 4)-nitrobenzenes is limited to these substrates only [24] (and references cited herein). Finally, attempts to replace the 1-nitro substituent in 1,4-dinitro-1*H*-imidazoles by another electron withdrawing group (e.g. -CN, $-SO_2Ar$, $-SO_2NR_2$) were either unsuccessful [30] or only partly successful [31,32]. A similar degenerated ring transformation process mechanism was observed in these cases but the reaction was generally not clean, not reproducible and yields of the desired 1-aryl-4-nitro-1*H*-imidazoles were usually low. Only a few known compounds have been prepared following this approach [30,31,32].

Antitrypanosomal activity

An overview of the *in vitro* assays is shown in Table 1. Of the 43 1-aryl-4-nitro-1*H*-imidazoles evaluated, 28 compounds showed anti-trypanosomal activity with good selectivity against *T. b. rhodesiense* (STIB900) with an IC_{50} in the micromolar range or below 1 μM for 15 compounds. Potent activity was observed for two compounds in particular, 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole (**16**) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (**31**) (IC_{50} 0.16 and 0.10 μM, respectively), comparable to the activity known for Megazol (IC50 0.10 μM). Several other compounds showed similar activity as compared to the new drug candidate Fexinidazole (IC50 2.57 μM). A number of analogs with a methyl substituent in position C2 on the imidazole ring (1-aryl-2-methyl-4- nitro-1H-imidazoles) did not show activity; similarly none of the pyrazole analogs showed any activity (results not shown).



Scheme 1: Synthesis of 1-aryl-4-nitro-1*H*-imidazoles. R=H, CH_3, R', R''= H, electron donating or electron withdrawing group, one or two substituents in ortho, meta or/and para positions.

From these *in vitro* data, the following structure/antitrypanosomal activity relationships can be drawn (Scheme 2): 1- good activity is observed with the presence of an electron withdrawing substituent in the meta or para position at the phenyl group, as compared to the parent compound namely 4-nitro-1-phenyl-1*H*-imidazole (**1**); the activity is about the same with F, Cl, Br or NO_2. In contrast, except in a few exceptions, the activity is decreased or lost with an electron donating substituent in the *meta* and *para* positions. 2- The activity is preserved with the addition of a second substituent in the para or other meta-position, independently on the nature of the substituent (electron withdrawing or electron donating group). 3- No to low activity is observed with a substituent present in the ortho position or the addition of a methyl substituent on the imidazole ring. A possible explanation for the loss of activity in the two latter cases lies in the lack of co-planarity of the two aromatic rings in those compounds; other derivatives can be almost planar (from quantum chemical calculations and x-ray measurements). 4- No to low activity is observed with a carboxyl ester substituent though we could expect some activity due to the electron withdrawing character of the substituent.

The two potent compounds 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole (**16**) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (**31**) were further evaluated in mouse models of HAT. In an acute infection model, both compounds were shown to be curative with a 100% cure rate at an oral dose of 25 mg/kg/day **16** or 50 mg/kg/day **31** administered for 4 mg/kg/day 31 administered for 4 days (Table 2). In the

Table 1: IC50s of 1-aryl-4-nitro-1*H*-imidazoles and reference molecules against *T. b. rhodesiense* (STIB900) and in L-6 rat myoblast cells

Compound No	subtituents	MW	*T. b. rhodesiense* (STIB900) [μg/ml] [a)]	*T. b. rhodesiense* (STIB900) [μM] [a)]	Cytotoxicity (L-6 rat myoblast cells) [μg/ml]	Reference to synthesis
*(**I**) 1-ary1-4-nitro-1H-imidazoles monosubtituted on the benzene ring*						
1	H	189.17	0.85	4.49	>90	23
2	2-F	207.16	0.59	2.85	>90	25
3	3-F	207.16	0.15	0.72	>90	25
4	4-F	207.16	0.52	2.53	>90	25
5	2-Cl	223.62	n.a.	n.a.	no data	25
6	3-Cl	223.62	0.16	0.72	>90	23, 25
7	4-Cl	223.62	0.16	0.72	35.68	23
8	2-Br	268.07	n.a.	n.a.	no data	b)
9	3-Br	268.07	0.24	0.90	>90	b)
10	4-Br	268.07	0.26	0.97	17.88	24
11	3-NO_2	234.17	0.18	0.77	>90	b)
12	3-CF_3	257.17	0.37	1.45	>90	b)
13	4-CF_3	257.17	0.18	0.70	>90	b)
14	2-CF_3O	273.17	5.67	20.76	>90	b)
15	3-CF_3O	273.17	0.48	1.76	>90	b)
16	4-CF_3O	273.17	0.04	0.16	>90	b)
17	3-Me	203.20	0.37	1.82	>90	23
18	4-Me	203.20	2.43	11.96	>90	23
19	3-MeO	219.20	0.42	1.92	>90	b)
20	4-MeO	219.20	0.70	3.19	66.80	23, 24
21	4-CO_2Bu	289.29	8.11	28.03	>90	b)

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Compound No	subtituents	MW	*T. b. rhodesiense* (STIB900) [μg/ml] [a)]	*T. b. rhodesiense* (STIB900) [μM] [a)]	Cytotoxicity (L-6 rat myoblast cells) [μg/ml]	Reference to synthesis
***(II)** 1-aryl-4-nitro-1H-imidazoles disubstituted on the benzene ring*						
22	2,3-diF	225.15	0.49	2.18	>90	**Error!**
23	2,4-diF	225.15	n.a.	n.a.	no data	25
24	2,5-diF	225.15	0.37	1.64	>90	25
25	2,6-diF	225.15	n.a.	n.a.	no data	25
26	3,4-diF	225.15	0.17	0.76	>90	25
27	3,5-diF	225.15	0.11	0.49	>90	25
28	2,3-diCl	258.06	0.49	1.90	38.66	25
29	2,4-diCl	258.06	n.a.	n.a.	no data	25
30	2,5-diCl	258.06	n.a.	n.a.	no data	25
31	3,4-diCl	258.06	0.03	0.10	>90	25
32	3,5-diCl	258.06	0.18	0.70	40.55	25
33	3-Cl-4-F	241.61	0.17	0.70	>90	b)
34	2-Br-4-Me	282.10	n.a.	n.a.	no data	b)
35	3-Me-4-Br	282.10	0.28	0.99	15.54	b)
36	3-Br-4-Me	282.10	0.56	1.99	>90	b)
37	2,4-diMe	217.23	n.a.	n.a.	no data	b)
38	3,4-diMe	217.23	0.46	2.12	>90	b)
39	2,4-diMeO	249.20	n.a.	n.a.	no data	b)
40	3,4-diMeO	249.20	n.a.	n.a.	no data	b)
41	3,4-O_2CH_2	233.18	0.22	0.94	>90	b)
42	3-CO_2Bu-4-Cl	323.74	45.75	141.32	>90	b)
43	3-CO_2H-4-Cl	267.63	25.57	95.54	>90	b)

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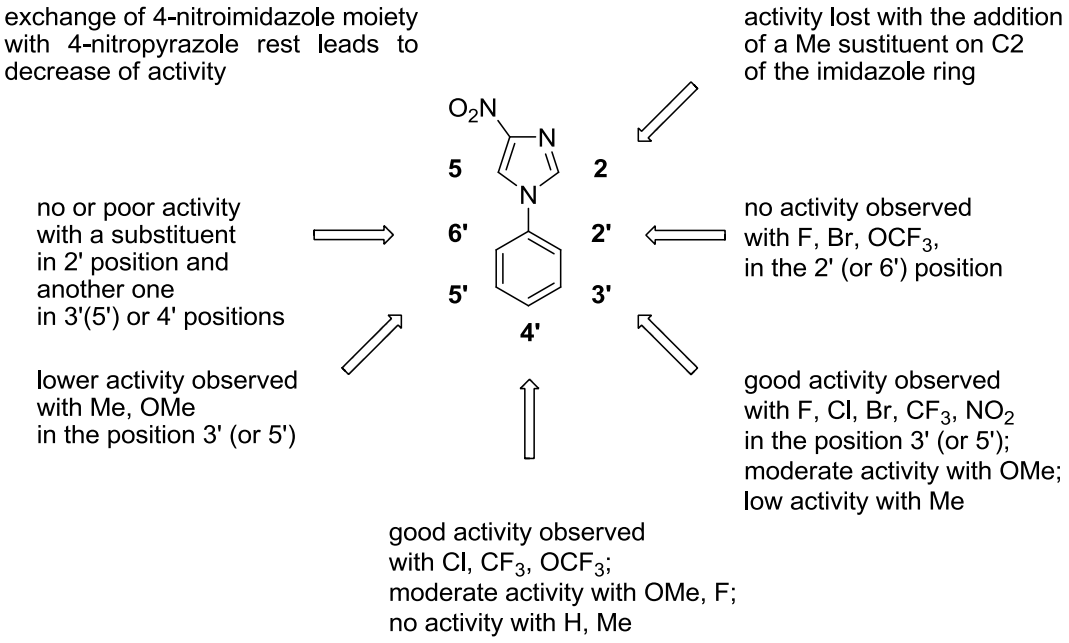
Compound No	subtituents	MW	*T. b. rhodesiense* (STIB900) [μg/ml] a)	*T. b. rhodesiense* (STIB900) [μM] a)	Cytotoxicity (L-6 rat myoblast cells) [μg/ml]	Reference to synthesis
***(III)** 1-aryl-2-methyl-4-nitro-1H-imidazoles mono or disubstituted on the benzene ring*						
44	4-CF_3O	287.20	3.42	11.89	>90	b)
45	4-CF_3	271.20	3.06	11.28	>90	b)
46	2-Br-4-Me	296.12	36.9	124.6	>90	b)
47	3-Cl-4-F	255.64	6.91	27.03	>90	b)
48	4-Br-3-Me	296.12	5.97	20.16	>90	b)
49	3-Br-4-Me	296.12	6.97	23.54	>90	b)
Reference molecules						
Fexinidazole			0.72	2.57	>90	-
Megazol			0.02	0.10	57	-
Melarsoprol			0.004	0.009	1.3	-
Eflornithine			0.90	3.80	12	-

a) n.a. is for not active). b) Prepared for the present study, synthetic details are provided in the experimental part.

stage 2 HAT infection model involving brain infection (also days (Table 2). In the stage 2 HAT infection model involving brain infection (also known as the "chronic CNS model"), 100% cure was achieved at an oral dose of 50 mg/kg 16 or 10 mg/kg **31** administered twice a day (bid) for five days, while a daily oral dose of 100 mg/kg administered for five days was partially curative (Table 3). Very few compounds are known to cure this established chronic CNS model except some arsenicals and selected experimental diamidines [33], and the relatively low curative dose of 50 mg/kg/day given bid over 5 days is quite remarkable. In fact the curative capacity of these compounds is comparable or even slightly better than Fexinidazole, also a nitroimidazole, currently in phase I clinical development for HAT [6]. The lowest curative dose for Fexinidazole in the late stage model is 5x100 (bid) mg/kg/day.

Interestingly, in this series compound **14** showed *in vitro* activity against *Trypanosoma cruzi*, the causative agent of Chagas disease; compound **33** showed *in vitro* activity against both *trypanosoma* species (*Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*) (IC_{50} against *T. cruzi* respectively 2.7 μM and 7.0 μM). Both compounds were further evaluated in animal models for Chagas disease; they are described here in relation to their genotoxicity profile only (see further below).



the activity is preserved or increased with the addition of a halogen atom as a second substituent in the positions 3'(5') or 4' (3',4'-diF; 3',5'-diF; 3',4'-diCl; 3',5'-diCl; 3'-Cl-4'-F; 3'-Br-4'-Me; 4'-Br-3'-Me); the activity is reduced with the presence of two electron donating groups (3',4'-diMe; 3',4'-OCH_2O-)

Scheme 2: Qualitative structure antiparasitic activity (SAR) analysis of substituted 1-aryl-4-nitro-1*H*-imidazoles

Table 2: Efficacy of selected 1-aryl-4-nitro-1*H*-imidazoles in the treatment of experimental acute infections with *T. b. rhodesiense* (STIB900) in mice

Compound	Dose (*days x* mg/kg)	Route [a)]	Cured/ infected	Mean survival days (MSD)
Control [b)]	-	-	0/4	**8**
31	*4x* 25	**p.o.**	0/4	**27.75**
31	*4x* 50	**p.o.**	4/4	**>60**
16	*4x* 25	**p.o.**	4/4	**>60**
16	*4x* 50	**p.o.**	4/4	**>60**

a) p.o.=oral application. b) Negative control: mice were infected but not treated.

Table 3: Efficacy of selected 1-aryl-4-nitro-1*H*-imidazoles in the treatment of experimental chronic CNS infection with *T. b. brucei* (GVR35) in mice

Study	Compound	Dose (*days x* mg/kg)	Route [a)]	Cured/ infected	Mean survival days (MSD)
1	Control [b)]	*1x* 40	**i.p.**	0/5	**55.8**
	31	*5x* 50	**p.o.**	1/5	**>120.4**
	31	*5x* 100	**p.o.**	2/5	**>149.2**
	16	*5x* 50	**p.o.**	0/5	**95.2**
	16	*5x* 100	**p.o.**	1/5	**>156.8**
2	Control [b)]	*1x* 40	**i.p.**	0/5	**57.2**
	31	*5x* (50 bid)	**p.o.**	3/4	**>155**
	31	*5x* (100 bid)	**p.o.**	5/5	**>180**
	16	*5x* (50 bid)	**p.o.**	5/5	**>180**
	16	*5x* (100 bid)	**p.o.**	3/3	**>180**

a) i.p.=intraperitoneal application; p.o.=oral application. bid: twice per day at a 8 hrs interval. b) Negative control: mice were infected and treated on day 21 with a single dose of diminazene aceturate (see further below paragraph 4.2.3).

Genotoxicity profile of four selected in vivo active compounds

Bacterial mutagenicity - Ames test

Bacterial mutagenicity of the four compounds **14**, **16**, **31** and **33** was assessed in the standard *Salmonella* tester strains recommended for mutagenicity screening by international guidelines for genotoxicity assessment: TA1535, TA100 and TA102, to detect base substitution point mutations and TA1537 and TA98 to detect frameshift point mutations. In addition, the assays were repeated in the corresponding strains

lacking one or both of the bacterial nitro-reductase genes (NR-deficient strains). By checking the difference in response between the normal Ames strains and its corresponding NR-deficient counterpart, the contribution of bacterial nitro-reduction to the observed mutagenicity can be estimated.

A summary of the Ames tests results is shown in Table 4; as an example, dose responses for compound **31** in two tester strains are shown in Figure 1 and Figure 2.

Table 4: Ames study

Lowest concentration at which mutagenic effect is observed (μg/plate) a)	**Compound No**			
	31	**16**	**33**	**14**
TA98, *detects frameshift mutations*				
TA98 –S-9	100	N	20	N
TA98NR –S-9	N	N	N	N
TA98 +S-9	100	N	100	N
TA98NR+S-9	N	N	N	N
TA100, *detects base-pair substitutions*				
TA100 –S-9	100	350	20	700
TA100NR –S-9	N	N	N	N
TA100 +S-9	20	350	20	700
TA100NR +S-9	N	N	N	N
TA102, *detects base-pair substitutions*				
TA102 –S-9	N*	N*	N	N
TA102NR –S-9	Not treated	Not treated	Not treated	Not treated
TA102 +S-9	N*	N*	N	N
TA102NR +S-9	Not treated	Not treated	Not treated	Not treated
TA1535, *detects base-pair substitutions*				
TA1535 –S-9	N	N	N	N
TA1535NR –S-9	Not treated	Not treated	Not treated	Not treated
TA1535 +S-9	N	N	N	N
TA1535NR +S-9	Not treated	Not treated	Not treated	Not treated
TA1537, *detects frameshift mutations*				
TA1537 –S-9	100	N	N	N
YG7167 (NR) –S-9	350	Not treated	Not treated	Not treated
TA1537 +S-9	N	N	N	N
YG7167 (NR) +S-9	Not treated	N	Not treated	Not treated

* Statistically significant increases in revertant numbers were observed at one or more concentrations although as they were not concentration related and were of a small magnitude, they were not considered to be indicative of mutagenic activity.
All positive controls used gave the expected significant increases in revertant colonies.
a) 'N' for 'negative', meaning there no mutagenic activity was observed at any dose tested (0.16, 0.8, 4, 20, 100, 350, 700 and 5000 μg/plate)

All four test compounds induced mutations in one or more of the standard tester strains used. However, in each case, mutagenicity was abolished in the corresponding nitro-reductase deficient strains, with the exception of compound **31** in TA1537, where mutagenic activity was reduced but not eliminated by the removal of the classical nitro-reductase in strain YG7167 (TA1537NR) (Table 4). This provides strong evidence that

the bacterial mutagenicity observed for **16**, **14** and **33** is due entirely to the action of bacterial specific nitro-reductases. Mutagenicity induced by **31** is also due, at least in part to bacterial nitro-reduction, as confirmed by the lack of activity in TA98NR, which lacks both the standard nitro-reductase and a supplementary nitro-reductase [34]. Frame-shift mutagenicity induced by **31** in strain TA1537 is influenced by the classical nitro-reductase but the residual activity seen in its sister strain lacking this nitro-reductase may be due to the action of the supplementary nitro-reductase, which is active in this strain.

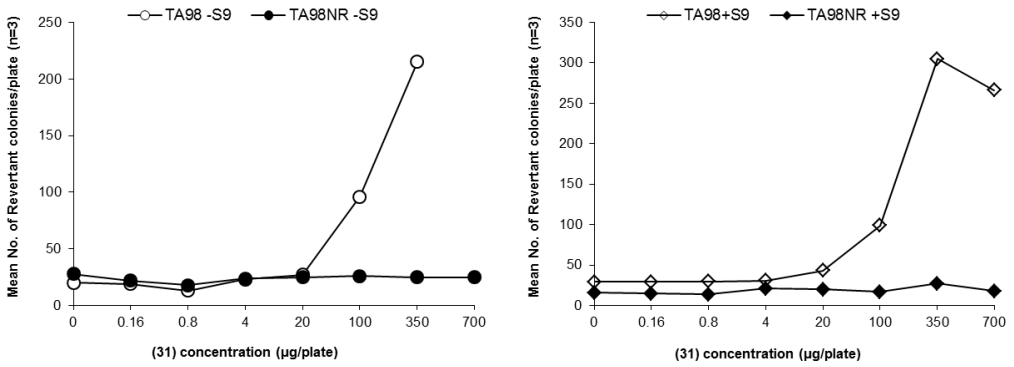


Figure 1: Mutagenic activity of compound **31** in the Ames test using *Salmonella typhymurium* strain TA98 and its nitro-reductase-deficient variant TA98NR, in the presence and absence of metabolic activation (+/- S9)

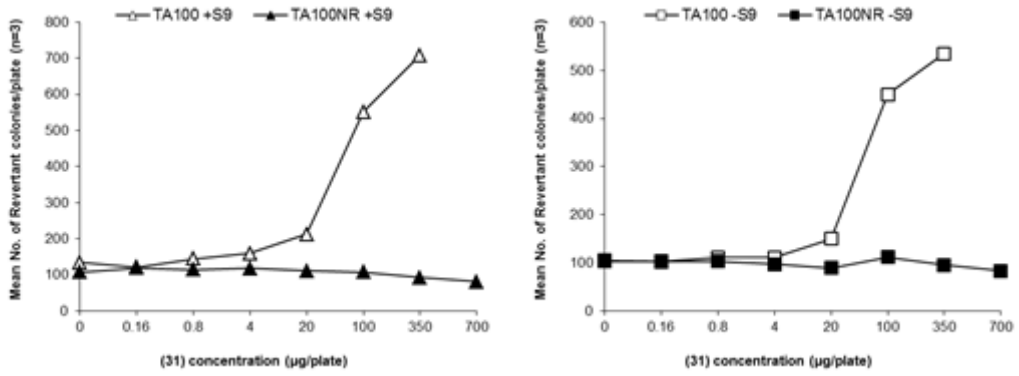


Figure 2: Mutagenic activity of compound **31** in the Ames test using *Salmonella typhymurium* strain TA100 and its nitro-reductase-deficient variant TA100NR, in the presence and absence of metabolic activation (+/- S9)

In vitro micronucleus test

Each test compound was also screened in the human peripheral lymphocyte micronucleus test that detects chromosomal damage and aneugenicity. Compound **16** did not induce significant increases in micronuclei in human peripheral lymphocytes

under the conditions of these assays, at concentrations that induced 57% cytotoxicity or less. The positive controls did induce statistically significant increases in the proportion of cells with micronuclei. Full data for **16** is shown in Table 5A-C.

Preliminary data for the other three compounds in this series are shown in Tables 6-8. Compound **31** showed no activity at concentrations inducing up to 62% cytotoxicity (Tanle 6A and B). Compound **14** showed no activity in the absence of S9. In the presence of S9 there were apparent small increases at 25 and 45 µg/mL compared to the concurrent vehicle control (Tables 7A and B). However, the values for the number of micronucleated cells at these concentrations were at the lower end of the normal historical control range (0-1.5%) for the testing laboratory concerned (as can be seen from comparisons with the data in Tables 5-8) and were attributed to an abnormally low vehicle control response.

Table 5: *In vitro* micronucleus test of compound **16**

Test concentration µg/mL	Cytotoxicity (%)	Mean MNBN[a)] (%)
A) -S9, 3 hours exposure, 21 hours recovery		
Vehicle	-	0.60
120.0	0	0.15
140.0	0	0.30
160.0	0	0.40
MMC[b)], 0.08	ND	11.25[c)]
B) -S9, 24 hours exposure, no recovery		
Vehicle	-	0.50
12.5	10	0.55
22.5	40	0.50
30.0	57	0.40
VIN[d)], 0.03	ND	6.06[c)]
C) +S9, 3 hours exposure, 21 hours recovery		
Vehicle	-	0.40
80.0	0	0.05
100.0	0	0.20
120.0	0	0.65
CPA[e)], 12.5	ND	2.45[c)]

[a)] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b)] Mitomycin C (MMC), positive control.
[c)] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d)] Vinblastine (VIN), positive control.
[e)] Cyclophosphamide (CPA), positive control that requires metabolic activation by S9.

Table 6: *In vitro* micronucleus test of compound **31**

Test concentration µg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) -S9, 20 hours exposure, 28 hours recovery		
Vehicle	-	0.3
343.6	3	0.3
536.9	62	0.7
2048	60	0.1
4NQO[b], 2.5	ND	4.8[c]
B) +S9, 3 hours exposure, 45 hours recovery		
Vehicle	-	0.5
100.0	5	0.4
200.0	20	0.3
250.0	56	0.3
CPA[d], 6.25	ND	7.8[c]

[a] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b] 4-Nitroquinoline –N-oxide (4NQO), positive control.
[c] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
[d] Cyclophosphamide (CPA), positive control.

Data for compound **33** are given in Tables 8A and B. In the absence of S9 a negative response was obtained. In the presence of S9, a small increase was seen at the middle test concentration of 200 µg/mL, but no increases were seen at the lower of higher test concentrations i.e. the increase was not dose-related. In addition the response was not consistent between the two replicate cultures. Thus this increase was not regarded as biologically significant.

In conclusion all four compounds were deemed negative in human peripheral lymphocyte micronucleus tests.

Redox potential

The single electron redox potential of compound **16** was -575 mV, which is substantially more negative than mammalian redox systems. For comparison, it is -516 mV for Metronidazole, -511 mV for Fexinidazole and significantly higher at -422 mV for Megazol). This low redox potential is consistent with the lack of activity observed in the *in vitro* micronucleus tests, indicating that mammalian cells cannot nitro-reduce these compounds to produce genotoxic chemical species under normal aerobic conditions.

Table 7: *In vitro* micronucleus test of compound **14**

Test concentration µg/mL	Cytotoxicity (%)	Mean MNBN[a] (%)
A) -S9, 20 hours exposure, 28 hours recovery		
Vehicle	-	0.2
175.0	9	0.2
400.0	31	0.3
475.0	69	0.3
4NQO[b], 2.5	ND	5.4[c]
B) +S9, 3 hours exposure, 45 hours recovery		
Vehicle	-	0.0
400.0	9	0.0
525.0	25	0.20[c]
550.0	45	0.30[c]
CPA[d], 12.5	ND	18.1[e]

a) 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
b) 4-Nitroquinoline –N-oxide (4NQO), positive control.
c) Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
d) Cyclophosphamide (CPA), positive control.
e) Statistically significant $p < 0.05$. MNBN = micronucleated binucleate cells, ND = not done.

Preliminary further evaluation of the lead compound 16

Considering the LogP and PSA physicochemical parameters alone, the lead compound **16** fits well to the selection criteria in terms of drugability and blood brain barrier permeability (logP(calc)= 2.3 (target value ~2); PSA(calc)= 72.9 (target value 60-80)). A preliminary further evaluation of the ADME (Absorption, Distribution, Metabolism and Excretion) and safety profile of the lead compound **16** was performed in *in vitro* assays and animal studies including *in vitro* liver microsomal (mouse and human) metabolic stability assays, *in vitro* permeability assays (Caco-2, MDR1-MDCK), *in vitro* safety pharmacology/receptor binding assays, a pharmacokinetics study in mice and a five-day repeated oral toxicity study in rats (all studies are listed in an annex, data are available on request); so far no critical issue or toxicity alerts could be identified which would preclude the further evaluation of this lead compound towards preclinical development. This assessment also confirmed the ability of the compound to cross the blood brain barrier which is a prerequisite for drugs intended to treat stage 2 HAT and is consistent with the high efficacy of the drug observed in the stage 2 HAT mouse model involving brain infection.

Table 8: *In vitro* micronucleus test of compound **33**

Test concentration µg/mL	Cytotoxicity (%)	Mean MNBN[a)] (%)
A) -S9, 20 hours exposure, 28 hours recovery		
Vehicle	-	0.4
150.0	25	0.6
200.0	27	0.7
300.0	44	0.4
4NQO[b)], 5.0	ND	3.5[c)]
B) +S9, 3 hours exposure, 45 hours recovery		
Vehicle	-	0.8
150.0	16	0.5
200.0	0	1.3[c)]
300.0	10	0.6
CPA[d)], 6.25	ND	10.0[e)]

[a)] 2000 cells scored for the vehicle control and 1000 cells scored for the test compound groups and the positive control.
[b)] 4-Nitroquinoline –N-oxide (4NQO), positive control.
[c)] Statistically significant $p < 0.001$. MNBN = micronucleated binucleate cells, ND = not done.
d) Cyclophosphamide (CPA), positive control.
e) Statistically significant $p < 0.05$. MNBN = micronucleated binucleate cells, ND = not done.

Conclusion

This series of 1-aryl-4-nitro-1*H*-imidazoles has demonstrated potent and selective antrypanosomal activity, including the exceptional capacity to cure a stringent model of second stage HAT, the chronic CNS model. Taken together with the absence of mammalian mutagenicity and the ADME and safety profile investigated so far, this confirms these compounds, and in particular the lead compound **16**, as promising leads for further development into a new oral treatment of human African Trypanosomiasis. It also corroborates the findings that it is possible to select compounds within the nitroimidazoles family that are pharmacologically active yet are unlikely to pose a genotoxic hazard to patients.

Although bacterial mutagenicity was observed in the standard Ames strains used, as is often the case for compounds containing nitroaromatic groups, this mutagenic activity was lost in strains lacking the classical bacterial nitro-reductase for three of the four compounds evaluated. No mutagenic activity was observed in the *in vitro* micronucleus test using human peripheral lymphocytes for any of the compounds tested, indicating that mammalian cells are unable to reduce these nitroaromatic groups to mutagenic products. To confirm the conclusion that these compounds are unlikely to represent a genotoxic risk for humans, a full regulatory genotoxicity assessment needs

to be completed including *in vivo* mammalian genotoxicity assays (e.g. *in vivo* micronucleus or chromosome aberration test).

Finally, it is clear that this family of 1-ary-4-nitro-1*H*-imidazoles merits further exploration for anti-microbial drug discovery, including the synthesis of new molecules with different substituents on the phenyl group (-SO_2R, -CO_2R) or a different aryl group (e.g. quinoline, pyridine, indole) or a different position of the nitro group on the imidazole ring (e.g. 2-methyl-5-nitroimidazole), which may lead to new antiparasitic activity of interest without genotoxic activity. For instance, several compounds already showed activity of interest against *Trypanosoma cruzi*, which may need further optimization before a drug lead for Chagas disease can be selected in this series.

Material and Methods

Chemistry

General

Melting points (not corrected) were determined in an open capillary or with a Boetius HMK apparatus; ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 (300 MHz for ^{1}H, 75.5 MHz for ^{13}C) or on a Varian 600 (600 MHz for ^{1}H, 150 MHz for ^{13}C) in DMSO-d_6 (unless otherwise specified) and with tetramethylsilane as the internal reference. The chemical shifts (δ) are reported in parts per million and the coupling constants (*J*) in hertz. Elementary analyses (EA) were performed using a Perkin-Elmer CHN automatic analyzer. Mass spectra were recorded using HPLC-MS Integrity Systems with a Termabeam Mass Detector (EI, 70 eV) (with introduction of samples in methanol) or on a GC/MS Perkin Elmer Clarus 600T (with injection of samples in acetone). UV-VIS spectra were recorded on a Hitachi U-2910 spectrometer in water containing 2.5-6% methanol as the solvent.

Synthesis

4.1.2.1. 1,4-Dinitro-1H-imidazoles were obtained by nitration of imidazoles following a known general procedure [35]. CAUTION: 1,4-dinitro-1*H*-imidazoles are potential self-reacting/explosive substances; for the risk associated with their synthesis and handling, see ref. [30,36].

4.1.2.1.1. 1,4-Dinitro-1H-imidazole: yield 70%, white prisms, m. p. 92-94°C, ^{1}H NMR (300 MHz) 8.97 (d, 1H, *J*=1.5 Hz, H-2_{imid}), 9.40 (d, 1H, *J*=1.5 Hz, H-5_{imid}), ^{13}C NMR (75.5 MHz): 115.9 (s, C-5_{imid}), 132.6 (s, C-2_{imid}), 144.3 (s, C-4_{imid}).

4.1.2.1.2. 2-Methyl-1,4-dinitro-1H-imidazole: yield 70%, white needles, m. p. 122-124 °C, ^{1}H NMR (300 MHz) 2.67 (s, 3H, -$CH_{3\ imid}$), 9.26 (s, 1H, H-5_{imid}), ^{13}C NMR (75.5 MHz) 16.2 (s, -$CH_{3\ imid}$), 116.9 (s, C-5_{imid}), 141.4 (s, C-2_{imid}), 142.7 (s, C-4_{imid}).

4.1.2.2. Anilines: Commercially available anilines were used without purification except in a few cases of dark liquids or darkish solids which were distilled or recrystallized prior to use. Other anilines were prepared according to published procedures. Preparation of butyl 5-amino-2-chlorobenzoate [CAS No: 135813-38-6] is described below.

4.1.2.2.1. 4-bromo-3-methylaniline [37]: yield 85%, m. p. 81-82 °C, white powder, ^{1}H NMR (300 MHz) 2.18 (s, 3H, -CH_3), 5.13 (s, 2H, -NH_2), 6.33 (dd, 1H, *J*=8.7 Hz, *J*=2.1 Hz, H-6), 6.52 (d, 1H, *J*=2.1 Hz, H-2), 7.12 (d, 1H, *J*=8.7 Hz, H-2), ^{13}C NMR (75.5 MHz) 22.5, 108.8, 113.5, 116.3, 132.0, 136.8, 148.2.

4.1.2.2.2. 3-bromo-4-methylaniline [38,39] yield 87%, dark yellow oil, ^{1}H NMR (300 MHz) 2.15 (s, 3H, -CH_3), 5.11 (s, 2H, -NH_2), 6.46 (dd, 1H, *J*=8.1 Hz, *J*=2.4 Hz, H-6), 6.78 (d, 1H, *J*=2.4 Hz, H-2), 6.93 (d, 1H, *J*=8.1 Hz, H-5), ^{13}C NMR (75.5 MHz) 21.2, 113.4, 116.8, 122.9, 124.2, 131.0, 148.1.

4.1.2.2.3. 3-bromo-5-methylaniline [40] yield 45%, after distillation colorless oil, b.p. 124-125°$C_{(6mmHg)}$ (lit. 150-154°$C_{(4\ mm\ Hg)}$), ^{1}H NMR (300 MHz) 2.13 (s_b, 3H, -CH_3), 5.26 (s_b, 2H, -HN_2), 6.33-6.34 (m, 1H, Ar-H), 6.45-6.46 (m, 1H, Ar-H), 6.53-6.55 (m, 1H, Ar-H), ^{13}C NMR (75.5 MHz) 20.8, 113.3, 113.3, 118.6, 121.9, 140.2, 150.2.

4.1.2.2.4. Butyl 5-amino-2-chlorobenzoate: A mixture of 2-chloro-5-aminobenzoic acid (3.0 g, 11 mmol), 1-butanol (20.25 g, 270 mmol) and sulfuric acid (1 ml) was heated under reflux for 5 hours in a flask equipped with a Dean-Stark apparatus. After addition of diethyl ether (60 ml), the resulting solution was washed with 3×15 ml of a saturated solution of sodium bicarbonate and then with water to neutral pH. 3-Butoxycarboxy-4-chloroaniline was extracted by washing with 5×15 ml of hydrochloric acid 10%. The aqueous layer was neutralized with solid sodium bicarbonate and the aniline extracted with diethyl ether 4×20 ml. The ethereal layer was separated, dried over magnesium sulfate and evaporated to dryness. 3-Butoxycarbonyl-4-chloroaniline was obtained as a yellowish oil. Yield 24%, ^{1}H NMR (300 MHz, $CDCl_3$) 0.97 (t, 3H, *J* = 7.2 Hz, -C<u>H</u>$_3$), 1.44-1.51 (m, 2H, -C<u>H</u>$_2$-CH_3), 1.72-1.77 (m, 2H, -C<u>H</u>$_2$-CH_2-CH_3), 3.67

(s_b, 2H, -N<u>H</u>$_2$), 4.32 (t, 2H, *J* = 6.6 Hz -O-C<u>H</u>$_2$-CH_2-CH_2-CH_3), 6.70 (dd, 1H, *J* = 8.6 Hz, *J* = 3.0 Hz, H-6), 7.09 (d, 1H, *J* = 3.0 Hz, H-2), 7.18 (d, 1H, *J* = 8.6 Hz, H-5). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.7, 19.3, 30.7, 65.3, 117.2, 118.9, 122.2, 130.9, 131.6, 145.0, 166.1. MS (*m/z*): 227 (M^+, 42%), 171 (100%), 154 (69%).

4.1.2.3. 1-Aryl-4-nitro-1H-imidazoles (general procedure). For compounds **1**-**7**, **10**, **17**, **18** and **22**-**32**, see reference to synthesis given in Table 1. 1-Aryl-4-nitro-1*H*-imidazoles were prepared following the general procedure we have developed in our laboratory [23] with some slight modifications. Equimolar amount of aniline derivative was treated with 1,4-dinitro-1*H*-imidazole in aqueous methanol at ambient temperature in the dark for several hours until complete disappearance of 1,4-dinitro-1*H*-imidazole monitored by TLC. In some cases, the mixture was heated under reflux to complete the reaction. On cooling, the desired crude 1-aryl-4-nitro-1*H*-imidazoles separated from the mixture by precipitation. After filtration and recrystallisation the pure product was obtained. Yields, solvents used for recrystallization and specific data are given below in the respective sections. Further details are available on request.

4.1.2.3.1. 1-(2-bromophenyl)-4-nitro-1H-imidazole (**8**): Yield 61%, light brown powder, m. p. 134-135.5 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 7.54 (td, 1H, *J*=7.5 Hz, *J*=1.5 Hz, Ar-H), 7.62 (td, 1H, *J*=7.5 Hz, *J*=1.5 Hz, Ar-H), 7.71 (dd, 1H, *J*=7.5 Hz, *J*=1.5 Hz, Ar-H), 7.79 (dd, 1H, *J*=7.5 Hz, *J*=1.5 Hz, Ar-H), 8.15 (d, 1H, *J*=1.5 Hz, H-2$_{imid.}$), 8.76 (d, 1H, *J*=1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 119.5, 122.7, 128.9, 129.0, 131.8, 133.5, 134.8, 138.0, 147.4 (s, 1C, C-4$_{imid.}$); EA: calcd. for $C_9H_6BrN_3O_2$ C 40.32, H 2.26, N 15.68; found C 40.24, H 2.30, N 15.68.

4.1.2.3.2. 1-(3-bromophenyl)-4-nitro-1H-imidazole (**9**): Yield 85%, yellowish powder, m. p. 113-114 °C (methanol/water); ^{1}H NMR (300 MHz) 7.53 (t, 1H, *J*=8.1 Hz, H-5'), 7.68 (ddd, 1H, *J*=8.1 Hz, *J*=1.8 Hz, *J*=0.9 Hz, Ar-H), 7.85 (ddd, 1H, *J*=8.1 Hz, *J*=1.8 Hz, *J*=0.9 Hz, Ar-H), 8.14 (t, 1H, *J*=1.8 Hz, H-2'), 8.53 (d, 1H, *J*=1.5 Hz, H-2$_{imid.}$), 9.06 (d, 1H, *J*=1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 119.7, 120.2, 122.5, 124.0, 131.3, 131.6, 135.6, 136.7, 148.1 (s, 1C, C-4$_{imid.}$); UV-VIS λ_{max} =300 nm; EA: calcd. for $C_9H_6BrN_3O_2$ C 40.32, H 2.26, N 15.68; found C 40.27, H 2.32, N 15.91.

4.1.2.3.3. 4-nitro-1-(3-nitrophenyl)-1H-imidazole (**11**): Yield 72%, dark yellow powder, m. p. 229-231 °C (glacial acetic acid); ^{1}H NMR (300 MHz) 7.88 (t, 1H, *J*=8.1 Hz, H-5'), 8.28-8.33 (m, 2H, H-4', H-6'), 8.65 (d, 1H, *J*=1.5 Hz, H-2$_{imid.}$), 8.72 (t, 1H, *J*=2.4 Hz, H-2'), 9.19 (d, 1H, *J*=1.5 Hz, H-5$_{imid.}$); ^{13}C NMR (75.5 MHz) 116.5, 120.0, 123.1, 127.6, 131.4, 135.9, 136.3 (s, 1C, C-1'), 148.24 (s, 1C, C-4$_{imid.}$), 148.51 (s, 1C,

C-3'); EA: calcd. for $C_9H_6N_4O_4$ C 46.16, H 2.58, N 23.93; found C 46.32, H 2.65, N 23.91.

4.1.2.3.4. 4-nitro-1-{3-(trifluoromethyl)phenyl}-1H-imidazole (**12**): Yield 79%, yellowish powder, m. p. 114-116 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 7.80-7.87 (m, 2H, Ar-H), 8.14-8.18 (m, 1H, Ar-H), 8.27-8.28 (m, 1H, Ar-H), 8.62 (d, 1H, *J*=1.6 Hz, H-$2_{imid.}$), 9.16 (d, 1H, *J*=1.6 Hz, H-$5_{imid.}$); ^{13}C NMR (75.5 MHz) 118. 4 (q, *J*=3.9 Hz, C-4'), 119.9 (s, C-$2_{imid.}$), 123.5 (q, J=272.8 Hz, -CF_3), 125.1 (q, *J*=3.7 Hz, C-2'), 125.3 (s, 1C), 130.7 (q, 1C, J=32.6 Hz, C-3'), 131.1 (s, 1C), 135.8 (s, 1C), 136.1 (s, 1C), 148.2 (s, C-$4_{imid.}$); UV-VIS λ_{max} =298 nm; MS: *m/z*: 257 (M^+, 35%), 172 (70%), 145 (100%); EA calcd. for $C_{10}H_6F_3N_3O_2$ C 46.70, H 2.35, N 16.34, found C 46.77, H 2.36, N 16.46.

4.1.2.3.5. 4-nitro-1-{4-(trifluoromethyl)phenyl}-1H-imidazole (**13**): Yield 69%, white powder, m. p. 144-145 °C (methanol/water); ^{1}H-NMR (300 MHz) 7.97 (d, 2H, *J*=8.4 Hz, Ar-H), 8.08 (d, 2H, *J*=8.4 Hz, Ar-H), 8.61 (d, 1H, *J*=1.6 Hz, H-2_{imid}), 9.13 (d, 1H, *J*=1.6 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 119.6 (s, C-$2_{imid.}$), 121.9 (s, C-2', C-6'), 123.7 (q, *J*=270.6 Hz -CF_3), 127.1 (q, *J*=3.7 Hz, C-3', C-5'), 128.7 (q, *J*=32.3 Hz, C-4') 135.7 (s, C-$5_{imid.}$), 138.5-138.5 (m, C-1') 148.3 (s, C-$4_{imid..}$); UV-VIS λ_{max} =307.5 nm (2.4% methanol in water); MS: *m/z*: 257 (M^+, 37%), 172 (69%), 145 (100%); EA calcd. for $C_{10}H_6F_3N_3O_2$ **C** 46.70, H 2.35, N 16.34, found C 46.93, H 2.34, N 16.16.

4.1.2.3.6. 4-nitro-1-{2-(trifluoromethoxy)phenyl}-1H-imidazole (**14**): Yield 71%, white plates, m. p. 87-89 °C (ethyl acetate/hexane), white needels m. p. 90.8-92 °C (diethyl ether); ^{1}H-NMR (300 MHz) 7.62-7.76 (m, 3H, Ar-H), 7.83-7.86 (m, 1H, Ar-H), 8.23 (d, 1H, *J*=1.5 Hz, H-2_{imid}), 8.81 (d, 1H, *J*=1.5 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 119.7 (q, 1C, *J*=259.2 Hz, -OCF_3), 122.2, 122.3, 128.1, 128.4, 128.8, 131.4, 137.8, 141.72-141.74 (m, 1C, C-2'), 147.7 (s, 1C, C-4_{imid}); UV-VIS λ_{max} =294 nm; MS: *m/z*: 273 (M^+, 48%), 188 (64%), 95 (100%). EA calcd for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 44.01, H 2.21, N 15.36.

4.1.2.3.7. 4-nitro-1-{3-(trifluoromethoxy)phenyl}-1H-imidazole (**15**): Yield 31%, white powder, m. p. 92-94 °C (ethanol); ^{1}H NMR (600 MHz) 7.49-7.51 (m, 1H, H-4'), 7.73 (t, 1H, *J*=8.4 Hz, H-5'), 7.91 (dd, 1H, *J*=8.4 Hz, *J*=1.2 Hz, H-2'), 7.98 (s_B, 1H, H-2'), 8.57 (d, 1H, *J*=1.2 Hz H-2_{imid}), 9.10 (d, 1H, *J*=1.2 Hz, H-5_{imid}); ^{13}C NMR (150 MHz) 114.6, 119.7 120.0 (q, 1C, *J*=257.8 Hz, -O<u>C</u>F_3), 120.2, 120.7, 131.7, 135.7, 136.8, 148.2, 149.0; EA calcd for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 44.18, H 2.19, N 15.34.

4.1.2.3.8. 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1H-imidazole (**16**): Yield 84%, light yellow plates, m. p. 129.5-131 °C (methanol/water); ^{1}H NMR (300 MHz): 7.63 (d, 2H, *J*=8.4 Hz, H-2', H-6'), 7.95-7.99 (m, 2H, H-3', H-5'), 8.51 (d, 1H, *J*=1.2 Hz, H-$2_{imid.}$), 9.05 (d, 1H, *J*=1.2 Hz, H-$5_{imid.}$); ^{13}C NMR (75.5 MHz) 120.0 (s, 1C), 120.0 (q, 1C, J=257.0 Hz, -O<u>C</u>F_3), 122.6 (s, 1C), 123.5 (s, 1C), 134.5 (s, 1C), 135.9 (s, 1C), 147.9 (s, 1C, C-4'), 148.2 (s, 1C, C-$4_{imid.}$); UV-VIS λ_{max} =216, 300 nm; MS: *m/z*: 273 (M^+, 47%), 188 (100%), 161 (51%), 95 (91%); EA calcd. for $C_{10}H_6F_3N_3O_3$ C 43.97, H 2.21, N 15.38, found C 43.83, H 2.09, N 14.87.

4.1.2.3.9. 1-(3-methoxyphenyl)-4-nitro-1H-imidazole (**19**): Yield 87%, bright yellow needels, m. p. 151-151.5 °C (methanol/water); ^{1}H NMR (300 MHz) 3.86 (s, 3H, -OCH_3), 7.03-7.08 (m, 1H, H-4'), 7.36-7.39 (m, 1H, H-6'), 7.40-7.41 (m, 1H, H-2'), 7.48 (t, 1H, *J*=8.1 Hz, H-5'), 8.51 (d, 1H, *J*=1.5 Hz, H-$2_{imid.}$), 9.03 (d, 1H, *J*=1.5 Hz, H-$5_{imid.}$); ^{13}C NMR (75.5 MHz) 55.6 (s, 1C, -OCH_3), 106.9 (s, C-2'), 113.0 (s, C-4'), 114.4 (s, C-6'), 119.6 (s, C-5_{imid}), 130.8 (s, C-5'), 135.6 (s, C-2_{imid}), 136.5 (s, C-1'), 148.0 (s, C-4_{imid}), 160.3 (s, C-6'); UV-VIS λ_{max} =303 nm; EA calcd. for $C_{10}H_9N_3O_3$ C 54.79, H 4.14; N 19.17, found: C 54.6, H 4.20, N 19.35.

4.1.2.3.10. butyl 4-(4-nitro-1H-imidazol-1-yl)benzoate (**21**): Yield 53%, bright yellow plates, m. p. 134-135.5 °C (methanol); ^{1}H NMR (600 MHz) 0.96 (t, 3H, *J* = 7.5 Hz, -C<u>H</u>$_3$), 1.45 (sk, 2H, J=7.5 Hz, -C<u>H</u>$_2$-CH_3), 1.70-1.75 (m, 2H, -C<u>H</u>$_2$-CH_2-CH_3), 4.31 (t, 2H, *J*=6.6 Hz, -O-C<u>H</u>$_2$-CH_2-), 7.97-7.99 (m, 2H, Ar-H), 8.09-8.11 (m, 2H, Ar-H), 8.59 (d, 1H, *J*=1.5 Hz, H-2_{imid}), 9.09 (d,-1H, *J*=1.5 Hz, H-5_{imid}); ^{13}C NMR (150 MHz) 13.5, 18.7, 30.2, 64.7, 119.4, 121.1 (s, 2C), 129.52, 130.73 (s, 2C), 135.59, 138.85, 148.32 (s, 1C, C-4_{imid}), 164.67 (s, 1C, C=O); EA calcd. for $C_{14}H_{15}N_3O_4$ C 58.13, H 5.23, N 14.53, found C 58.45, H 5.18, N 14.49.

4.1.2.3.11. 1-(3-chloro-4-fluorophenyl)-4-nitro-1H-imidazole (**33**): Yield 64%, bright yellow prisms, m. p. 115-116 °C (methanol); ^{1}H NMR (300 MHz) 7.63-7.69 (m, 1H, Ar-H), 7.86-7.88 (m, 1H, Ar-H), 8.19-8.20 (m, 1H, Ar-H), 8.48 (s_b, 1H, H-2_{imid}), 9.02 (s_b, 1H, H-5_{imid}), ^{13}C NMR (75.5 MHz) 117.9 (d, 1C, *J*=22.6 Hz, C-5'), 119.9 (s, 1C, C-5_{imid}), 120.8 (d, 1C, *J*=19.1 Hz, C-3'), 122.1 (d, 1C, *J*=7.8 Hz, C-6'), 123.8 (s_b, 1C, C-Ar), 132.5 (d, 1C, *J*=3.1 Hz, C-1'), 135.8 (s, 1C, C-2_{imid}), 148.0 (s, 1C, C-4_{imid}), 156.8 (d, 1C, *J*=247.7 Hz, C-4'); UV-VIS λ_{max} =299 nm; MS: *m/z*: 241 (M^+, 38%), 156 (100%), 129 (87%); EA calcd. for $C_9H_5ClFN_3O_2$ C 44.74, H 2.09, N 17.39, found C 44.77, H 2.14, N 18.32.

4.1.2.3.12. 1-(2-bromo-4-methylphenyl)-4-nitro-1H-imidazole (**34**): Yield 56%, yellowish thin plates, m. p. 144-145 °C (methanol); ^{1}H NMR (300 MHz) 2.41 (s, 3H, -CH_3), 7.41 (dd, 8.0 Hz, *J*=0.9 Hz, H-5'), 7.58 (d, 1H, *J*=8.0 Hz, H-6'), 7.75 (d, 1H, *J*=0.9 Hz, H-3'), 8.12 (d, 1H, *J*=1.5 Hz, H-2_{imid}), 8.73 (d, 1H, *J*=1.5 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 20.3 (s, 1C, -CH_3), 119.1 (s, 1C, C-2'), 122.8 (s, 1C, C-5_{imid}), 128.4 (s, 1C, C-6'), 129.5 (s, 1C, C-5'), 132.3 (s, 1C, C-1'), 133.6 (s, 1C, C-3'), 138.1 (s, 1C, C-4'), 142.2 (s, C-2_{imid}), 147.4 (s, 1C, C-4_{imid}); EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.55, H 2.86, N 14.85.

4.1.2.3.13. 1-(4-bromo-3-methylphenyl)-4-nitro-1H-imidazole (**35**): Yield 88%, yellowish powder, m. p. 161-163 °C (methanol/water); ^{1}H NMR (300 MHz) 2.42 (s, 3H, CH_3), 7.60 (dd, 1H, *J*=8.7 Hz, *J*=2.4 Hz, H-6'), 7.76 (d, 1H, *J*=8.7 Hz, H-5'), 7.87 (d, 1H, *J*=2.4 Hz, H-2'), 8.48 (d, 1H, *J*=1,5 Hz, H-2_{imid}), 8.98 (d, 1H, *J*=1,5 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 22.4, 119.4, 120.3, 123.5, 123.7, 133.3, 135.4, 139.3, 148.1; UV-VIS λ_{max} =229, 303 nm; EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.59, H 2.91, N 15.24.

4.1.2.3.14. 1-(3-bromo-4-methylphenyl)-4-nitro-1H-imidazole (**36**): Yield 70%, bright yellow powder m. p. 147-148 °C (methanol/water); ^{1}H NMR (300 MHz) 2.40 (s, 3H, CH_3), 7.53 (d, 1H, *J*=8.4 Hz, H-5'), 7.74 (dd, 1H, *J*=8.4 Hz, *J*=2.1 Hz, H-6'), 8.12 (d, 1H, *J*=2.1 Hz H-2'), 8.50 (d, 1H, *J*=0.9 Hz, H-2_{imid}), 9.02 (d, 1H, *J*=0.9 Hz, H-2_{imid}); ^{13}C NMR (75.5 MHz) 21.9, 119.6, 120.2, 124.5, 124.7, 131.8, 134.3, 135.5, 137.8, 148.0; UV-VIS λ_{max} = 303 nm; EA calcd. for $C_{10}H_8BrN_3O_2$ C 42.58, H 2.86, N 14.90, found C 42.80, H 2.93, N 14.94.

4.1.2.3.15. 1-(2,4-dimethylphenyl)-4-nitro-1H-imidazole (**37**): Yield 78%, yellowish prisms, m. p. 123-124.5 °C (methanol); ^{1}H NMR (300 MHz) 2.16 (s, 3H, -CH_3), 2.36 (s, 3H, -CH_3), 7.20 (d_b, 1H, *J*=7.8 Hz, H-5'), 7.27 (s_b, 1H, H-3'), 7.33 (d, 1H, *J*=7.8 Hz, H-6'), 8.06 (d, 1H, *J*=1.5 Hz, H-2_{imid}), 8.67 (d, 1H, *J*=1.5 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 17.1 (s, -CH_3), 20.6 (s, -CH_3), 122.5 (s, C-5_{imid}), 126.3 (s, 1C), 127.5 (s, 1C), 131.7 (s, 1C), 132.6 (s, C-2_{imid}), 133.0 (s, C-1'), 137.9 (s, 1C), 139.4 (s, 1C), 147.5 (s, C-4_{imid}); EA calcd. for $C_{11}H_{11}N_3O_2$ C 60.82, H 5.10, N19.34, found C60.70, H 5.05, N 19.37.

4.1.2.3.16. 1-(3,4-dimethylphenyl)-4-nitro-1H-imidazole (**38**): Yield 77%, white powder, m. p. 114.5-116 °C (methanol); ^{1}H NMR (300 MHz) 2.27 (s, 3H, -CH_3), 2.30 (s, 3H, -CH_3), 7.31 (d, 1H, *J*=8.1 Hz, H-5'), 7.50 (dd, 1H, *J*=8.1 Hz, *J*=2.4 Hz, H-6'), 7.60 (d, 1H, *J*=2.4 Hz, H-2'), 8.41 (d, 1H, *J*=1.4 Hz, H-2_{imid}), 8.91 (d, 1H, *J*=1.4 Hz, H-

5_{imid}); ^{13}C NMR (75.5 MHz) 18.8 (s, -CH_3), 19.3 (s, -CH_3), 118.3 (s, 1C), 119.4 (s, 1C), 122.0 (s, 1C), 130.5 (s, 1C), 133.2 (s, C-1'), 135.3 (s, 1C), 136.9 (s, Ar-CH_3), 138.2 (s, Ar-CH_3), 147.9 (s, C-4_{imid}); UV-VIS λ_{max} = 308 nm; EA calcd. for $C_{11}H_{11}N_3O_2$ C 60.82, H 5.10, N 19.34, found C 60.78, H 4.90, N 19.43.

4.1.2.3.17. 1-(2,4-dimethoxyphenyl)-4-nitro-1H-imidazole (**39**): Yield 66.5%, grey powder m. p. 161.5-162 °C (methanol); ^{1}H NMR (300 MHz) 3.85 (s_b, 6H, 2×-OCH_3), 6.67 (dd, 1H, *J*=0.9 Hz, *J*=0.3 Hz, H-5'), 6.82 (d, 1H, *J*=0.3 Hz, H-3'), 7.46 (d, 1H, *J*=0.9 Hz, H-6'), 8.02 (d 1H, *J*=0.2 Hz, H-2_{imid}), 8.56 (d, 1H, *J*=0.15 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 55.7 (s, -OCH_3), 56.2 (s, -OCH_3), 99.6 (s, 1C), 105.2 (s, 1C), 117.6 (s, C-1'), 122.5 (s, C-5_{imid}), 127.0 (s, 1C), 137.9 (s, C-2_{imid}), 147.1 (s, C-4_{imid}), 153.6 (s, Ar-OCH_3), 161.1 (s, Ar-OCH_3); EA calcd. for $C_{11}H_{11}N_3O_4$ C 53.01, H 4.45, N 16.86, found C 53.01, H 4.39, N 16.61.

4.1.2.3.18. 1-(3,4-dimethoxyphenyl)-4-nitro-1H-imidazole (**40**): Yield 82.5%, yelow powder, m. p. 167.5-168.5 °C (methanol); ^{1}H NMR (300 MHz) 2.14 (s, 3H, -$CH_{3\ imid}$), 3.82 (s, 3H, -OCH_3), 3.86 (s, 3H, -OCH_3), 6.68 (dd, 1H, *J*=8.7 Hz, *J*=2.4 Hz, H-5'), 6.82 (d, 1H, *J*=2.4 Hz, H-3'), 7.58 (d, 1H, *J*=8.7 Hz, H-6'), 8.37 (s, 1H, H-5_{imid}); ^{13}C NMR (300 MHz) 12.7 (s, -$CH_{3\ imid}$), 55.7 (s, -OCH_3), 56.1 (s, -OCH_3), 99.5 (s, 1C), 105.3 (s, 1C), 117.1 (s, 1C, C-5_{imid}), 123.7 (s, 1C), 128.8 (s, 1C), 145.9 (s, 1C, C-4_{imid}), 146.0 (s, 1C, C-1'), 154.8 (s, Ar-OCH_3), 161.6 (Ar-OCH_3); EA calcd for $C_{12}H_{13}N_3O_4$ C 54.75, H 4.98, N 15.96, found C 54.86, H 4.86, N 16.05.

4.1.2.3.19. 1-(benzo[d][1,3]dioxol-5-yl)-4-nitro-1H-imidazole (**41**): Yield 58%, grey powder, m. p. 206-207 °C (acetone); ^{1}H NMR (300 MHz) 6.15 (s, 2H, -CH_2-), 7.09 (d, 1H, *J*=8.4 Hz, H-5'), 7.26 (dd, 1H, *J*=2.4 Hz, H-6'), 7.26 (dd, 1H, *J*=2.4 Hz, H-6'), 7.47 (d, 1H, *J*=2.4 Hz, H-2'), 8.36 (d, 1H, *J*=1.7 Hz, H-2_{imid}), 8.89 (d, 1H, *J*=1.7 Hz, H-5_{imid}); ^{13}C NMR (75.5 MHz) 102.2, 103.2, 108.6, 115.0, 120.0, 129.7, 135.7, 147.4, 147.8, 148.2. MS: *m/z*: 233 (M^+, 100.0%); EA calcd. for $C_{10}H_7N_3O_4$ C 51.51, H 3.03, N 18.02, found C 51.64, H 3.00, N 17.95.

4.1.2.3.20. butyl 2-chloro-5-(4-nitro-1H-imidazol-1-yl)benzoate (**42**): Yield 37%, yellowish powder, m. p. 88-90 °C (methanol); ^{1}H NMR (600 MHz) 0.94 (t, 3H, *J* = 7.5 Hz, -C<u>H</u>$_3$), 1.41-1.47 (m, 2H, -C<u>H</u>$_2$-CH_3), 1.70-1.75 (m, 2H, -C<u>H</u>$_2$-CH_2-CH_3), 4.34 (t, 2H, *J* = 6.6 Hz, -O-C<u>H</u>$_2$-CH_2-CH_2-CH_3), 7.81 (d, 1H, *J* = 9.0 Hz, H-5'), 8.02 (dd, 1H, *J* = 9.0 Hz, *J* = 3.0 Hz, H-6'), 8.20 (d, 1H, *J* = 3.0 Hz, H-2'), 8.53 (d, 1H, *J* = 1.2 Hz, H-2_{imid}), 9.06 (d, 1H, *J* = 1.2 Hz, H-5_{imid}). ^{13}C NMR (150 MHz) 13.5, 18.6, 30.0, 65.5, 119.9, 123.3, 125.5, 131.0, 132.0, 132.1, 134.4, 135.8, 148.1 (s, 1C, C-4_{imid}), 164.39

(s,1C, C=O). EA calcd. for $C_{14}H_{14}ClN_3O_4$ C 51.94, H 4.36, N 12.98, found C 52.12, H 4.44, N 12.78.

4.1.2.3.21. 2-chloro-5-(4-nitro-1H-imidazol-1-yl)benzoic acid (**43**): Yield 89%, light brown prisms, m. p. 266-268 °C (methanol), ^{1}H NMR (600 MHz) 7.77 (d, 1H, *J* = 8.4 Hz, H-3), 7.98 (dd, 1H, *J* = 8.4 Hz, *J*=3.0 Hz, H-4), 8.20 (d, 1H, *J* = 3.0 Hz, H-6), 8.53 (d, 1H, *J* = 1.2 Hz, H-2_{imid}), 9.07 (d, 1H, *J*=1.2 Hz, H-5_{imid}), 13.77 (s_b, 1H, -COOH). ^{13}C NMR (150 MHz) 119.8, 123.2, 124.9, 131.0, 131.9, 133.2, 134.2, 135.7, 148.1 (s,1C, C-4_{imid}), 165.8 (s, 1C, C=O); EA calcd. for $C_{10}H_6ClN_3O_4$ C 44.88, H 2.26, N 15.70, found C 44.96, H 2.31, N 15.92.

4.1.2.3.22. 2-methyl-4-nitro-1-{4-(trifluoromethoxy)phenyl}-1H-imidazole (**44**): Yield 73.5%, bright yellow powder, m. p. 130-132 °C (methanol-chloroform); ^{1}H NMR (300 MHz) 2.32 (s, 3H, -$CH_{3\ imid}$), 7.59-7.62 (m, 2H, Ar-H), 7.74-7.79 (m, 2H, Ar-H), 8.61 (s, 1H, C-5_{imid}). ^{13}C NMR (75.5 MHz) 13.4 (s 1C, -$CH_{3\ imid}$), 120.0 (q, *J*=257.3 Hz, -CF_3), 122.2 (s, 2C), 122.7 (s, 1C, C-2_{imid}), 128.1 (s, 2C), 134.6 (s, 1C, C-1'), 144.8 (s, 1C, C-5_{imid}), 146.2 (s, 1C, C-4_{imid}), 148.5 (s_b, 1C, C-4'); EA calcd. for $C_{11}H_8F_3N_3O_2$ C 46.00, H 2.81, N 14.63, found C 45.66, H 2.70, N 14.35.

4.1.2.3.23. 2-methyl-4-nitro-1-{4-(trifluoromethyl)phenyl}-1H-imidazole (**45**): Yield 66%, yellow powder, m. p. 96-98 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz) 2.37 (s, 3H, -$CH_{3\ imid}$), 7.86 (d, 2H, *J*=8.4 Hz, Ar-H), 8.00 (d, 2H, J=8.4 Hz, Ar-H), 8.67 (s, 1H, H-5_{imid}); ^{13}C NMR (75.5 MHz) 13.5 (s, 1C, -$CH_{3\ imid}$), 122.5 (s, C-2_{imid}), 123.7 (q, *J*=272.1 Hz, -CF_3), 126.8 (m, C-2', C-3', *C*-5', C-6'), 129.0 (d, *J*=1.3 Hz, C-1'), 144.7 (s, 1C, C-5_{imid}), 146.3 (s, 1C, C-4_{imid}); EA calcd. for $C_{11}H_8F_3O_2N_3$ C 48.72, H 2.97, N 15.49, found C 48.66, H 2.63, N 14.33.

4.1.2.3.24. 1-(2-bromo-4-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**46**): Yield 40%, yellow powder, m. p. 126-128 °C (ethyl acetate/hexane), ^{1}H NMR (300 MHz) 2.14 (s, 3H, -$CH_{3\ imid}$), 2.42 (s, 3H, -CH_3), 7.24 (dd, 1H, *J*=7.8 Hz, *J*=0.9 Hz, H-5'), 7.59 (d, 1H, *J*=7.8 Hz, H-6'), 7.76 (d, 1H, *J*=0.9 Hz, H-3'), 8.54 (s, 1H, H-5_{imid}); ^{13}C NMR (75.5 MHz) 12.8 (s, -$CH_{3\ imid}$), 20.3 (s, -CH_3), 120.4, 123.1, 129.0, 129.7, 132.1, 133.6, 142.6, 145.3, 146.2 (s, 1C, C-4_{imid}); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 45.01, H 3.46, N 14.09.

4.1.2.3.25. 1-(3-chloro-4-fluorophenyl)-2-methyl-4-nitro-1H-imidazole (**47**): Yield 72%, white powder, m. p. 177-178 °C (methanol/water); ^{1}H NMR (300 MHz) 2.32 (s, 3H, -CH_3), 7.63-7.72 (m, 2H, Ar-H), 7.99-8.02 (m, 1H, Ar-H), 8.59 (s, 1H, H-5_{imid}); ^{13}C NMR (75.5 MHz) 13.3 (s, 1C, -$CH_{3\ imid}$), 117.7 (d, 1C, *J*=22.5 Hz, C-5'), 120.4 (d, 1C,

J=19.0 Hz, C-3'), 122.8 (s, 1C, C-5_{imid}), 127.1 (d, 1C, J=8.2 Hz, C-Ar), 128.6 (s, 1C, C-Ar), 132.6 (d, 1C, *J*=3.5 Hz, C-1'), 145.0 (s, 1C, C-2_{imid}), 146.0 (s, 1C, C-4_{imid}); EA calcd. for $C_{10}H_7ClFN_3O_2$ C 46.99, H 2.76, N 16.44, found C 47.02, H 2.97, N 16.97.

4.1.2.3.26. 1-(4-bromo-3-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**48**): Yield 64%, yellowish powder, m. p. 156-158 °C (methanol/water); ^{1}H NMR (300 MHz)2.33 (s, 3H, $CH_{3\ imid}$), 2.42 (s, 3H, CH_3), 7.38 (dd, 1H, *J*=8.7 Hz, *J*=2.7 Hz, H-6') 7.62 (d, 1H, *J*=2.7 Hz, H-2'), 7.79 (d, 1H, *J*=8.7 Hz, H-5'), 8.54 (s, 1H, H-5_{imid}); ^{13}C NMR (75.5 MHz) 13.4 (s, 1C, -$CH_{3\ imid}$), 22.4 (s, 1C, -CH_3), 125.0 (s, 2C), 128.2 (s, 1C), 133.1 (s, 1C), 135.0 (s, 1C), 139.1 (s, 1C), 144.6 (s, 1C), 146.1 (s, 1C); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 44.75, H 3.38, N 14.29.

4.1.2.3.27. 1-(3-bromo-4-methylphenyl)-2-methyl-4-nitro-1H-imidazole (**49**): Yield 69%, light brown powder, m. p. 150-152 °C (methanol/water); ^{1}H NMR (300 MHz) 2.31 (s, 3H, $CH_{3\ imid}$), 2.43 (s, 3H, CH_3), 7.53 (dd, 1H, *J*=8.1 Hz, *J*=1,8 Hz, H-6'), 7.57 (d, 1H, *J*=8.1 Hz, H-5'), 7.90 (d, 1H, *J*=1.8 Hz, H-2'), 8.57 (s, 1H, H-5_{imid}); ^{13}C NMR (75.5 MHz) 13.4 (s, 1C, $CH_{3\ imid}$), 22.1 (s, 1C, CH_3), 122.7 (s, 1C) 124.3 (s, 1C), 125.1 (s, 1C), 129.2 (s, 1C), 131.6 (s, 1C), 129.2 (s, 1C), 131.6 (s, 1C), 134.5 (s, 1C), 138.9 (s, 1C), 144.8 (s, 1C, C-5_{imid}), 146.0 (s, 1C, C-4_{imid}); EA calcd. for $C_{11}H_{10}BrN_3O_2$ C 44.62, H 3.40, N 14.19, found C 44.60, H 3.49, N 14.57.

Antiprotozoal activity testing

Drug preparation

For the *in vitro* assays, a 10 mg/ml stock solution in dimethyl sulfoxide (DMSO) of the test compounds was prepared. For the *in vivo* studies, the test compounds were dissolved in dimethyl sulfoxide (DMSO), the resulting solution was further diluted with water up to 10% in volume. Drugs were administered to mice by gavage (oral administration) or intraperitoneal injection (i.p. administration).

In vitro trypanocidal and cytotoxicity assays

The compounds were tested in Minimum Essential Medium (50 µl) with Earle's salts, supplemented [41] with the following modifications: 2-mercaptoethanol 0.2 mM, Na-pyruvate 1 mM, hypoxanthine 0.5 mM and 15% heat-inactivated horse serum. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. Then $3x10^4$/ml bloodstream forms of *T. b. rhodesiense* STIB900 in 50 µl medium was added to each well and the plate was incubated at 37°C under a 5% CO_2

atmosphere for 70h. 10 µl of the viability marker Alamar blue (12.5 mg resazurin [Sigma] dissolved in 100 ml phosphate buffered saline) was then added to each well and the plates incubated for an additional 2-4 hours to determine cell viability [42]. The assay was assessed by reading the fluorescence in each well at an excitation wavelength of 536 ηm and at an emission wavelength of 588 ηm. The IC_{50}s were calculated from the sigmoidal inhibition curves using SoftmaxPro software.

For the cytotoxicity assessment, $4x10^4$/ml L-6 rat skeletal myoblast cells were seeded in 96 well plates. Test compounds were prepared and added as above. Incubations and assessment of cell viability were carried out as for parasite cultures.

***In vivo* trypanocidal assays**

Acute infection with *Trypanosoma brucei rhodesiense* (STIB900): Groups of 4 mice were infected by intraperitoneal injection with 10^4 bloodstream forms of *T. b. rhodesiense* (STIB900) and treated with the test compounds or reference drugs once or twice daily for 4 consecutive days, starting on day 3 post-infection [33]. A control group was infected but not treated. Parasitaemia was monitored using smears of tail-snip blood twice a week after treatment for two weeks followed by once a week until 60 days post-infection. Mice were considered cured if there was no parasitaemia relapse detected in tail blood over the 60 days observation period.

Chronic CNS infection with *Trypanosoma brucei brucei* (GVR35): Groups of 5 mice were infected intraperitoneally with $2x10^4$ bloodstream forms of *T. b. brucei* (GVR35) and treated with the test compounds or reference drugs once or twice daily from day 21 post-infection for 5 days [33,43]. A control group was treated on day 21 with a single dose of diminazene aceturate at 40 mg/kg i.p., which is subcurative as it clears the trypanosomes only in the hemolymphatic system but not in the CNS leading to a subsequent reappearance of trypanosomes in the blood. Parasitemia was monitored twice in the first week after treatment followed by once a week until 180 days post infection. Mice were considered cured when there was no parasitemia relapse detected in tail blood over the 180 days observation period [33,43].

All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

Genotoxicity assays

Drug preparation

For all tests, stock solutions were prepared by formulating the test compound in DMSO under subdued lighting conditions with the aid of vortex mixing, warming at 37°C and ultrasonication, immediately prior to assay to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 4 hours of initial formulation.

Bacterial mutagenicity tests

Strains: *Salmonella typhimurium* strains TA1535, TA1537, TA100, TA98 and TA102 were obtained from the UK NCTC. TA100NR and TA98NR, which lack the classical *Salmonella typhimurium* nitro-reductase, were obtained from Novartis Pharma AG (Switzerland) and the University of York CRU (UK) respectively. It is known that TA98 and TA98NR are also deficient in a second 'supplementary' nitro-reductase [34]. TA1535NR and derivatives of TA1535, TA1537 and TA102 containing knock-outs of the classical nitro-reductase gene were constructed and kindly supplied by Dr Masami Yamada, National Institute of Health Sciences, Tokyo (Japan).

Ames tests: Standard bacterial plate incorporation assays were carried out, using triplicate plating, essentially as described by [44]. Tests were carried out with and without rat liver post-mitochondrial fraction plus co-factors (S9 mix) to provide a mammalian metabolic activation system. The S9 fraction was obtained from Molecular Toxicology Incorporated USA and was prepared from Sprague-Dawley rats pretreated with the mixed cytochrome P 450 enzyme inducer Aroclor 1254. After incubation at 37 °C for three days, plates were scored for mutant colonies using a Seescan Colony Counter (Seescan plc) plate reader.

In the absence of rat liver S9, the positive controls used were for TA98 and TA98NR 4-nitoquinoline-1-oxide and for TA1535 and TA1535NR, TA100 and TA100NR, sodium azide. For TA1537, 9-aminoacridine was used and for TA102, mitomycin C. In the presence of S9, 2-aminoanthracene was used for all strains. 2-Nitrofluorene and nitrofurantoin were used as additional positive controls to check the effects of loss of the nitro-reductase enzymes in the NR strains on the detection of nitro-containing compounds. In the presence of S9, the positive controls used were benzo(a)pyrene for the TA98 strains and 2-aminoantracene for the TA100 strains.

***In vitro* micronucleus tests**

Lymphocytes were obtained from healthy, non-smoking female donors. Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into a sufficient volume of HEPES-buffered RPMI medium containing 20% (v/v) heat inactivated fetal calf serum and 50 μg/mL gentamycin. The mitogen, phytohaemagglutinin (PHA), reagent grade, was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37 ± 1°C and rocked continuously for 48 hours prior to treatment. Quadruple cultures were used for the negative control and duplicate cultures for the test compounds and the positive control. Tests were carried out with and without rat liver S9-mix.

Preliminary tests were carried out to determine the effects of the test compounds to determine concentrations that induced cytotoxicity. Data from concentrations exhibiting high cytotoxicity (>55%) were excluded from the analysis.

a) Protocol used for compound **16**

The test compound was added at 48 hours following culture initiation (stimulation by PHA). Cells were exposed to the test compound for 3 hours followed by a 21-hour recovery period (+/-S9). In addition (–S9), a further group was exposed for 24 hours with no further recovery time, to explore the effects of extended exposure. Cytochalasin-B (6 μg/mL per culture), was added at the time of treatment. This generates binucleate cells by preventing cytokinesis, without preventing nuclear division. Scoring binucleate cells ensures that the cells scored have passed through one cell division. Several drops of cells suspended in fixative were spread onto multiple clean, dry microscope slides. After the slides were dried the cells were stained for 5 minutes in filtered 4% (v/v) Giemsa in pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips for scoring. One thousand binucleate cells were scored per replicate for the presence of micronuclei, the scoring of such cells ensures that the micronuclei seen have resulted from the last nuclear division. Slides were also examined for the proportions of mononucleate and binucleate cells per culture.

Measurements were made to check changes in the osmolality of the culture in the presence of the highest concentration of the test compounds and also for any pH changes. Studies have shown that significant changes in osmolality and pH can induce false positive results in these assays.

The positive controls used were mitomycin C (0.08 µg/mL) and vinblastine (0.03 µg/mL) for the cultures without S9 and cyclophosphamide (12.5 µg/mL), which requires metabolism by S9 to generate clastogenic metabolites, for cultures with S9.

b) Screening protocol used for compounds **14**, **31** and **33**

As above, but a 20 hour treatment was used in the absence of rat liver S9, with a 28 hour recovery period. In the presence of S9, a 3 hour treatment period was used, with a 45 hour recovery period. For compound **33**, quadruple cultures were used for the vehicle control and duplicate cultures for the test compound. For compounds **31** and **14**, duplicate cultures were scored for the vehicle control and single cultures for the test compounds. The positive controls used were 4-Nitroquinoline-N-oxide (2.5 or 5.0 µg/mL) in the absence of S9 and cyclophosphamide (6.25 or 12.5 µg/mL) in the presence of S9.

Redox potential measurement

One-electron reduction potentials were determined by pulse radiolysis following an established procedure [22].

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Contributors

B Bourdin Trunz identified the 1-aryl-4-nitro-1H-imidazoles of the present study as potential new drug candidates for HAT, and coordinated the chemistry work, the parasitology and genotoxicity studies with partners as part of DNDi's nitroimidazoles

project. J Suwinski and R Je˛drysiak designed and synthesized the molecules, the anrorc coupling reaction was developed in the laboratory of J Suwinski; B Bourdin Trunz, J Suwinski and R Jedysiak contributed to the structureeactivity relationship (SAR) analysis. D Tweats acted as a toxicology expert consultant for the design and interpretation of the genotoxicity studies. M Kaiser and R Brun designed, conducted and interpreted the parasitology studies. E Torreele was the overall project leader of DNDi's nitroimidazoles project. B Bourdin Trunz drafted the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interests.

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The Ames tests and the *in vitro* cytogenetic experiments were contracted out to Covance Laboratories Ltd, Harrogate, UK.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.01.071.

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CHAPTER 5

Novel 3-Nitro-1H-1,2,4-triazole-based Aliphatic and Aromatic Amines as anti-Chagasic Agents

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[a]Abbreviations: T. cruzi, Trypanosoma cruzi; T. brucei, Trypanosoma brucei; HAT, human African trypanosomiasis; Nfx, nifurtimox (4-(5-nitrofurfurylindenamino)-3-methylthio-morpholine-1,1-dioxide); Bnz, benznidazole (N-benzyl-2-(2-nitro-1Himidazol- 1-yl)acetamide); NTR, type I nitroreductase; TbNTR, T. brucei NTR; DNDi, Drugs for Neglected Diseases initiative; SI, selectivity index; SARs, structure-activity relationships; E1/2, reduction potential; tet, tetracycline.

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Abstract

A series of novel 2-nitro-1H-imidazole- and 3-nitro-1H-1,2,4-triazole-based aromatic and aliphatic amines were screened for anti-trypanosomal activity and mammalian cytotoxicity by the Drugs for Neglected Diseases initiative (DNDi). Out of 42 compounds tested, eighteen 3-nitro-1,2,4- triazoles and one 2-nitroimidazole displayed significant growth inhibitory properties against *T. cruzi* amastigotes (IC50 ranging from 40 nM to 1.97 μM), without concomitant toxicity towards the host cells (L6 cells), having selectivity indices (SI) 44 to 1320. Most (16) of these active compounds were up to 33.8-fold more potent than the reference drug benznidazole, tested in parallel. Five novel 3-nitro-1,2,4-triazoles were active against bloodstream form (BSF) *T. b. rhodesiense* trypomastigotes (IC50 at nM levels and SI 220 to 993). An NADH-dependent nitroreductase (TbNTR) plays a role in the anti-parasitic activity, since BSF *T. b. brucei trypomastigotes* with elevated TbNTR levels were hypersensitive to tested compounds. Therefore, a novel class of affordable 3-nitro-1,2,4-triazole-based compounds with antitrypanosomal activity has been identified.

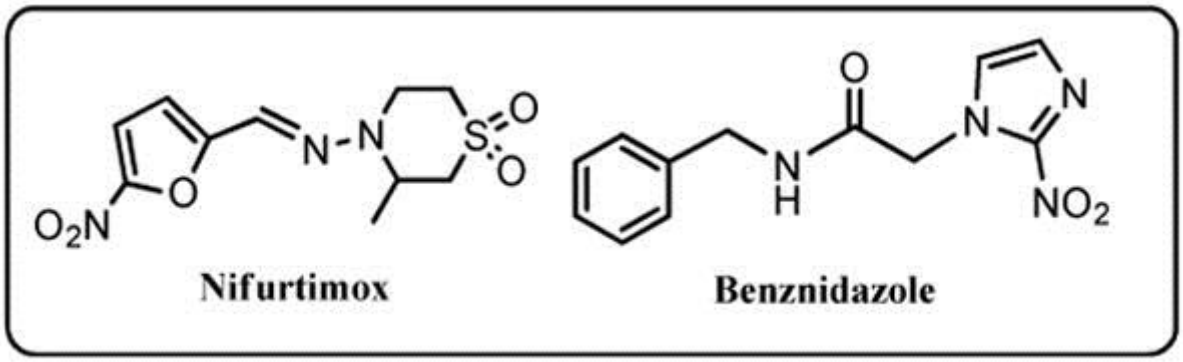
Keywords

nitrotriazoles; amines; T. cruzi; Chagas disease; antitrypanosomal agents

Introduction

The protozoan parasites *Trypanosoma cruzi* (*T. cruzi*)[a], *Trypanosoma brucei* (*T. brucei*), and various Leismania species, also referred to as trypanosomatids, are the causative agents of Chagas disease, Human African Trypanosomiasis (HAT) and different forms of leishmaniasis, respectively. Over 10 million people are infected by *T. cruzi* and 50,000 to 80,000 by *T. b. gambiense* or *T. b. rhodesiense*, resulting in more than 40,000 deaths per year.[1] Chagas disease is transmitted by blood sucking triatomine insects and occurs mainly in Latin America. Although over the past 20 years the number of incidences has declined, primarily due to vector control initiatives,[2] the number of cases in non-endemic regions such as the United States is on the rise.[3] Reasons for this rise include population migration, drug usage and medical practices. With no immediate prospect for vaccines, chemotherapy is the only way to fight the parasite in the patient.

Chart 1

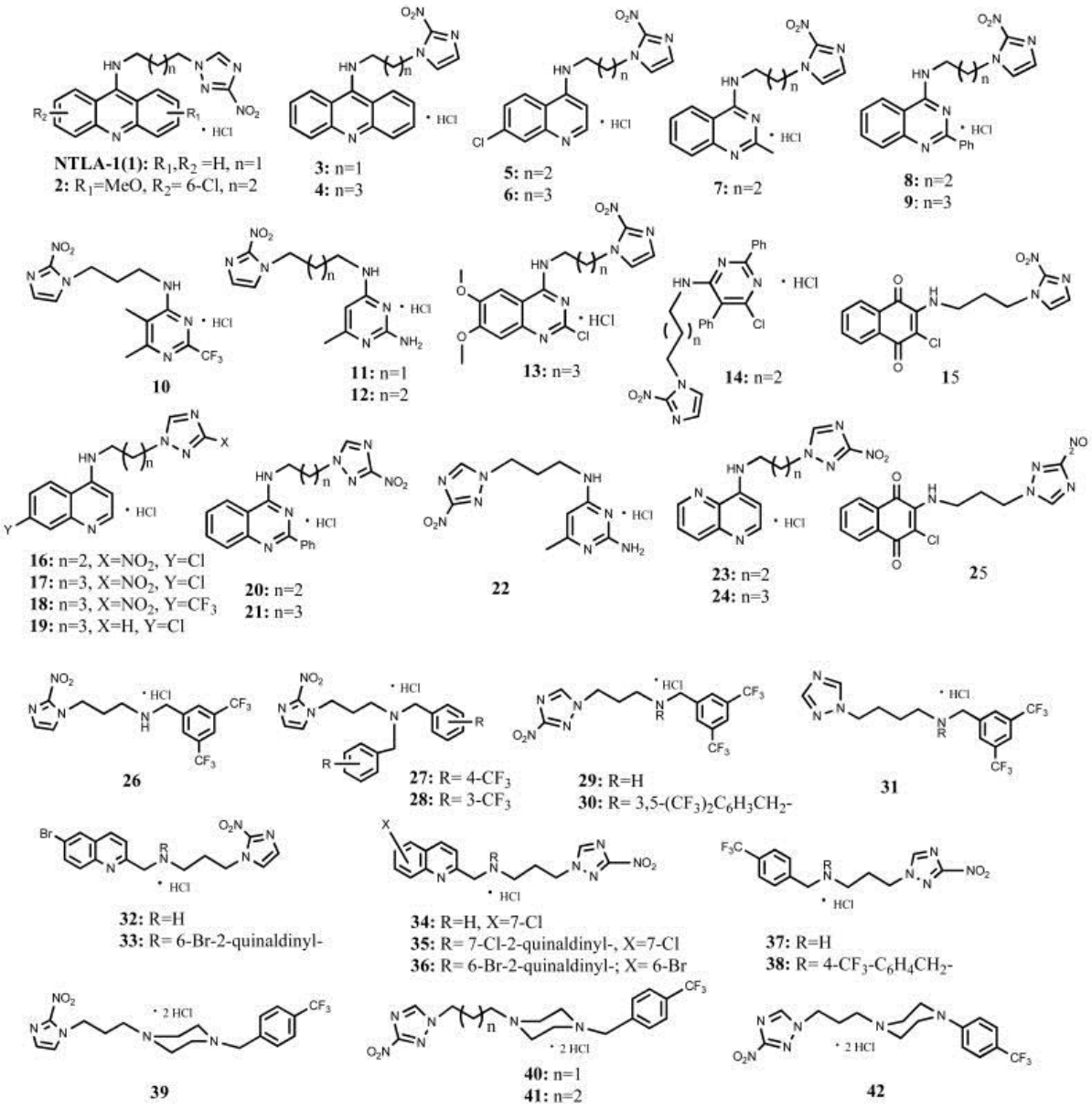


Currently two nitroheterocycle prodrugs, Nifurtimox (4-(5-nitrofurfurylidenamino)-3-methylthio-morpholine-1,1-dioxide) (Nfx) and Benznidazole (N-benzyl-2-(2-nitro 1Himidazol- 1-yl)acetamide) (Bnz) (Chart 1), are used to treat Chagas disease.[4] However, their use is problematic as both can cause side effects, have limited efficacy, while some strains are refractory to treatment.[5] In addition, the large quantities of medication required render it expensive, and the recommended long course of treatment is often not completed, resulting in the development of resistance. Therefore, the need for new drugs to treat this disease is urgent.

As with most nitroheterocyclic compounds, Nfx and Bnz both function as prodrugs and must undergo activation before mediating their cytotoxic effects. Initially it was proposed that the trypanocidal action of Nfx was due to its ability to induce oxidative stress within the parasite[5-7] and several trypanosomal flavoproteins have been shown to mediate the 1- electron reduction of this prodrug's conserved nitro-group that subsequently promotes formation of superoxide anions via a futile cycle.[7-9] However, although this reaction does occur in parasite cells, the available functional data suggests that it does not occur at levels that are toxic to the trypanosome.[10] Recently, an alternative reduction pathway has been elucidated involving the activity of a type I nitroreductase (NTR).[11] This enzyme can mediate a series of 2 electron reduction reactions of both Nfx and Bnz resulting in fragmentation of the heterocyclic ring and production of toxic metabolites.[10,12]

Recent reports about several new nitroheterocycles having trypanocidal activities with no or low toxicity, [13-18] in conjunction with the fact that the activation of nitroheterocyclic prodrugs can be catalyzed by the type I NTR, which is normally absent in most eukaryotes, with trypanosomes being a major exception, have led to a renewed interest in the use of these compounds as antiparasitic agents.

Chart 2



In collaboration with the Drugs for Neglected Diseases initiative (DNDi), we have found that 9-[(3-nitro-1H-1,2,4-triazolyl)-propylamino]acridine hydrochloride (NTLA-1 or NLA-6, 1; Chart 2), a compound that was originally designed as a DNA-targeting anticancer agent,[19,20] and which was screened against *T. cruzi*, *T. b. rhodesiense* and *L. donovani*, was significantly and selectively active against *T. cruzi* amastigotes in infected L6 myoblasts, without showing toxicity for the host cells.[21] Thus, NTLA-1 demonstrated an IC50 of 140 nM for the parasite and a selectivity index (SI = IC50 for L6 cells/IC50 for *T. cruzi*) of 146.[21] NTLA-1, given at just 2 mg/kg/day, for 50 days in mice infected with *T. cruzi*, in the acute phase of infection, resulted in a rapid and

persistent drop in peripheral parasite levels and in a fraction of cures (20%).[22] Importantly, there was an absolute correlation between treatment efficacy as determined parasitologically and the increase in the fraction of *T. cruzi*-specific CD8+ T cells with a T central memory phenotype in the peripheral blood of treated mice.[22] However, NTLA-1, which inhibits topoisomerase I and II,[20] demonstrated toxicity at 15 mg/kg given i.p. for 30 days. Therefore, a more thorough investigation was initiated for the development of less toxic and more efficacious nitrotriazole- and nitroimidazole-based compounds as trypanocidal agents. Here we describe the synthesis and in vitro evaluation of 3-nitro-1H-1,2,4-triazole-based and 2- nitroimidazole-based aromatic and aliphatic amines as antiparasitic agents.

Chemistry

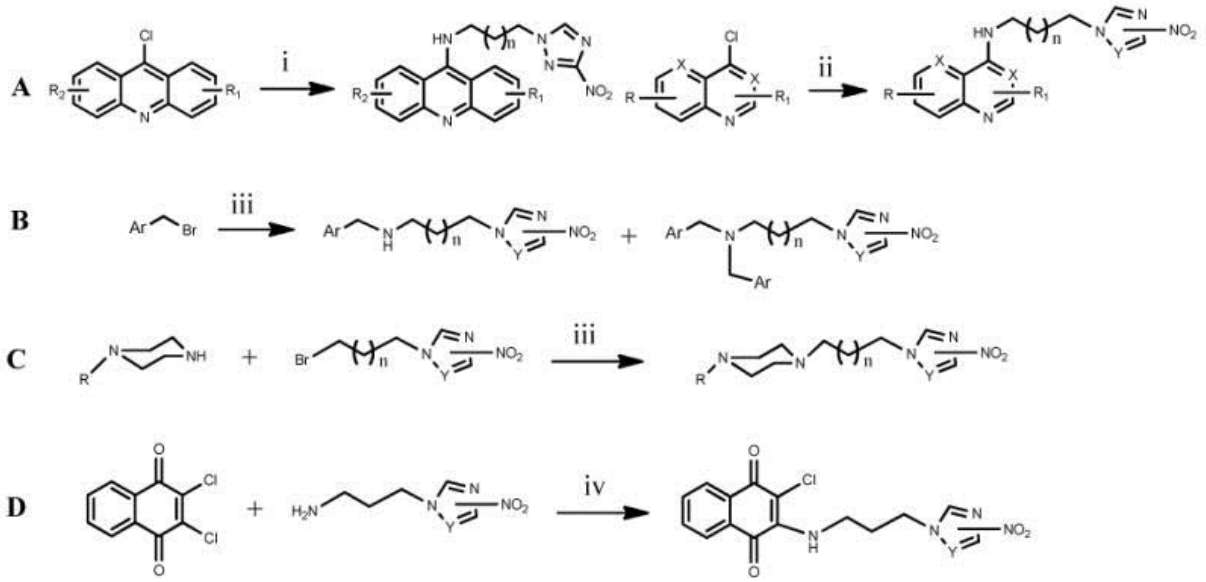
The structure of all compounds is depicted on Chart 2. Their synthesis is straightforward and based on well-established chemistry, outlined in Scheme 1. Aromatic amines **1-14** and **16-24** were synthesized by coupling the appropriate nitrotriazole- or nitroimidazole alkyl amine[19] with the appropriate chloro- or fluoro-aromatic chromophore[23] by nucleophilic aromatic substitution (Scheme 1A). The yields were in general moderate to good with the exception of **18**. Aliphatic secondary and tertiary amines **26-38** were synthesized via the same reaction by nucleophilic attack of the appropriate nitrotriazole- or nitroimidazole alkyl amine to a chosen bromide in the presence of K_2CO_3 (Scheme 1B). In most cases the monoalkylated product was the dominant one, since 1 equivalent of the required halide was used. Piperazine derivatives **39-42** were synthesized similarly, by nucleophilic attack of the appropriate, commercially available mono-substituted piperazine to the appropriate nitrotriazole- or nitroimidazole alkyl bromide[24] (Scheme 1C). Finally, enamines **15** and **25** were synthesized from 2,3-dichloro-1,4-naphthoquinone and 2-nitroimidazole-propylamine or 3-nitro-1,2,4-triazole-propylamine, respectively, by nucleophilic substitution (Scheme 1D).

Results and Discussion

Anti-proliferative effects of nitrotriazole and nitroimidazole compounds

The in vitro growth inhibitory properties of all compounds against *T. b. rhodesiense* bloodstream form trypomastigotes, *T. cruzi* amastigotes (in infected L6 myoblasts),

Schema 1



i)EtOH, reflux, 12 h; ii)propanol, reflux 12-30 h; iii)K_2CO_3 (9 eq), CH_3CN, RT, 48 h; iv) CH_2Cl_2, RT.
n=1 or 2; X= C or N; Y=C (2-NO_2) or N (3-NO_2); Other substituents vary.

axenically cultured *L. donovani* amastigotes and rat skeletal myoblasts (L6 cells) were evaluated by using standard drug screens[25]. From resultant dose response curves, IC50 values in μM were determined (Table 1). The criteria for activity were set as follows: For *T. b. rhodesiense,* compounds that gave an IC50 < 0.5 μM, were designated as 'active', while those yielding an IC50 = 0.5-6.0 μM or an IC50 > 6.0 μM were designated 'moderately active' and 'inactive', respectively. For T. cruzi, IC50 < 4.0 μM, 'active'; IC50 = 4.0-60 μM, 'moderately active'; IC50 > 60 μM, 'inactive'. For *L. donovani*, IC50 < 1 μM, 'active'; IC50 = 1.0-6.0 μM, 'moderately active'; IC50 > 6.0 μM, 'inactive'. Based on these criteria, all but compounds **19** and **31** were active or moderately active against *T. cruzi*, about 66 % of all compounds were active or moderately active against *T. brucei rhodesiense*, and only 6 compounds (~ 14%) were active or moderately active against *L. donovani* parasites. However, for a compound to be considered for further in vivo investigation, the growth inhibitory effect against the mammalian cell line L6 has to be evaluated from which a measure of a compound's cytotoxicity can be deduced. Thus, the selectivity index (SI), namely the ratio of IC50 against L6 cells to IC50 against each parasite, is also an important parameter. This SI must be ≥ 100 for *T. b. rhodesiense*, ≥ 50 for *T. cruzi* and ≥ 20 for *L. donovani* axenic amastigotes.

Based on the above, only 6 compounds (**16-18, 24, 36, 38**) were active and selective against

Table 1: In vitro screening data against three different trypanosomatids

Compound	All values as µM							Compound Type[e]
	T.b.rhodesiense[a]		*T. cruzi*[b]		*L.donovani axen.*[c]		L6 cells[d]	
	IC-50	SI	IC-50	SI	IC-50	SI	IC-50	
1	0.61	34	0.140	147	35.4	1	20.50	Nitro-Trz
2	0.134	74	0.151	66	10.2	1	9.94	Nitro-Trz
3	0.996	<1	0.996	<1	9.09	<1	0.309	Nitro-im
4	0.60	3	0.926	2	14.8	<1	1.68	Nitro-im
5	1.40	18	1.04	24	54.4	<1	25.0	Nitro-im
6	1.87	36	3.51	19	39.1	2	66.8	Nitro-im
7	3.46	9	2.10	15	68.3	<1	31.6	Nitro-im
8	3.92	22	1.97	45	10.1	9	87.8	Nitro-im
9	2.76	12	3.63	9	6.17	5	32.5	Nitro-im
10	15.2	>16	4.28	>55	20.1	>12	>236	Nitro-im
11	10.3	>28	7.08	>41	>95.7	~3	>287	Nitro-im
12	11.8	>23	41.8	>7	98.1	>3	>275	Nitro-im
13	2.26	7	0.968	17	3.63	5	16.5	Nitro-im
14	15.5	1	9.67	1	12.4	1	11.7	Nitro-im
15	0.62	6	30.1	<1	0.516	8	3.9	Nitro-im
16	0.309	463	0.607	236	45.8	3	143	Nitro-Trz
17	0.193	708	0.140	976	64.5	2	137	Nitro-Trz
18	0.117	973	0.305	373	137.0	1	114	Nitro-Trz
19	21.8	>14	92.1	>3	>299	~1	>299	Trz
20	1.42	68	1.48	66	9.48	10	97.0	Nitro-Trz
21	0.562	137	1.74	44	8.69	9	77.0	Nitro-Trz
22	2.19	>131	33.7	>8	>95.4	~3	>286	Nitro-Trz
23	2.22	60	1.03	129	182	1	133	Nitro-Trz
24	0.435	220	0.461	209	>275	<1	95.8	Nitro-Trz
25	0.882	8	15.6	<1	6.53	1	7.2	Nitro-Trz
26	13.8	10	24.5	6	>69.4	~2	140	Nitro-im
27	23.0	>7	3.77	>46	6.49	>27	>172	Nitro-im
28	21.6	4	8.02	10	4.27	19	79.6	Nitro-im
29	7.84	18	0.169	816	11.1	12	138	Nitro-Trz
30	8.17	10	1.96	40	4.87	16	78.8	Nitro-Trz
31	32.3	>7	123	>2	172.0	>1	>223	Trz
32	8.56	16	6.05	23	80.3	2	137	Nitro-im
33	14.2	7	4.67	20	18.7	5	93.6	Nitro-im
34	1.05	136	0.311	460	63.8	2	143	Nitro-Trz
35	0.92	78	0.358	200	9.58	7	71.6	Nitro-Trz
36	0.463	100	0.320	144	9.33	5	46.0	Nitro-Trz
37	3.61	34	0.320	383	159	1	122	Nitro-Trz
38	0.271	339	0.145	634	0.348	264	91.9	Nitro-Trz
39	15.3	>13	20.7	>9	>191	~1	>191	Nitro-im
40	1.38	>138	0.340	>562	58.2	>3	>191	Nitro-Trz
41	1.20	100	0.412	287	44.5	3	118	Nitro-Trz
42	5.33	10	0.040	1320	25.9	2	52.8	Nitro-Trz
Melarsoprol	0.01*							
Benznidazole			1.35*					
Miltefosine					0.44*			
	active							
	moderate active							
	active but low specificity							

[a]STIB 900 trypomastigotes; [b]Tulahuen C4 amastigotes; [c]MHOM-ET-67/L82 amastigotes; [d]Cytotoxicity measurements; [e]Nitro-Trz: 3-nitro-1.2.4-triazole; Nitro-Im: 2-nitro-1,3-imidazole; The IC50 values are the means of two independent assays, the individual values vary less than a factor 2. *Median values from 43 measurements in parallel with each compound. SI = IC50 in L6 cells / IC50 in parasites. Compounds in green have been previously synthesized.

T. b. rhodesiense, whereas 18 compounds (**1, 2, 16-18, 20, 23, 24, 27, 29, 34-38, 40-42**) were active and selective against *T. cruzi* (Table 1). Only one compound, **38**, was active and selective against *L. donovani*. Therefore, the antichagasic activity of these compounds is of the greatest interest based on the number of active molecules.

Evaluation of Structure-Activity Relationships: Analysis of the nitroheterocyclic ring

As a large set of the compounds showed significant anti-*T. cruzi* activity, we were able to conduct a detailed structure-activity relationship. Analysis of the trypanocidal activity in relation to the nitroheterocyclic ring revealed that compounds (**3-9, 13, 21, 27, 30**) that were active against *T. cruzi* (IC50< 4 μM) but not sufficiently specific (SI < 50), were exclusively 2-nitroimidazole derivatives except for compounds **21** and **30**. Similarly, moderately active compounds with low specificity against *T. cruzi* (**11, 12, 14, 15, 22, 25, 26, 28, 32, 33, 39**) were seen mainly in the 2-nitroimidazole series. In contrast, all 3-nitrotriazoles, with the exception of **22** and **25**, demonstrated significant in vitro anti-*T. cruzi* activity coupled with excellent selectivity (Table 1). In all cases where an active/moderately active trypanocidal effect was observed, irrespective of SI values, the 3-nitrotriazole derivatives (**1, 16, 17, 20, 21, 29, 38**) always had a greater effect (1.3 - 45 fold) on parasite growth as compared to their 2-nitroimidazole counterparts (**3, 5, 6, 8, 9, 26, 27**), and no toxicity: compare **1** with **3**, **16** with **5**, **17** with **6** etc (Table 1). Similar results are seen even with the moderately active and not specific 3-nitrotriazole **25** (a naphthoquinone derivative) which is 2-fold more potent than its 2-nitroimidazole analog **15**.

To determine whether the nitro-group was important in the anti-parasitic activity of the triazoles, two non-nitro compounds (**19** and **31**) were synthesized and their growth inhibitory properties against *T. cruzi* compared with that of their nitro-analogs **17** and **29** (note: **31** has an extra methylene group as compared to **29**). In both cases, the removal of the nitro-group led to inactivity (IC50 > 60 μM) and the IC50 value was significantly increased (658- and 730-fold, respectively) compared to the nitro-containing analog (Table 1). The anti-HAT activity of **19** and **31** was also reduced compared to that of **17** and **29**, but to a lesser degree. Therefore, the nitro group present on the triazole ring is essential in mediating the anti-parasitic activity of these compounds.

Table 2: Biological and physical properties of analogs active against *T. cruzi* amastigotes

Compound	T. cruzi IC-50 (μM)	SI	Bzn/Comp[a]	clogP	pKa	Lipinski Rule of 5	PSA (Å²)
1	0.14	147	9.6	3.20	9.20	S	101.45
2	0.15	66	8.9	4.16	8.84	S	110.68
8	1.97	45	0.7	4.56	5.06	S	101.45
16	0.61	236	2.2	2.43	7.31	S	101.45
17	0.14	976	9.6	2.95	7.31	S	101.45
18	0.31	373	4.4	3.22	7.53	S	101.45
20	1.48	66	0.9	4.05	5.06	S	114.34
21	1.74	44	0.8	4.52	5.06	S	114.34
23	1.03	129	1.3	0.99	6.81	S	114.34
24	0.46	208	2.9	1.51	6.81	S	114.34
29	0.17	816	8.0	3.51	9.44	S	88.56
34	0.31	460	4.3	2.60	8.76	S	101.45
35	0.36	200	3.8	5.55	6.87	V (2)	105.55
36	0.32	144	4.2	5.88	6.87	V (2)	105.55
37	0.32	383	4.2	2.63	9.65	S	88.56
38	0.15	634	9.3	5.62	8.79	V (2)	79.77
40	0.34	>562	4.0	2.86	8.33	S	83.01
41	0.41	287	3.3	3.38	8.52	S	83.01
42	0.04	1320	33.8	3.03	7.85	S	83.01
Bnz	1.35*		1.0	1.32		S	92.74

[a]Bzn/Comp: IC50 of Bnz / IC50 of Comp. PSA: Polar surface area. *Median values from 43 measurements in parallel with each compound. All physical properties were predicted by using the Marvin Calculator (www.chemaxon.com).

Analysis of aromatic amines

A closer look at the SARs for all antichagasic compounds is given in Table 2. In the subclass of 3-nitrotriazole bearing aromatic amines (**1, 2, 16-18, 20, 21, 23, 24**), activity decreases in the following order: acridines (**1, 2**) ≥ quinolines (**16-18**) > 1,5-naphthyridines (**23, 24**) > quinazolines (**20, 21**). The 2-nitroimidazole linked quinazoline derivative **8** demonstrates similar activity with the 3-nitrotriazole analogs **20** and **21**.

An extra methylene group in the linkage in compound **2** and the chloro- substituent in the acridine ring increased lipophilicity and toxicity, compared to its analog **1**, but did not decrease activity. It is assumed that the acridine compounds **1** and **2** demonstrate increased toxicity and lack of sufficient selectivity due to DNA-intercalation[19] and topoisomerase I/II inhibition.[20] Thus, compound 1, which was tested in vivo for Chagas, could not be given at sufficient doses for an extended period of time, due to the observed toxicity.[22]

Comparing the quinoline analogs **16** and **17**, it is observed that increased lipophilicity in **17**, due to an extra methylene group in the linkage, slightly increased toxicity (Table 1);

however, at the same time activity was also increased, resulting in an improved SI (by a factor of 4) compared to **16**. Comparing the analogs **17** and **18**, it is observed that the replacement of chlorine in **17** with a trifluoromethyl group increases lipophilicity and toxicity in **18**, however the activity remains at low nM concentrations, slightly less than that in **17**, but still better than the one in **16**. All three quinoline compounds show excellent selectivity, significantly higher than the threshold of 50 we have set, and are candidates for in vivo studies.

Table 3. The effect of type I Nitroreductase (TbNTR) on the activity of selected compounds against bloodstream-form *T. brucei brucei* parasites.

ID No	T.b. brucei[a]	TbNTR[b]	TbNTR[b]	Ratio	$E_{1/2}$[c]
	IC-50 (μM)	-tet	+tet	-tet/+tet	(V)
8	7.47 ± 0.71	7.58±0.19	0.95±0.11	8.00	-1.03
17	0.17 ± 0.04	0.44±0.06	0.10±0.04	4.00	-1.18
20	2.63 ± 0.25	4.48±0.19	0.07±0.02	64.00	-1.04
23	> 10	nd	nd	nd	nd
29	7.83 ± 0.32	11.08±2.50	0.76±0.16	14.00	-1.07
38	0.21 ± 0.01	0.20±0.01	0.10±0.02	2.00	-1.06
40	> 10	nd	nd	nd	nd
41	> 10	nd	nd	nd	-1.04
42	2.30 ± 0.10	2.63±0.12	0.21±0.01	13	nd
Nifurtimox[d]		1.71 ± 0.06	0.13±0.04	13	-0.88

[a] Bloodstream form wild type T. brucei brucei parasites; [b] bloodstream form T. b. brucei, engineered to over-express type I nitroreductase in the presence of tetracycline (tet). [C] Reduction potential of each compound was measured in DMSO (except for 17, in CH3CN) by cyclic voltammetry relative to Ag/AgCl. d The E1/2 value is taken from ref. [25].

Comparing the quinazoline systems **20** and **21** (Tables 1 and 2), it is observed that in this case an extra methylene group in the linkage of **21** did not improve the antichagasic activity but increased lipophilicity and thus toxicity, lowering thus the SI from 66 to 44. Similar results, but significantly more prominent, can be seen with the 2-nitroimidazole-based quinazoline systems **8** and **9**, which are the corresponding analogs of **20** and **21**, respectively; in this case **9** was totally inactive, whereas **8** is more comparable with **21** rather than **20** with regard to its antichagasic activity and selectivity (Table 2).
Finally, in the case of the two naphthyridine compounds **23** and **24**, the beneficial effect of an extra methylene group in the linkage of **24** is reflected in its improved activity and selectivity, despite its increased toxicity (Tables 1 and 2).

It is worth mentioning that while alteration in the length of the linkage between the nitrotriazole/imidazole ring and aromatic chromophore in the aromatic amines can not always predict the direction of changes in the antichagasic activity, it is clear in all cases (**2, 17, 18, 21, 24**) that four methylene groups in the linkage favor anti-HAT activity (Table 1).

Analysis of aliphatic amines

The 3-nitrotriazole-based benzylamines **29, 37** and **38** are all active against *T. cruzi* and demonstrate very good SI values (Table 2). The dibenzylated derivative **38** is significantly more lipophilic and thus more toxic than the monobenzylated analog **37**, violating twice the Lipinski rule of 5 (Table 2). However, its increased antichagasic activity balances out its toxicity, so it appears with a better SI value than **37** (Tables 1 and 2). Interestingly, **38** is the only compound active across all parasites tested (Table 1). Compound **29**, although more lipophilic (due to 2 trifluoromethyl groups) than **37**, appears less toxic, perhaps because the trifluoromethyl groups being in meta positions offer a better compound-stability compared to **37**.

The 3-nitrotriazole-based quinaldinamines **34, 35** and **36** demonstrate similar antichagasic activity and their SI corresponds inversely to their clogP value and toxicity (Table 1 and 2). All three analogs have similar activity with the p-trifluoromethylbenzylamine **37**, but the monoalkylated chloro-quinaldine analog **34** demonstrates a superior SI value, presumably due to its decreased toxicity compared to **37**, despite the fact that both **34** and **37** have similar clogP values. As was expected, the dialkylated analogs **35** and **36** also violate the Lipinski rule of 5.

The piperazine systems (**40**-**42**) showed significant antichagasic activity in vitro (Table 1). However, the 1-phenyl-piperazine **42** showed about 10-fold increased activity (IC50 at low nM concentrations) compared to the 1-benzyl-piperazines **40** and **41**. Although the lipophilicity of **42** was between that of **40** and **41**, its toxicity was higher than both of them. Despite an increased toxicity (Table 1), the SI of **42** was 1320, the highest of all tested compounds, making **42** a good candidate for in vivo studies. Comparing the substituted benzylpiperazine derivatives **40** and **41**, it is observed that an extra methylene in **41**, in the linkage between 3-nitrotriazole and the piperazine ring, decreased potency and increase lipophilicity and toxicity, resulting in a lower SI value compared to **40** (Table 2).

It can be observed that all compounds with antichagasic activity in Table 2 have a polar surface area < 140 and >60 $Å^2$, which means good cell-membrane permeability and presumably absence of neurotoxicity since they can not cross the blood-brain barrier. In addition, all but compounds **8**, **20** and **21** (all 2-phenylquinazolines), demonstrate a better antichagasic activity (1.3-33.8 fold) than the reference compound benznidazole, tested in parallel. It appears that increased antichagasic activity is observed with increased basicity in the amines of Table 2.

Evaluating the mechanism of action of nitrotriazoles

As was mentioned earlier, nitroheterocyclic prodrugs must undergo enzyme-mediated activation within the pathogen to have cytotoxic effects. These enzymes are most likely nitroreductases, although other reducing enzymes specific to the parasite, such as trypanothione reductase[8,26] or NADH-fumarate reductase[27], could be involved. Both Nfx and Bnz are activated by the NADH-dependent, oxygen insensitive, mitochondrially localized, bacterial-like, type I NTR, and down-regulation of this enzyme explains how resistance emerges.[10-12] Therefore, we investigated the role of recombinant *T. brucei* NTR (TbNTR) in the activation of selected nitrotriazoles and the nitroimidazole **8** (Fig. 1; supplemental material), as well as the susceptibility of bloodstream form *T. brucei brucei*, engineered to overexpress tetracycline-inducible TbNTR, to these compounds (Table 3). The reduction potentials (E1/2) of the active compounds towards bloodstream form *T. b. brucei* were also measured by cyclic voltametry, to elucidate if there is any correlation between enzymatic activity and redox properties, and are shown in Table 3. Compounds from all sub-categories (aromatic and aliphatic amines, as well as piperazinic derivatives) have been chosen for these studies.

With regard to anti-HAT activity, it is observed that compounds **17** and **38** that were very active against *T. b. rhodesiense* (Table 1), were similarly active against bloodstream form *T. b. brucei* (Table 3), whereas compounds that were inactive (8, 29, 42) or moderately active (**20, 23, 40, 41**) against *T. b. rhodesiense* (Table 1), were in general more inactive against bloodstream form *T. b. brucei* (Table 3). With regard to the tetracycline (+tet)-inducible TbNTR overexpression system, it is observed that parasites induced to overexpress TbNTR are more susceptible to all nitrotriazoles/nitroimidazole tested, with compounds being moderately active against bloodstream form *T. b. brucei* showing a greater difference than the most active **17** and **38**. As a general rule of thumb, if a -tet/+tet ratio is >5, then it is assumed that the major

growth inhibitory activity of a compound is via NTR activation. For compounds with a -tet/+tet ratio <5, alternative systems may be involved or the NTR generated reduction products are extremely trypanocidal.

There was no correlation between trypanocidal activity and enzymatic activity (see supplemental material). Furthermore, no conclusive data were obtained by comparing the enzymatic activity with reduction potentials ($E_{1/2}$), although there was a trend suggesting an increasing activity at more negative $E_{1/2}$ values, values that possibly lie outside the normal range of mammalian redox systems. If this is true, then the mutagenic potential of these compounds may be low,[28] something that has been confirmed in limited Ames studies with **16, 20** and **29** (data not shown).

In conclusion, nine nitrotriazole-based compounds (**16-18, 24, 29, 34, 40-42**) have been identified from Table 2 as potential candidates for in vivo studies in *T. cruzi* infected mice, and further development against Chagas. All of them have demonstrated significant antichagasic activity at low to intermediate nmolar concentrations, SI values of ≥200, and satisfy the Lipinski's rule of 5. In addition, compound **38** may also warrant additional attention as it displays significant anti-parasitic activity against *T. cruzi*, both *T. brucei* subspecies and L. major with high selectivity, although this compound does violate two of the Lipinski's rule of 5.

Experimental

All starting materials and solvents were purchased from Sigma-Aldrich (Milwaukee, WI), were of research-grade quality and used without further purification. Solvents used were anhydrous and the reactions were carried out under a nitrogen atmosphere and exclusion of moisture. Melting points were determined by using a Mel-Temp II Laboratory Devices apparatus (Holliston, MA) and are uncorrected. Elemental Analyses were obtained by Midwest Microlab, LLC (Indianapolis, IN). Proton NMR spectra were obtained on a Varian Inova-500 or a Bruker Avance-III-500 spectrometer at 500 MHz and are referenced to Me4Si or to the corresponding protonated solvent, if the solvent was not CDCl3. Highresolution electrospray ionization (HRESIMS) mass spectra were obtained on a Agilent 6210 LC-TOF mass spectrometer at 11000 resolution. Thin-layer chromatography was carried out on aluminum oxide N/UV_{254} or polygram silica gel G/UV_{254} coated plates (0.2 mm, Analtech, Newark, DE). Chromatography was carried out on preparative TLC alumina GF (1000 microns) or silicagel GF (1500 microns) plates (Analtech). All the amines were purified by preparative TLC chromatography on

alumina plates (≥ 95% purity). The results from elemental analysis for C, H and N were within 0.4 of the theoretical value.

The synthesis of compounds **1, 3-7, 10** and **15** has been described before.[19, 23, 29-31] Compounds **2** and **25** were synthesized in a similar manner with **1**[19] and **15**,[31] respectively.

General Synthetic Procedure of aromatic amines

For compounds **8-14**, **16-24**: The appropriate chloro-aromatic starting material (commercially available in most cases) (1.24 mmol) was coupled with 2-nitro-1H--imidazolyl-alkylamine (1.24 mmol)[19] or 3-nitro-1,2,4-triazolyl-alkylamine (1.24 mmol),[19] by refluxing in absolute propanol (7-10 ml) for 12 -30 h. In the case of compounds **16, 17** and **19**, the 4,7-dichloroquinoline was first converted to 7-chloro-4-fluoroquinoline[23] before coupling. In the case of compound **19**, 4-(1H-1,2,4-triazol-1-yl)butylamine was first synthesized as in ref. [19], to be then coupled with 7-chloro-4-fluoroquinoline. In the case of compound **18, 4** fluoro-7-trifluoromethyl quinoline could not be synthesized from the corresponding 4-chloro-7-trifluoromethylquinoline. In most cases the hydrochloride salt of the final product was precipitated upon cooling of the reaction mixture and separated by filtration. In some cases, the free amine of the desired product was isolated by preparative TLC on alumina, dissolved in ethyl acetate and converted to its HCl salt by treating with 1 M HCl in diethyl ether. In the case of compounds **23** and **24**, the starting material 4- chloro-1,5-naphthyridine was synthesized in 4 steps as described previously.[29]

General Synthetic Procedure of mono- and dialkylated aliphatic amines 26-38

The appropriate bromide (1.035 mmol) was added dropwise (15 min) to a solution of 2-nitro-1H-imidazolyl-alkylamine (1.035 mmol) or 3-nitro-1H-1,2,4-triazolyl-alkylamine (1.035 mmol)[19] in the presence of potassium carbonate (9.52 mmol) in dry acetonitrile (15 mL) and the reaction mixture was stirred under a nitrogen atmosphere, at room temperature for 48 h. In the case of 31, 4-(1H-1,2,4-triazol-1-yl)butylamine was used. The reaction mixture was then filtered, the solids were washed with acetonitrile, the organic filtrate was evaporated and the residue extracted from water-chloroform. The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was separated by preparative TLC on alumina plates with ethyl acetate: petroleum ether mixture. Monoalkylated and dialkylated products were

obtained in the same reaction at varying ratios for each case. The separated products were dissolved in ethyl acetate and converted to their HCl salts by treating with HCl gas in dry ether (1 M solution).

Piperazine derivatives (**39-42**) were synthesized from the commercially available appropriate monoalkylated piperazines (1.44 mmol) and the appropriate 2-nitro-1Himidazolyl- alkylbromide or 3-nitro-1H-1,2,4-triazolyl-alkylbromide (1.485 mmol)[24] in the presence of potassium carbonate (13.24 mmol) in dry acetonitrile (25 mL) as above.

6-Chloro-2-methoxy-N-[4-(3-nitro-1H-1,2,4-triazol-1-yl)butyl]acridin-9-amine hydrochloride (2)

Yellow powder (35%): mp 214-216 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.58 (s, 1H), 8.44 (d, *J*=9.5 Hz, 1H), 7.78 (m, 3H), 7.68 (dd, *J*=8.0, 2.0 Hz, 1H), 7.50 (d, *J*=9.0 Hz, 1H), 4.40 (t, *J*=6.5 Hz, 2H), 4.18 (t, *J*=7.0, 2H), 4.01 (s, 3H), 2.11 (m, 2H), 1.99 (m, 2H). HRESIMS calcd for $C_{20}H_{20}ClN_6O_3$ m/z $[M+H]^+$ 427.1286, found 427.1286.

N-[3-(2-Nitro-1*H*-imidazol-1-yl)propyl]-2-phenylquinazolin-4-amine hydrochloride (8)

Off white powder (44%): mp 174-176 °C (dec.); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.34 (d, *J*=8.5 Hz, 1H), 8.27 (d, *J*=7.5 Hz, 2H), 8.07 (t, *J*=7.5 Hz, 1H), 7.98 (d, *J*=8.0 Hz, 1H), 7.81-7.76 (m, 2H), 7.69 (t, *J*=8.0 Hz, 2H), 7.59 (s,1H), 7.13 (s, 1H), 4.68 (t, *J*=7.0 Hz, 2H), 4.04 (t, *J*=7.0 Hz, 2H), 2.45 (quintet, *J*=7.0 Hz, 2H). HRESIMS calcd for $C_{20}H_{19}N_6O_2$ *m/z* $[M+H]^+$ 375.1570, found 375.1569.

N-[4-(2-Nitro-1*H*-imidazol-1-yl)butyl]-2-phenylquinazolin-4-amine hydrochloride (9)

Off white powder (51%). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.34-8.30 (m, 3H), 8.05 (t, *J*=7.5 Hz, 1H), 7.96 (d, *J*=8.5 Hz, 1H), 7.80-7.75 (m, 2H), 7.69 (t, *J*=8.0 Hz, 2H), 7.49 (s, 1H), 7.08 (s, 1H), 4.55 (t, *J*=7.0 Hz, 2H), 3.99 (t, *J*=7.0 Hz, 2H), 2.06 (quintet, *J*=7.0 Hz, 2H), 1.93 (quintet, *J*=7.0 Hz, 2H). HRESIMS calcd for $C_{21}H_{21}N_6O_2$ *m/z* $[M+H]^+$ 389.1721, found 389.1729.

6-Methyl-4-N-[3-(2-nitro-1*H*-imidazol-1-yl)propyl]-pyrimidin-2,4-diamine hydrochloride (11)

Orange solid (34%): mp 178 °C (dec); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.53 (s, 1H), 7.17 (s, 1H), 5.89 (s, 1H), 4.55 (t, *J*=7.0 Hz, 2H), 3.52 (t, *J*=6.5, 2H), 2.25 (s, 3H), 2.19 (m, 2H). HRESIMS calcd for $C_{11}H_{16}N_7O_2$ *m/z* $[M+H]^+$ 278.1366, found 278.1364.

6-Methyl-4-N-[4-(2-nitro-1*H*-imidazol-1-yl)butyl]-pyrimidin-2,4-diamine hydrochloride (12)

Off white powder (45%): mp 225-226 °C (dec); ^{1}H NMR (500 MHz, CD_3OD) δ: 7.50 (s, 1H), 7.15 (s, 1H), 5.86 (s, 1H), 4.51 (t, *J*=7.5 Hz, 2H), 3.49 (t, *J*=6.5 Hz, 2H), 2.23 (s, 3H), 1.92 (quintet, *J*=7.5 Hz, 2H), 1.66 (quintet, *J*=7.5 Hz, 2H). HRESIMS calcd for $C_{12}H_{18}N_7O_2$ *m/z* $[M+H]^+$ 292.1522, found 292.1530.

2-Chloro-6,7-dimethoxy-N-[4-(2-nitro-1*H*-imidazol-1-yl)butyl]quinazolin-4-amine (13)

Light yellowish powder (21%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.23 (s, 1H), 7.17 (s, 1H), 7.14 (s, 1H), 6.91 (s, 1H), 5.93 (br t, 1H), 4.54 (t, *J*=7.5 Hz, 2H), 4.00 (s, 3H), 3.98 (s, 3H), 3.80-3.76 (m, 2H), 2.00 (quintet, *J*=7.5 Hz, 2H), 1.82 (quintet, *J*=7.5 Hz, 2H). HRESIMS calcd for $C_{17}H_{20}ClN_6O_4$ *m/z* $[M+H]^+$ 407.1229, found 407.1236.

6-Chloro-N-[4-(2-nitro-1*H*-imidazol-1-yl)butyl]-2,5-diphenylpyrimidin-4-amine hydrochloride (14)

pale white powder (72%): mp 79-81 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.21 (d, *J*=7.5 Hz, 2H), 7.68-7.56 (m, 6H), 7.46 (s, 1H), 7.39 (d, *J*=7.5 Hz, 2H), 7.08 (s, 1H), 4.49 (t, *J*=7.0 Hz, 2H), 3.66 (t, *J*=7.0, 2H), 1.91 (quintet, *J*=7.5 Hz, 2H), 1.71 (quintet, *J*=7.5 Hz, 2H).

HRESIMS calcd for $C_{23}H_{22}ClN_6O_2$ *m/z* $[M+H]^+$ 449.1487, found 449.1488.

7-Chloro-N-[3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]quinolin-4-amine hydrochloride (16)

White powder (84%): mp 240-242 °C; ^{1}H NMR (500 MHz, D_2O) δ: 8.61 (s, 1H), 8.28 (d, *J*=7.0 Hz, 1H), 7.89 (d, *J*=9.0 Hz, 1H), 7.82 (s, 1H), 7.57 (d, *J*=9.0 Hz, 1H), 6.76 (d, *J*=7.0 Hz, 1H), 4.55 (t, *J*=6.5 Hz, 2H), 3.74 (t, *J*=6.5, 2H), 2.48 (m, 2H). HRESIMS calcd for $C_{14}H_{14}ClN_6O_2$ *m/z* $[M+H]^+$ 333.0867, found 333.0866.

7-Chloro-N-[4-(3-nitro-1*H*-1,2,4-triazol-1-yl)butyl]quinolin-4-amine hydrochloride (17)

White powder (67%): mp 210-220 °C (dec); ^{1}H NMR (500 MHz, D_2O) δ: 8.58 (s, 1H), 8.20 (d, *J*=7.0 Hz, 1H), 8.03 (d, *J*=9.0 Hz, 1H), 7.78 (s, 1H), 7.68 (d, *J*=9.0 Hz, 1H), 6.68 (d, *J*=7.0 Hz, 1H), 4.39 (t, *J*=6.5 Hz, 2H), 3.58 (t, *J*=7.0, 2H), 2.07 (m, 2H), 1.77 (m, 2H). HRESIMS calcd for $C_{15}H_{16}ClN_6O_2$ *m/z* $[M+H]^+$ 347.1023, found 347.1019.

N-[4-(3-Nitro-1*H*-1,2,4-triazol-1-yl)butyl]-7-(trifluoromethyl)quinolin-4-amine hydrochloride (18)

White powder (14%). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.64 (s, 1H), 8.57 (d, *J*=8.5 Hz, 1H), 8.50 (d, *J*=7.0 Hz, 1H), 8.16 (s, 1H), 7.95 (d, *J*=8.5 Hz, 1H), 4.44 (t, *J*=6.5 Hz, 2H), 3.69 (t, *J*=7.0 Hz, 2H), 2.13 (m, 2H), 1.86 (m, 2H). HRESIMS calcd for $C_{16}H_{16}F_3N_6O_2$ *m/z* $[M+H]^+$ 381.1281, found 381.1286.

7-Chloro-N-[4-(1H-1,2,4-triazol-1-yl)butyl]quinolin-4-amine (19)

White powder (43%): mp 125-127 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.54 (d, *J*=5.30 Hz, 1H), 8.10 (s,1H), 8.01 (s, 1H), 7.96 (d, *J*=2.1 Hz,1H), 7.73 (d, *J*=9.0, 1H), 7.37 (dd, *J*=8.9, 2.1 Hz, 1H), 6.39 (d, *J*=5.4 Hz, 1H), 5.32 (br s, 1H), 4.30 (t, *J*=6.8 Hz, 2H), 3.37 (m, 2H), 2.10 (m, 2H), 1.79 (m, 2H). HRESIMS calcd for $C_{15}H_{17}ClN_5O_2$ *m/z* $[M+H]^+$ 302.1167, found 302.1169.

N-[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]-2-phenyl-quinazolin-4-amine hydrochloride (20)

Yellow powder (71%): mp 246-248 °C (dec); ^{1}H NMR (500 MHz, CD_3SOCD_3) δ: 8.91 (s, 1H), 8.49 (br d, *J*=7.0 Hz,1H), 8.33 (d, *J*=7.5 2H), 8.08 (br s, 1H), 8.03 (br s, 1H), 7.75 (br s, 2H), 7.65 (br t, *J*=7.0 Hz, 2H), 4.52 (t, *J*=6.5 Hz, 2H), 3.90 (br m, 2H), 2.38 (t, *J*=6.5 Hz, 2H). HRESIMS calcd for $C_{19}H_{18}N_7O_2$ *m/z* $[M+H]^+$ 376.1522, found 376.1523.

N-[4-(3-Nitro-1*H*-1,2,4-triazol-1-yl)butyl]-2-phenyl-quinazolin-4-amine hydrochloride (21)

Yellow powder (69%): mp >250 °C; ^{1}H NMR (500 MHz, CD_3SOCD_3) δ: 8.90 (s, 1H), 8.57 (d, *J*=8.0 Hz,1H), 8.39 (d, *J*=7.5 2H), 8.16 (d, *J*=8.0 Hz, 1H), 8.04 (t, *J*=7.5 Hz, 1H), 7.76 (t, J=7.0, 2H), 7.68 (t, *J*=7.5 Hz, 2H), 4.41 (t, *J*=7.0 Hz, 2H), 3.86 (br q, *J*=6.0 Hz, 2H), 2.012 (quintet, *J*=7.5 Hz, 2H), 1.78 (quintet, J= 7,0 Hz, 2H). HRESIMS calcd for $C_{20}H_{20}N_7O_2$ *m/z* $[M+H]^+$ 390.1679, found 390.1681. Calculated analysis for $C_{20}H_{20}ClN_7O_2$: C, 56.41; H, 4.73; N, 23.02; Cl, 8.33. Found: C, 56.06; H, 5.01; N, 22.84; Cl, 9.06.

6-Methyl-4-N-[3-(2-nitro-1*H*-1,2,4-triazol-1-yl)propyl]-pyrimidin-2,4-diamine hydrochloride (22)

Off white powder (58%): mp 204-206 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.60 (s, 1H), 5.85 (s, 1H), 4.42 (t, *J*=6.5 Hz, 2H), 3.53 (t, *J*=6.5, 2H), 2.28 (m, 2H), 2.24(s, 3H). HRESIMS calcd for $C_{10}H_{15}N_8O_2$ *m/z* $[M+H]^+$ 279.1318, found 279.1319.

N-[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]-1,5-naphthyridin-4-amine hydrochloride (23)

Yellowish powder (50%): mp 215-217 °C (dec); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.94 (d, *J*=3.5 Hz,1H), 8.65 (s, 1H), 8.47 (d, *J*=7.0 Hz, 1H), 8.27 (d, *J*=8.5 Hz,1H), 7.95 (dd, *J*=8.5, 4.5 Hz, 1H), 7.09 (d, *J*=7.0 Hz, 1H), 4.54 (t, *J*=6.5 Hz, 2H), 3.80 (t, *J*=7.0, 2H), 2.48 (m, 2H). HRESIMS calcd for $C_{13}H_{14}N_7O_2$ *m/z* $[M+H]^+$ 300.1209, found 300.1206.

N-[4-(3-Nitro-1*H*-1,2,4-triazol-1-yl)butyl]-1,5-naphthyridin-4-amine hydrochloride (24)

Off white powder (61%). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.97 (d, *J*=4.0 Hz,1H), 8.64 (s, 1H), 8.43 (d, *J*=7.0 Hz, 1H), 8.26 (d, *J*=8.5 Hz,1H), 7.95 (dd, *J*=8.5, 4.5 Hz, 1H), 7.06 (d, *J*=7.5 Hz, 1H), 4.44 (t, *J*=7.0 Hz, 2H), 3.72 (t, *J*=7.0, 2H), 2.12 (m, 2H), 1.85(m, 2H). HRESIMS calcd for $C_{14}H_{16}N_7O_2$ *m/z* $[M+H]^+$ 314.1360, found 314.1362.

2-Chloro-3-{[3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]amino}-1,4-dihydronaphthalene-1,4-dione (25)

Dark red powder (74%): mp 137-138 °C; ^{1}H NMR (500 MHz, CD_3COCD_3) δ: 8.69 (s, 1H), 8.06 (d, *J*=8.0 Hz,1H), 8.02 (d, *J*=8.5 Hz, 1H), 7.84 (t, *J*=8.0 Hz,1H), 7.75 (t, *J*=8.0 Hz, 1H), 6.96 (br s, 1H), 4.61 (t, *J*=7.0 Hz, 2H), 4.04 (t, *J*=7.0 Hz, 2H), 2.44 (m, 2H). HRESIMS calcd for $C_{15}H_{13}ClN_5O_4$ *m/z* $[M+H]^+$ 362.0651, 364.0627, found 362.0654, 364.0632.

{[3,5-bis(Trifluoromethyl)phenyl]methyl}[3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]amine hydrochloride (29)

White powder (60-64%): mp 140-142 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.68 (s, 1H), 8.20 (s, 2H), 8.12 (s, 1H), 4.52 (m, 2H), 4.44 (s, 2H), 3.27 (t, *J*=8.0 Hz, 2H), 2.40 (m, 2H). HRESIMS calcd for $C_{14}H_{14}F_6N_5O_2$ *m/z* $[M+H]^+$ 398.1052, found 398.1054.

bis({[3,5-bis(Trifluoromethyl)phenyl]methyl})[3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl] amine hydrochloride (30)

White powder (8.5%): mp 138-140 °C (dec.); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.61 (s, 1H), 8.06 (s, 4H), 8.00 (s, 2H), 4.60-4.48 (br m, 6H), 3.29 (br m, 2H), 2.54 (br m, 2H). HRESIMS calcd for $C_{23}H_{18}F_{12}N_5O_2$ *m/z* $[M+H]^+$ 624.1263 found 624.1279.

{[3,5-bis(Trifluoromethyl)phenyl]methyl}[4-(1H-1,2,4-triazol-1-yl)butyl]amine hydrochloride (31)

White powder (37%): mp 120-123 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 9.00 (s, 1H), 8.33 (s, 1H), 8.19 (s, 2H), 8.12 (s, 1H), 4.41 (br s, 4H), 3.17 (br t, *J*=5.8 Hz, 2H), 2.03

(m, 2H), 1.76 (m, 2H). HRESIMS calcd for $C_{15}H_{17}F_6N_4$ *m/z* $[M+H]^+$ 367.1352, found 367.1338.

[(6-Bromoquinolin-2-yl)methyl][3-(2-nitro-1*H*-imidazol-1-yl)propyl]amine hydrochloride (32)

White powder (22%): mp 158-160 °C (dec.); ^{1}H NMR (400 MHz, CD_3OD) δ: 8.33 (d, *J*=8.4 Hz, 1H), 8.19 (s, 1H), 7.99 (d, *J*=9.6 Hz, 1H), 7.89 (d, *J*=8.8 Hz, 1H), 7.53 (s, 1H), 7.52 (d, *J*=9.6 Hz, 1H), 7.17 (s, 1H), 4.61 (t, *J*=7.2 Hz, 2H), 4.59 (s, 2H), 3.34 (br t, 2H), 2.41 (m, 2H). HRESIMS calcd for $C_{16}H_{17}BrN_5O_2$ *m/z* $[M+H]^+$ 390.0566, 392.0545 found 390.0569. 392.0551.

bis[(6-Bromoquinolin-2-yl)methyl][3-(2-nitro-1*H*-imidazol-1-yl)propyl]amine hydrochloride (33)

Pinkish powder (18%). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.36 (d, *J*=8.4, 2H), 8.2 (s, 2H), 7.91 (s, 4H), 7.57 (d, *J*=8.4 Hz, 2H), 7.47 (s, 1H), 7.09 (s, 1H), 4.95 (s, 4H), 4.63 (t, *J*=7.6 Hz, 2H), 3.66 (t, *J*=8.0 Hz, 2H), 2.60 (m, 2H). HRESIMS calcd for $C_{26}H_{23}Br_2N_6O_2$ *m/z* $[M+H]^+$ 609.0249, 611.0229, 613.0208, found 609.0251, 611.0233, 613.0210.

[(7-Chloroquinolin-2-yl)methyl][3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]amine hydrochloride (34)

Beige powder (29%): mp 135 °C (dec.); **1**H NMR (500 MHz, CD_3OD) δ: 8.68 (s, 1H), 8.42 (d, *J*=8.5 Hz, 1H), 8.12 (s, 1H), 7.99 (d, *J*=9.0 Hz, 1H), 7.64 (d, *J*=8.5 Hz, 1H), 7.54 (d, *J*=8.5 Hz, 1H), 4.64 (s, 2H), 4.55 (t, *J*=6.5 Hz, 2H), 3.35 (t, *J*=8.0 Hz, 2H), 2.50 (m, 2H). HRESIMS calcd for $C_{15}H1_6ClN_6O_2$ *m/z* $[M+H]^+$ 347.1018, 349.0994, found 347.1003. 349.0985.

bis[(7-Chloroquinolin-2-yl)methyl][3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]amine hydrochloride (35)

Off white powder (17%): mp 104-106 °C (dec.); **1**H NMR (500 MHz, CD_3OD) δ: 8.61 (s, 1H), 8.55 (d, *J*=8.5 Hz, 2H), 8.14 (s, 2H), 8.03 (d, *J*=8.5 Hz, 2H), 7.69 (d, *J*=8.0 Hz, 4H), 4.91 (s, 4H), 4.52 (t, *J*=6.5 Hz, 2H), 3.56 (br t, 2H), 2.04 (m, 2H). HRESIMS calcd for $C_{25}H_{22}C_{l2}N_7O_2$ *m/z* $[M+H]^+$ 522.1212, found 522.1216.

bis[(6-Bromoquinolin-2-yl)methyl][3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl]amine hydrochloride (36)

Off white powder (16%): mp 128-130 °C (dec); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.60 (s, 1H), 8.41 (d, *J*=8.5 Hz, 2H), 8.22 (s, 2H), 7.92 (br s, 4H), 7.63 (d, *J*=8.5 Hz, 2H), 4.95 (s, 4H), 4.52 (t, *J*=6.5 Hz, 2H), 3.64 (t, *J*=8.0 Hz, 2H), 2.62 (m, 2H). HRESIMS

calcd for $C_{25}H_{22}Br_2N_7O_2$ *m/z* $[M+H]^+$ 611.0235, 612.0181, 613.0215 found 611.0254, 612.0233, 613.0230.

[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]({[4-(trifluoromethyl)phenyl]methyl})amine hydrochloride (37)

White powder (12%): mp 127-128 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.65 (s, 1H), 7.79 (d, *J*=8.5 Hz, 2H), 7.71 (d, *J*=8.0 Hz, 2H), 4.50 (t, *J*=6.5 Hz, 2H), 4.33 (s, 2H), 3.22 (t, *J*=8.0 Hz, 2H), 2.38 (m, 2H). HRESIMS calcd for $C_{13}H_{15}F_3N_5O_2$ *m/z* $[M+H]^+$ 330.1172 found 330.1179.

[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]bis({[4-(trifluoromethyl)phenyl]methyl})amine hydrochloride (38)

White powder (26%): mp 184-186 °C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.59 (s, 1H), 7.78 (d, *J*=8.0 Hz, 4H), 7.70 (d, *J*=7.5 Hz, 4H), 4.53 (br s, 4H), 4.44 (t, *J*=6.0 Hz, 2H), 3.25 (br s, 2H), 2.52 (br m, 2H). HRESIMS calcd for $C_{21}H_{20}F_6N_5O_2$ *m/z* $[M+H]^+$ 488.1516 found 488.1513. Calculated analysis for $C_{21}H_{20}F_6ClN_5O_2$: C, 48.13; H, 3.85; N, 13.37; Cl, 6.77. Found: C, 48.21; H, 3.93; N, 13.29; Cl, 6.88.

1-[3-(2-Nitro-1*H*-imidazol-1-yl)propyl]-4-{[4-(trifluoromethyl)phenyl]methyl}piperazine dihydrochloride (39)

White powder (67%): mp 185-187 °C; ^{1}H NMR (500 MHz, D_2O) δ: 7.85 (d, *J*= 7.5 Hz, 2H), 7.70 (d, *J*=7.5 Hz, 2H), 7.49 (s, 1H), 7.22 (s, 1H), 4.58 (br t, *J*=7.0 Hz, 2H), 4.53 (s, 2H), 3.63 (br s, 8H), 3.34 (br s, 2H), 2.37 (br s, 2H). HRESIMS calcd for $C_{18}H_{23}F_3N_5O_2$ *m/ z* $[M+H]^+$ 398.1798, found 398.1803. Calculated analysis for $C_{18}H_{24}F_3C_{l2}N_5O_2$: C, 45.95; H, 5.15; N,14.89; Cl, 15.08. Found: C, 45.85; H, 5.05; N, 14.59; Cl, 15.12.

1-[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]-4-{[4 (trifluoromethyl)phenyl]methyl} piperazine dihydrochloride (40)

White powder (74%): mp 233-235 °C (dec); ^{1}H NMR (500 MHz, D_2O) δ: 8.65 (s, 1H), 7.84 (d, *J*=8.0 Hz, 2H), 7.67 (d, *J*=8.0 Hz, 2H), 4.51 (t, *J*=6.0 Hz, 2H), 4.39 (s, 2H), 3.48 (br s, 8H), 3.27 (t, *J*=8.0 Hz, 2H), 2.41 (m, 2H). HRESIMS calcd for $C_{17}H_{22}F_3N_6O_2$ *m/z* $[M+H]^+$ 399.1751, found 399.1761. Calculated analysis for $C_{17}H_{23}F_3C_{l2}N_6O_2$: C, 43.30; H, 4.92; N, 17.83; Cl, 15.05. Found: C, 43.26; H, 4.91; N, 17.71; Cl, 15.42.

1-[4-(3-Nitro-1*H*-1,2,4-triazol-1-yl)butyl]-4-{[4 (trifluoromethyl)phenyl]methyl} piperazine dihydrochloride (41)

White powder (16%): mp 223-225 °C (dec); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.67 (s, 1H), 7.80 (s, 4H), 4.44 (t, J=7.0 Hz, 2H), 4.41 (s, 2H), 3.80-3.40 (br m, 10H), 2.06 (quintet, *J*=7.5 Hz, 2H), 1.86 (m, 2H). HRESIMS calcd for $C_{18}H_{24}F_3N_6O_2$ *m/z* $[M+H]^+$ 413.1907, found 413.1909.

1-[3-(3-Nitro-1*H*-1,2,4-triazol-1-yl)propyl]-4-[4-(trifluoromethyl)phenyl]piperazine dihydrochloride (42)

White powder: mp 225 °C (dec.); ^{1}H NMR (500 MHz, CD_3OD) δ: 8.68 (s, 1H), 7.56 (d, *J*=8.5 Hz, 2H), 7.14 (d, *J*=8.5 Hz, 2H), 4.52 (t, *J*=6.5 Hz, 2H), 4.04 (d, *J*=13 Hz, 2H), 3.72 (d, *J*=11.5 Hz, 2H), 3.35 (t, *J*=8.0 Hz, 2H), 3.27-3.20 (m, 4H), 2.48 (quintet, *J*=6.5 Hz, 2H). HRESIMS calcd for $C_{16}H_{20}F_3N_6O_2$ *m/z* $[M+H]^+$ 385.1595, found 385.1606; calcd for $C_{16}H_{19}F_3N_6NaO_2$ *m/z* $[M+Na]^+$ 407.1414, found 407.1419. Calculated analysis for $C_{16}H_{21}F_3C_{l2}N_6O_2$: C, 42.01; H, 4.63; N, 18.38; Cl, 15.51. Found: C, 42.29; H, 4.68; N, 18.79; Cl, 15.39.

***In vitro* biological evaluation**

In vitro activity against *T. cruzi, Trypanosoma b. rhodesiense, Leishmania donovani* axenic amastigotes and cytotoxicity assessment using L6 cells (rat skeletal myoblasts) was determined using a 96-well plate format as previously described.25 Data were analyzed with the graphic program Softmax Pro (Molecular Devices, Sunnyvale, CA, USA), which calculated IC50 values by linear regression from the sigmoidal dose inhibition curves.

***In vitro T. brucei brucei* antiproliferating assays and susceptibility studies**

T. brucei brucei bloodstream form parasites were seeded at 1 × 103 ml-1 in 200 μL of growth medium containing different concentrations of a nitrotriazole or nifurtimox. Where appropriate, induction of the TbNTR was carried out by adding tetracycline (1 μg/mL). After incubation for 3 days at 37 °C, 20 μL of Alamar blue was added to each well and the plates incubated for a further 16 h. The cell density of each culture was determined as described before11 and the IC50 established.

Enzymatic activity studies

Recombinant TbNTR was prepared and assayed as previously described.16 The activity of purified his-tagged TbNTR was assessed spectrophotometrically at 340 nm using various nitrotriazole substrates (100 μM) and NADH (100 μM) and expressed as nmol NADH oxidized min-1 mg-1 of enzyme.

Cyclic Voltametry

Reduction potentials (E1/2) were measured by cyclic voltametry and evaluated relative to the Ag/AgCl reference electrode. Supporting electrolyte was 0.1M of tetrabutyl ammonium hexafluorophosphate (TBAPF6), 98% purity from Sigma Aldrich. The working electrode was carbon mesh and the counter electrode Pt wire. The typical scan rate was 100 mV/sec.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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CHAPTER 6

Novel 3-Nitro-1H-1,2,4-triazole-based Amides and Sulfonamides as Potential anti-Trypanosomal Agents

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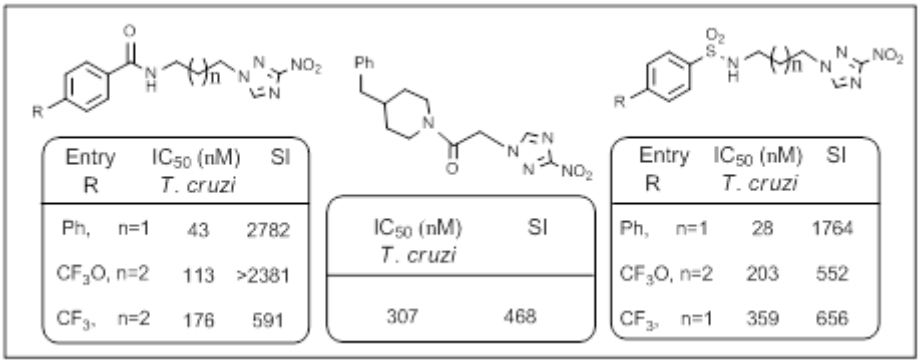
Abstract

A series of novel 3-nitro-1H-1,2,4-triazole-(and in some cases 2-nitro-1H-imidazole)-based amides and sulfonamides were characterized for their in vitro anti-trypanosomal and antileishmanial activities as well as mammalian toxicity. Out of 36 compounds tested, 29 (mostly 3-nitro-1H-1,2,4-triazoles) displayed significant activity against *T. cruzi* intracellular amastigotes (IC50 ranging from 28 nM to 3.72 μM) without concomitant toxicity to L6 host cells (selectivity 66 to 2782). Twenty three of these active compounds were more potent (up to 58 fold) than the reference drug benznidazole, tested in parallel. In addition, 9 nitrotriazoles which were moderately active (0.5 μM ≤ IC50 < 6.0 μM) against *T. b. rhodesiense* trypomastigotes, were 5 to 31 fold more active against bloodstream-form *T. b. brucei* trypomastigotes engineered to overexpress NADH-dependent nitroreductase (TbNTR). Finally, 3 nitrotriazoles displayed a moderate activity against the axenic form of Leishmania donovani. Therefore, 3-nitro-1H-1,2,4-triazole-based amides and sulfonamides are potent anti-trypanosomal agents.



Keywords

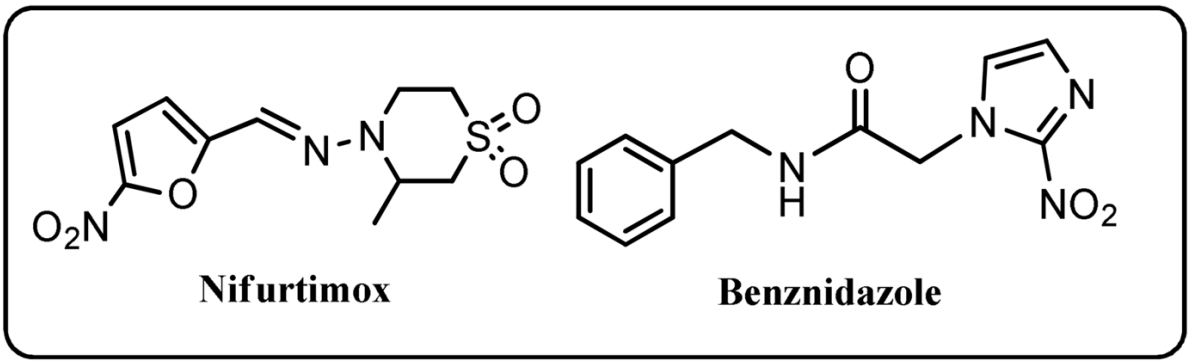
nitrotriazoles; amides; sulfonamides; *T. cruzi*; Chagas disease; anti-trypanosomal agents

INTRODUCTION

The trypanosomatids protozoan parasites *Trypanosoma cruzi* (*T. cruzi*), *Trypanosoma brucei* (*T. brucei*), and various Leishmania species are the causative agents of Chagas disease, human African trypanosomiasis (HAT) and different forms of leishmaniasis, respectively. Over 20 million people are infected by *T. cruzi*, *T. brucei* and Leishmania, resulting in 100,000 of deaths per year.[1] Chagas disease is transmitted by blood sucking triatomine insects and occurs mainly in Latin America. Despite the fact that in the past 20 years the number of incidences for both Chagas and HAT has significantly declined,

primarily due to vector control initiatives,[2] the number of cases in non-endemic regions such as the United States, Australia, Europe and Japan is on the rise.[3] Reasons for this rise include population migration, drug usage and medical practices. With no immediate prospect for vaccines, chemotherapy is the only way to fight the parasite in the patient. Currently, two nitroheterocycle prodrugs, nifurtimox (4-(5-nitrofurfurylidenamino)-3-methylthio-morpholine-1,1-dioxide) (Nfx) and benznidazole (N-benzyl-2(2-nitro-1Himidazol- 1-yl)acetamide) (Bnz) (Chart 1), are used to treat Chagas disease.[4] However, their use is problematic as both can cause side effects, have limited efficacy, while some strains are refractory to treatment.[5] In addition, the large quantities of medication required render it expensive, and the recommended long course of treatment is often not completed, resulting in the development of resistance. Therefore, the need for new, affordable and safer drugs to treat this disease is urgent.

Chart 1.



Most nitroheterocyclic compounds function as prodrugs and must undergo activation before mediating their cytotoxic effects. Initially it was proposed that the trypanocidal action of Nfx was due to its ability to induce oxidative stress through 1-electron reduction of its nitrogroup and the subsequent formation o superoxide anions via a futile cycle.[5–9] Several trypanosomal flavoproteins have been shown to mediate 1-electron reduction in vitro. However, more recent studies have shown that the above process does not occur to such a degree to cause toxicity to the parasites[10] and that a type I nitroreductase (NTR) [11] is responsible for Nfx and Bnz trypanocidal activity. This enzyme mediates a series of 2 electron reduction reactions resulting in the fragmentation of the heterocyclic ring and production of toxic metabolites.[10, 12] The fact that the activation of nitroheterocyclic prodrugs can be catalyzed by the type I NTR, which is

absent from most eukaryotes, with trypanosomes being a major exception, have led to a renewed interest in the use of such compounds[13–18] as antiparasitic agents.

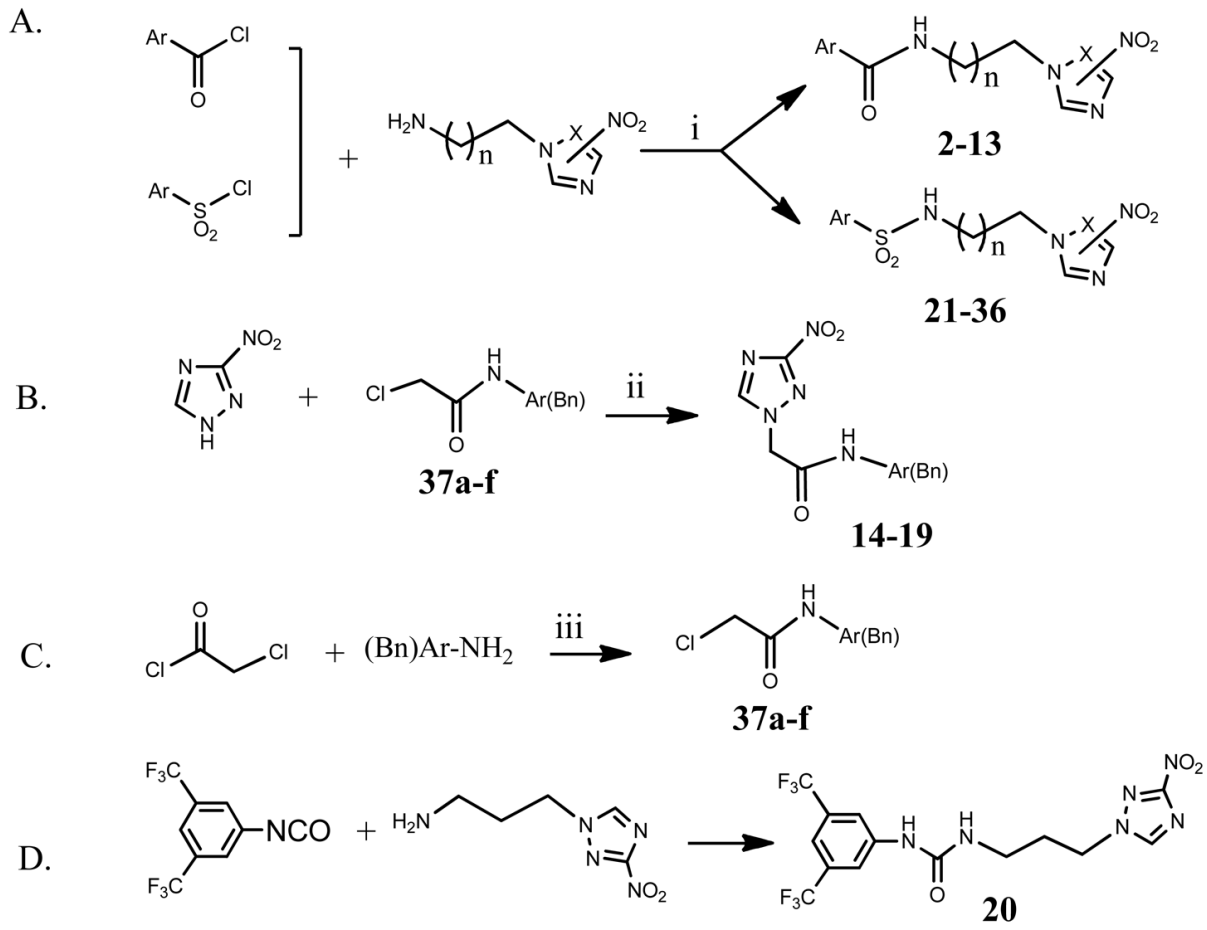
We have recently reported[19] that 3-nitro-1H-1,2,4-triazole-based aromatic and aliphatic amines demonstrate excellent in vitro activity against intracellular *T. cruzi* amastigotes and in some cases activity against *T. b. rhodesiense* and *T. b. brucei* parasites. We have also shown that 3-nitrotriazole-based amines are activated by type I nitroreductase and that blood stream form *T. b. brucei* parasites overexpressing NTR are hypersensitive to these compounds. Moreover, these compounds were significantly less toxic in host cells compared to parasites, and up to 34 fold more potent than the reference compound benznidazole.[19] Interestingly, the 3-nitrotriazole-based amines that were evaluated in the Ames test, were found negative for mutagenicity, in contrast to their 2-nitroimidazole analogs (unpublished data). Treatment of *T. cruzi*-infected mice with one aromatic amine, NTLA-1,[19,20] given at just 2 mg/kg/day × 50 days, resulted in a rapid and persistent drop in peripheral parasite levels and in a fraction of cures.21 Importantly, there was an absolute correlation between treatment efficacy as determined parasitologically and the increase in the fraction of *T. cruzi*-specific CD8+ T cells with a T central memory phenotype in the peripheral blood of treated mice.[21] Several other 3-nitrotriazole-based amines are currently being investigated in vivo for antichagasic activity. Encouraged by these results, we have expanded our investigation to the classes of 3-nitro-1H-1,2,4-triazole-based amides and sulfonamides.
Here we describe the synthesis and in vitro evaluation of such compounds as antitrypanosomal agents.

CHEMISTRY

The structure of all compounds is depicted on Table 1. Their synthesis is straightforward and based on well-established chemistry, outlined in Scheme 1. Compound **1** has been described before.[22] Amides **2–13** and sulfonamides **21–36** were synthesized at room temperature by nucleophilic substitution of the appropriate arylcarbonyl/arylsulfonyl chloride by the appropriate nitrotriazole/nitroimidazole alkyl amine[23] in the presence of triethyl amine (Scheme 1A). For compounds **3, 5, 22, 26, 30** and **32** the hydrochloride salt of 2-(3- nitro-1H-1,2,4-triazole)ethylamine was used because the free amine was too water soluble to be extracted by an organic solvent after its synthesis. Amides **14–19** were synthesized as depicted in Scheme 1B, according to

the literature[24]. First, 3-nitro-1,2,4-triazole was converted to its potassium salt by treating with KOH in acetonitrile under mild heating and then this mixture was added to a solution of the appropriate α-chloroacetamide **37a–f** in acetonitrile for a nucleophilic substitution, which occurred under refluxing conditions (8 h). The 2-chloro-N-arylacetamides **37a–f** were synthesized through nucleophilic acyl substitution of an appropriate arylamine with 2-chloroacetyl chloride in dry dichloromethane[24] (Scheme 1C). The yields of the final compounds in Table 1 were in general good to very good with the exception of some compounds (**14, 19, 22, 26, 29, 30–32**) with yields < 50%. However, the yields are higher if they are calculated on the basis of recovered starting material, since on many

Scheme 1.



i) Et_3N (2 eq), CH_2Cl_2, RT, 12 h; n = 1–3; X= C, 2-NO_2; X= N, 3-NO_2; when n =1, the HCl salt was used instead of the free amine with 4 eq of Et_3N. ii) KOH, CH_3CN, mild heating. iii) Et_3N, CH_2Cl_2.

nucleophilic substitution of the appropriate arylcarbonyl/arylsulfonyl chloride by the appropriate nitrotriazole/nitroimidazole alkyl amine[23] in the presence of triethyl amine (Scheme 1A). For compounds **3, 5, 22, 26, 30** and **32** the hydrochloride salt of 2-(3-

nitro-1H-1,2,4-triazole)ethylamine was used because the free amine was too water soluble to be extracted by an organic solvent after its synthesis. Amides **14–19** were synthesized as depicted in Scheme 1B, according to the literature[24]. First, 3-nitro-1,2,4-triazole was converted to its potassium salt by treating with KOH in acetonitrile under mild heating and then this mixture was added to a solution of the appropriate α-chloroacetamide **37a–f** in acetonitrile for a nucleophilic substitution, which occurred under refluxing conditions (8 h). The 2-chloro-N-arylacetamides **37a–f** were synthesized through nucleophilic acyl substitution of an appropriate arylamine with 2-chloroacetyl chloride in dry dichloromethane[24] (Scheme 1C). The yields of the final compounds in Table 1 were in general good to very good with the exception of some compounds (**14, 19, 22, 26, 29, 30–32**) with yields < 50%. However, the yields are higher if they are calculated on the basis of recovered starting material, since on many occasions unreacted chloride was isolated from the reaction mixture. Finally, the urea **20** was formed by addition of 3-(3-nitro-1H-1,2,4- triazolyl)propylamine to 3,5 bis(trifluoromethyl)phenyl isocyanate.

RESULTS AND DISCUSSION

Anti-Trypanosomal activity of nitrotriazole/nitroimidazole-based amides and sulfonamides

The in vitro growth inhibitory properties of all compounds against bloodstream form *T. b. rhodesiense* trypomastigotes, *T. cruzi* amastigotes (in infected L6 myoblasts), axenically cultured *L. donovani* amastigotes and rat skeletal myoblasts (L6 cells) were evaluated by using standard drug screens.[25] From resultant dose response curves, IC50 values in μM were determined (Table 1). The criteria used for activity take into account the complex life cycles of the parasites and the fact that *T. cruzi* and *L. donovani* are, in contrast to *T. b. rhodesiense*, intracellular parasites. These criteria were established by the TDR's "compound screeners network", published in a review[26] and are as follows: For *T. b. rhodesiense*, compounds that gave an IC50 < 0.5 μM, were designated as 'active', while those yielding an IC50 = 0.5–6.0 μM or an IC50 > 6.0 μM were designated 'moderately active' and 'inactive', respectively. For T. cruzi, IC50 < 4.0 μM, 'active'; IC50 = 4.0–60 μM, 'moderately active'; IC50 > 60 μM, 'inactive'. For *L. donovani*, IC50 < 1 μM, 'active'; IC50 = 1.0–6.0 μM, 'moderately active'; IC50 > 6.0 μM, 'inactive'.

On the basis of these criteria, all but compound **32** were active or moderately active against *T. cruzi*, 16 compounds (47 %) were active or moderately active against *T. b. rhodesiense*, and only 3 compounds (~ 8%) were moderately active against *L. donovani* parasites (Table 1). However, for a compound to be considered for further in vivo investigation, the growth inhibitory effect against the mammalian cell line L6 has to be evaluated from which a measure of a compound's cytotoxicity can be deduced. Thus, the selectivity index (SI), namely the ratio of IC50 against L6 cells to IC50 against each parasite, is also an important parameter and both IC50 and SI values are used to rank compounds.[26] This SI must be ≥ 100 for T.b. rhodesiense, ≥ 50 for *T. cruzi* and ≥ 20 for *L. donovani* axenic amastigotes.

On the basis of the above, only 9 compounds (**4–6, 13, 23, 24, 28, 29 and 34**) were moderately active/active and selective against *T. b. rhodesiense,* whereas 30 compounds (83%), namely **1–17, 21–31 and 34–36** were active (with the exception of **30** which was moderately active) and selective against *T. cruzi* (Table 1). Compounds **17** and **18**, which were moderately active against *L. donovani* have also an acceptable selectivity. Therefore, as in the case of 3-nitrotriazole-based amines,[19] the majority of these 3-nitrotriazole-based amides/sulfonamides act as antichagasic agents.

Evaluation of SARs: Analysis of the nitroheterocyclic ring

On the basis of our previous experience that the 2-nitroimidazole-based aromatic and aliphatic amines tend to be significantly less potent as anti-trypanosomal agents and more toxic to the host cells than their 3-nitrotriazole analogs[19], we focused more on the synthesis and evaluation of 3-nitrotriazole-based amides/sulfonamides. Therefore, only two 2- nitroimidazole-based amides (**1** and **2**) and one sulfonamide (**21**) were included. Because of the very limited number of such compounds, no solid conclusions can be obtained regarding the effect of the nitroheterocyclic ring on the anti-trypanosomal activity of these classes. However, it is apparent that all of these compounds were inactive against *T. b. rhodesiense,* and in general, they were less potent anti-chagasic agents than their closely related 3- nitrotriazoles or benznidazole (compare **1** with **3**, **4** and **7; 2** with **5** and **6; 21** with **23**) (Table 1).

Analysis of amides in which the 3-nitrotriazole ring is linked through the amino group

Comparing the antichagasic activity of the N-(3-nitrotriazolyl-alkyl)benzamides **3–8**, it is observed that activity increases with the length of the linker between the 3-nitrotriazole ring and amido group (Table 1, compare **3** with **4** and **7; 5** with **6**). The same rule applies for the activity against *T. b. rhodesiense* as well. Replacing the trifluoromethyl group in **3** with the trifluoromethoxy group, resulted in decreased activity and selectivity against *T. cruzi* in **5**; however the opposite effect was observed in the case of compounds **4** and **6**. Interestingly, the more lipophilic **6**, was slightly less toxic to L6 cells compared to the less lipophilic **5** and, because of its increased potency against *T. cruzi*, resulted in a very high selectivity of > 2381. It is also worthy mentioning that the trifluoromethoxy group increased lipophilicity to the same degree as two methylene groups (Table 1). However, this increased lipophilicity was not always translated to increased antichagasic or anti-HAT activity and the length of the linker played a more important role. The addition of an extra trifluoromethyl group in the phenyl ring of **8** resulted also in decreased antichagasic activity and selectivity, as well as in inactivity against T.b. rhodesiense (Table 1). Exchanging the phenyl group with a pyridino- in **9**, significantly decreased the activity and selectivity against *T. cruzi* but did not have any dramatic effect on the moderate activity against *T. b. rhodesiense* (compare **7** with **9**).

Quinoline-2-carboxamides **10** and **11** demonstrated exceptional in vitro activity against T. cruzi and very good selectivity. The additional methylene in the linker of **11** naturally increased lipophilicity of this compound and led to a decreased selectivity (Table 1). Going from the quinoline-2-carboxamide **10** to the quinoxaline analog **12**, we observe a decrease in the antichagasic activity and selectivity, and complete inactivity against *T. b. rhodesiense* (Table 1). A significant drop in logP value compared to **10** (Table 1) may be related to this inactivity. Finally, the 4-phenylbenzamide **13** was the most potent derivative against T. cruzi, with an IC50 of 43 nM (36 times more potent than benznidazole) and selectivity of 2782, the highest selectivity observed in all compounds. Compound **13** was also moderately active against *T. b. rhodesiense* (Table 1).

All the 3-nitrotriazole-based amides in which the nitrotriazole ring was linked through the amino group (**3–13**), with the exception of **9**, were 1.9–36 fold more potent than benznidazole against *T. cruzi* amastigotes (Table 1).

Table 1. In Vitro Biological and Physical Properties of 3-Nitrotriazole-Based Amides/Sulfonamides

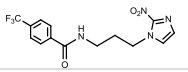
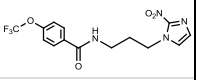
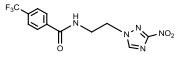
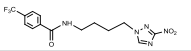
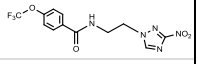
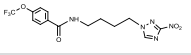
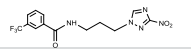
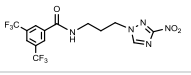
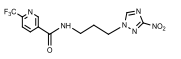
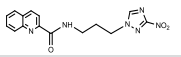
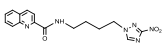
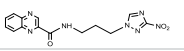
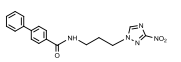
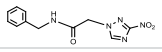
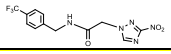
Comp.	*T.b.rhod.* [a] IC_{50} (µM)	SI	*T. cruzi* [b] IC_{50} (µM)	SI	*L.don. axen.* [c] IC_{50} (µM)	SI[d]	Cytotox. L6 [e] IC_{50} (µM)	IC_{50} Bnz/ IC_{50} Comp	logP	PSA (Å^2)	Chemical Structure
Melars.	0.012 ± 0.001										Reference
Bnz			1.562 ± 0.011								Reference
Miltef.					0.382 ± 0.005						Reference
Podoph.							0.022				Reference
1	21.374		6.053	29	13.77		176.6	0.3	2.5	92.7	
2	46.648		3.715	74	36.03		274	0.4	3.1	102.0	
3	3.161		0.438	>625	33.44		>273.6	3.6	2.1	105.6	
4	0.501	208	0.176	591	7.93		104.3	8.9	2.7	105.6	
5	1.986	>131	0.73	>357	29.25		>260.9	2.1	2.6	114.9	
6	1.391	>193	0.113	>2381	12.98		>268	13.8	3.2	114.9	
7	3.761		0.353	>826	37.32		>292	4.4	2.2	105.6	
8	16.4	11.4	0.642	290.7	12.34		186.6	2.4	3.0	105.6	
9	3.546		3.459	>84	96.51		>291	0.5	1.3	118.5	
10	3.22		0.138	1579	19.94		217.98	11.3	1.8	118.5	
11	4		0.132	691	13.41		91.5	11.8	2.3	118.5	
12	34.862		0.807	>379	62.69		>306	1.9	1.0	131.4	
13	0.587	199	0.043	2782	8.37		117	36.3	2.9	105.6	
14	9.96		3.383	102	28.12		344.83	0.5	1.0	105.6	
15	6.474		0.970	133	10.39		128.88	1.6	1.8	105.6	

Table 1. continued

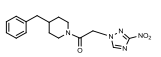
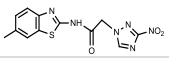
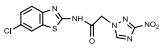
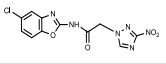
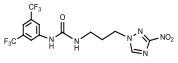
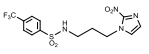
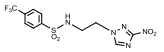
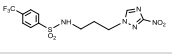
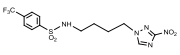
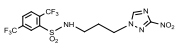
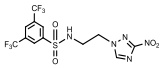
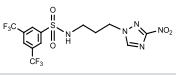
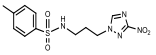
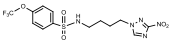
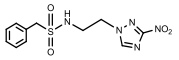
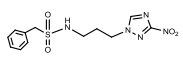
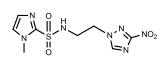
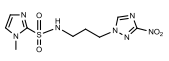
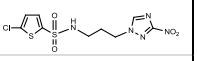
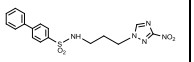
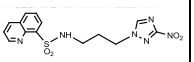
Comp.	*T.b.rhod.* [a] IC$_{50}$ (µM)	SI	*T. cruzi* [b] IC$_{50}$ (µM)	SI	*L.don. axen.* [c] IC$_{50}$ (µM)	SI[d]	Cytotox. L6 [e] IC$_{50}$ (µM)	IC$_{50}$ Bnz/ IC$_{50}$ Comp	logP	PSA (Å^2)	Chemical Structure
16	3.404		0.307	468	51.37		143.77	5.1	2.2	96.8	
17	11.51	14.8	1.799	94.4	5.91	28.7	169.8	0.9	2.6	118.5	
18	48.45	3.4	6.588	24.8	5.82	28	163.1	0.2	2.7	118.5	
19	34.42	<1	7.876	3.3	4.68	26.3	8.49	0.2	1.9	131.7	
20	6.03	4	0.734	33	11.43		24.18	2.1	3.1	117.7	
21	27.51		1.659	106	15.55		175.99	0.9	2.3	109.8	
22	2.79		0.803	248.5	32.38		199.5	1.9	1.8	122.7	
23	0.504	467	0.359	656	13.09		235.33	4.4	1.9	122.7	
24	0.354	240	0.71	120	7.79		84.91	2.2	2.4	122.7	
25	10.313		0.644	178	46.09		114.77	2.4	2.8	122.7	
26	36.7	3	1.677	66.2	33.26		111.1	0.9	2.7	122.7	
27	11.3	9.6	0.322	337.6	20.74		108.7	4.9	2.8	122.7	
28	2.54	121	0.412	746.8	38.15	8.1	307.7	3.8	1.5	122.7	
29	0.477	234.9	0.203	551.7	7.8	14.4	112	7.7	3.0	131.9	
30	8.39	>38.3	6.463	>48	112.86		>321.5	0.2	0.7	122.7	
31	6.49	>47.4	2.237	>137.6	79.38		>307.7	0.7	0.8	122.7	
32	35.88	>9.3	83.39	4	>332.2		>332.2	0.0	-0.3	140.5	

Table 1. continued

Comp.	*T.b.rhod.* [a] IC_{50} (µM)	SI	*T. cruzi* [b] IC_{50} (µM)	SI	*L.don. axen.* [c] IC_{50} (µM)	SI[d]	Cytotox. L6 [e] IC_{50} (µM)	IC_{50} Bnz/ IC_{50} Comp	logP	PSA (Å^2)	Chemical Structure
33	21.9	>14.5	20.57	>15	223.17		>317.5	0.1	-0.2	140.5	
34	1.99	122	0.438	556	33	7.4	243.5	3.6	1.7	122.7	
35	1.049		0.028	1764	7.54		50	55.8	2.7	122.7	
36	6.519		0.4	519	32.87		208	3.9	1.2	135.6	
	active		moderately active			active but cytotoxic, low specificity					

[a]T.brucei rhodesiense, strain STIB 900, trypomastigotes. [b]T. cruzi, strain Tulahuen C4, amastigotes. [c]Axenic L. donovani, strain MHOM-ET-67/L82,amastigotes. [d]SI is the ratio IC50 in L6 cells/IC50 in each parasite. [e]Cytotoxicity in L6 cells. Reference drugs: melarsoprol (Melars), benznidazole (Bnz), miltefosine (Miltef), podophylotoxin (podoph). The IC50 value of each reference is the mean from 36 measurements in parallel with each
compound (SD was 0.001, 0.011, and 0.005 for Melars, Bnz, and Miltef, respectively). PSA = polar surface area. All physical properties werepredicted by using the Marvin Calculator (www.chemaxon.com).

Analysis of amides in which the 3-nitrotriazole ring is linked through the carbonyl group

A small number of amides (**14–19**) in which the 3-nitrotriazole ring is linked through the carbonyl group were also synthesized for comparison with benznidazole. Compound **14** was 0.5 fold less potent against *T. cruzi* amastigotes than its 2-nitroimidazole-bearing analog, benznidazole (Table 1), perhaps due to its decreased lipophilicity (logP 0.95 versus 1.32 for benznidazole). Indeed, the more lipophilic amides **15** and **16** were also more potent antichagasic agents than benznidazole (Table 1).

Interestingly, despite their relatively high lipophilicity, the benzothiazole acetamides **17** and **18** and the benzoxazole acetamide **19** were less potent against *T. cruzi* amastigotes compared to benznidazole. Similarly, all three compounds were inactive against *T. b. rhodesiense* (Table 1). However, compounds **17–19** demonstrated a moderate antileishmanial activity and could be considered as initial scaffolds for further investigation for such drugs.

To further expand the class of amides, we have evaluated one urea (**20**). Although urea **20** was similarly active against *T. cruzi* with the analogous amide **8**, it was significantly

more toxic, resulting in an unacceptable selectivity of 33 (Table 1). Lipophilicity alone could not account for the toxicity of **20**, since both **8** and **20** have similar logP values (Table 1).

Analysis of N-(3-nitrotriazole-alkyl) arene-sulfonamides

Evaluating sulfonamides **21–36**, it is observed that all but the methyl-imidazole sulfonamides **32** and **33,** were potent antichagasic agents. Looking at Table 1, it is apparent that compounds **32** and **33** were the only ones with negative logP values and PSA > 140 A, indicative of poor penetration through cell membranes.

The 2-nitroimidazole-based sulfonamide **21** was a more potent antichagasic agent than the analogous amides **1** and **2,** but still slightly less active than the reference drug benznidazole (Table 1). These results imply that perhaps further evaluation of 2-nitroimidazole-based sulfonamides as antichagasic agents is worthwhile. However, as was mentioned previously, both 2-nitroimidazole-based amides and sulfonamides were not effective anti-HAT agents compared to their 3-nitrotriazole-based analogs.

As in the case of N-(3-nitrotriazolyl-alkyl)benzamides, activity of N-(3-nitrotriazolyl-alkyl) benzene sulfonamides **22–24** against *T. b. rhodesiense*, proportionally increases with the length of the linker between the 3-nitrotriazole ring and sulfamido group (Table 1). The same rule, however, does not apply here for activity against *T. cruzi*, although it is clear that two methylene-linker corresponds to the lowest activity (Table 1).

In general, sulfonamides were slightly less potent antichagasic agents compared to their analogous amides (compare **22** with **3; 24** with **4; 29** with **6**). However, sulfonamides **27** and**35** were more potent than amides **8** and **13**, respectively, against *T. cruzi* (Table 1). A second trifluoromethyl group on the phenyl ring (**25, 26** and **27**) resulted in inactivity against *T. b. rhodesiense*, independently of its position on the ring (**25, 26, 27**), with the linker length (**26**) being the most determinant parameter. However, the effect of the second trifluoromethyl group on the antichagasic activity of sulfonamides was not clear (Table 1). Replacing the trifluoromethyl group in **24** with a trifluoromethoxy group in **29** increased the activity and selectivity against *T. cruzi* but slightly reduced the activity and selectivity against *T. b. rhodesiense*. Membrane permeability issues, due to a greater PSA value in **29**, may be the reason for this slight reduction in anti-HAT activity (Table 1).

Replacing the trifluoromethyl group in **23** with a methyl group in **28** resulted in slightly decreased activity and slightly increased selectivity against *T. cruzi*, perhaps due to a

slight decrease in lipophilicity (Table 1). However, this slight decrease in lipophilicity of **28** had a more dramatic decrease in both activity and selectivity against of *T. b. rhodesiense* (Table 1). Exchanging the tolyl group in **28** with a benzyl group in **31** further decreased the logP value and resulted in lower activity and selectivity against both *T. cruzi* and *T. b. rhodesiense* (Table 1). Finally, shortening the linker of **31** by one methylene group in **30** significantly decreased the activity against *T. cruzi* and *T. b. rhodesiense* and resulted in unacceptable selectivity (Table1). Interestingly, both benzyl

Table 2. Effect of TbNTR Expression on the Activity of Selected Compounds against Bloodstream-Form T. brucei brucei Parasites

ID No	*T.b. rhod.* [a] IC_{50} (μM)	*T.b. Brucei* [b] IC_{50} (μM)	TbNTR[c] -tet	TbNTR[c] +tet	Ratio -tet/+tet
3	3.16	1.3 ± 0.4	1.26 ± 0.27	0.09 ± 0.02	14
4	0.50	0.9 ± 0.1	1.30 ± 0.28	0.13 ± 0.01	10
6	1.39	3.6 ± 0.7	1.05 ± 0.05	0.10 ± 0.00	11
7	3.76	> 10	nd[d]	nd	nd
8	16.4	>10	nd	nd	nd
9	3.55	7.9 ± 0.2	nd	nd	nd
10	3.22	> 10	nd	nd	nd
11	4.00	> 10	nd	nd	nd
12	34.86	> 10	nd	nd	nd
13	0.59	0.3 ± 0.0	0.28 ± 0.02	0.05 ± 0.01	6
14	9.96	> 10	nd	nd	nd
15	6.47	8.5 ± 0.2	nd	nd	nd
16	3.40	> 10	nd	nd	nd
20	6.03	1.0 ± 0.0	0.81 + 0.07	0.18 + 0.02	5
22	2.79	> 10	nd	nd	nd
23	0.50	3.4 ± 0.6	7.83 ± 0.50	0.25 ± 0.01	31

Table 2. (continued)

ID No	*T.b. rhod.* [a] IC_{50} (μM)	*T.b. Brucei* [b] IC_{50} (μM)	TbNTR[c] -tet	TbNTR[c] +tet	Ratio -tet/+tet
25	10.31	> 10	nd	nd	nd
26	36.7	7.9 ± 0.1	nd	nd	nd
27	11.3	6.6 ± 0.1	nd	nd	nd
28	2.54	4.0 ± 0.3	5.63 + 2.40	0.24 + 0.02	23
34	1.99	2.3 ± 0.1	4.34 ± 0.05	0.23 + 0.01	19
35	1.05	0.5 ± 0.0	0.44 ± 0.03	0.07 ± 0.01	6
36	6.52	> 10	nd	nd	nd
Nfx[e]			1.71 ± 0.06	0.13 ± 0.04	13

[a]STIB 900 trypomastigotes. [b]Bloodstream-form wild-type T. brucei brucei (Lister 427, clone 221a) parasites. [c]Bloodstream-form T. brucei brucei parasites, engineered to overexpress type I nitroreductase in the presence (+tet) or absence (−tet) of tetracycline. [d]Not determined. [e]Nifurtimox (positive control). [f]Benznidazole (positive control). [g]Melarsoprol (negative control).

sulfonamides **30** and **31** were less potent antichagasic agents than benznidazole.

As in the case of 4-phenylbenzamide **13**, the 4-phenylbenzene sulfonamide **35** was the most potent antichagasic compound in the series of sulfonamides, with an IC50 of 28 nM (~56 times more potent than benznidazole) and selectivity of 1764. Sulfonamide **35** was more potent against *T. cruzi* than the analogous amide **13,** but less active than **13** against T. b. rhodesiense. In addition, increased toxicity of **35** to L6 host cells, independently of lipophilicity, resulted in decreased selectivity as compared to **13** (Table 1).

Replacing the phenyl ring with a chloro-thiophene in **34**, slightly decreased the potency against *T. cruzi* and had a more significant impact in selectivity due to an increase in toxicity (compare **28** with **34**). However, the activity against *T. b. rhodesiensie* and selectivity of **34** was similar to that of **28** (Table 1). Replacing the benzene ring with an 8-quinoline in **36**, did not affect the antichagasic potency but resulted in increased toxicity and decreased selectivity as compared to **28**. The decreased anti-HAT activity

of **36** compared to **28**, may be related to a decreased lipophilicity and an increased PSA value (Table 1).

The involvement of type I nitroreductase in the activation of 3-nitrotriazole-based amides/sulfonamides

Nitroheterocyclic prodrugs must undergo enzyme-mediated activation within the pathogen to have cytotoxic effects, a reaction catalyzed by nitroreductases. Both Nfx and Bnz are activated by the NADH-dependent, oxygen insensitive, mitochondrially localized, bacteriallike, type I NTR, and down-regulation of this enzyme resulted in resistance to these compounds.[10–12]

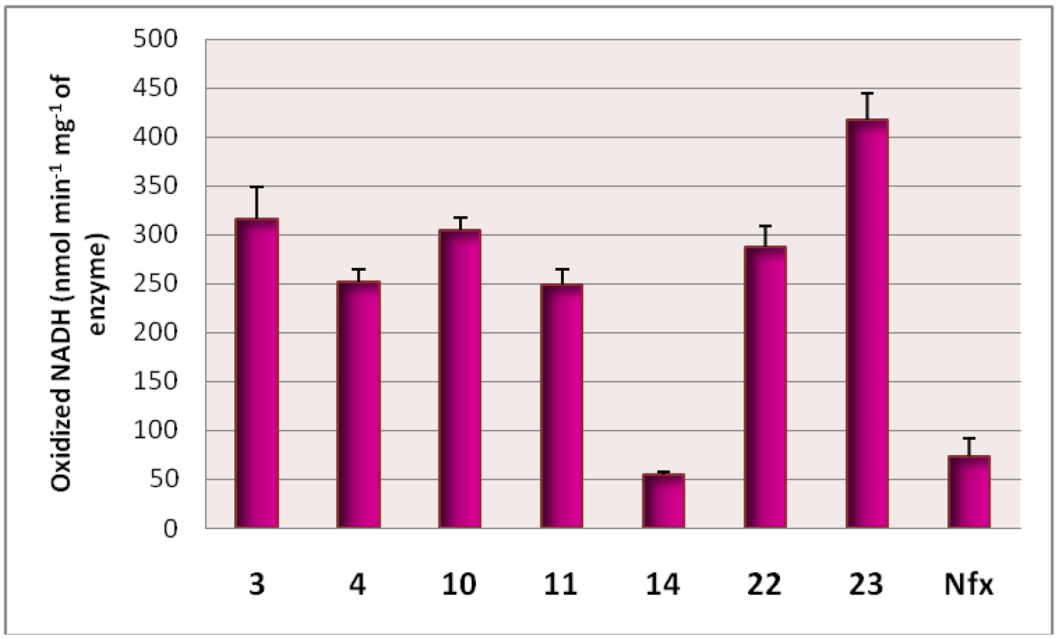


Figure 1. Activity of recombinant TbNTR toward different amides/sulfonamides and nifurtimox (Nfx).

Several compounds from all sub-categories in Table 1 have been evaluated for anti-HAT activity against bloodstream form *T. b. brucei* (Table 2). With few exceptions (**3, 13, 20, 35**), most compounds demonstrated a greater IC50 value or were inactive against *T. b. brucei* compared to *T. b. rhodesiense* (Table 2). Compounds with an IC50 ≤ 5 μM against *T. b. brucei* were tested in a parasite line engineered to overexpress tetracycline-inducible TbNTR, in order to examine the involvement of this enzyme in their activation (Table 2).

It is observed that parasites overexpressing tetracycline-inducible TbNTR were more susceptible to all such compounds (**3, 4, 6**, **13, 20, 23**, **28, 34** and **35)** as compared to

wildtype parasites, with −tet/+tet (non-induced/induced) ratio ranging from 5 to 31 (Table 2). This implies that the major growth inhibitory activity of these compounds is via type I NTR activation. It is also observed in Table 2 that the least active compounds against wild type *T. b. brucei* (**6, 23, 28** and **34**) showed a greater −tet/+tet ratio than the most active compounds **13** and **35**.

Selected compounds from Table 1 were tested as substrates of purified type I TbNTR. As shown in Fig. 1, all of the tested compounds were preferred substrates of the nitroreductase and there is, in general, a good correlation between enzymatic activity and activity against *T. b. rhodesiense*.

To exclude the possibility that these compounds may exert some of their anti-trypanosomal activity via trypanothione reductase (TR) inhibition,[8,27] we have tested selected compounds (**3, 6, 10, 15, 16, 21, 23**) against this enzyme. None of the compounds showed an inhibitory activity against TR at concentrations < 100 μM (unpublished results, private communication with Dr. Mary O'Sullivan, Canisius College, Buffalo, NY).

CONCLUSIONS

From the above results and discussion, it is concluded that, like the 3-nitrotriazole-based aromatic and aliphatic amines, 3-nitrotriazole-based amides and sulfonamides exert exceptional in vitro antichagasic and anti-HAT activities. All tested compounds satisfy the Lipinski rule of 5 and at least 19 of them (**3–8, 10–13, 16, 22, 23, 27–29, 34–36**) have been identified (Table 1) as potential candidates for in vivo studies in *T. cruzi* infected mice. All of the 19 compounds have demonstrated significant antichagasic activity at low to intermediate nmolar concentrations and selectivity > 200. In addition, all of them were 2–56 fold more potent as antichagasic agents than benznidazole (Table 1). Compounds **4, 13, 23, 24** and **29** also deserve further in vivo investigation as anti-HAT agents, whereas compounds **17–19** should be used as initial scaffolds for further investigation of antileishmania drugs.

EXPERIMINTAL

All starting materials and solvents were purchased from Sigma-Aldrich (Milwaukee, WI), were of research-grade quality and used without further purification. Solvents used were anhydrous and the reactions were carried out under a nitrogen atmosphere and

exclusion of moisture. Melting points were determined by using a Mel-Temp II Laboratory Devices apparatus (Holliston, MA) and are uncorrected. Elemental Analyses were obtained by Midwest Microlab, LLC (Indianapolis, IN). Proton NMR spectra were obtained on a Varian Inova-500 or a Bruker Avance-III-500 spectrometer at 500 MHz and are referenced to Me4Si or to the corresponding protonated solvent, if the solvent was not CDCl3. Highresolution electrospray ionization (HRESIMS) mass spectra were obtained on a Agilent 6210 LC-TOF mass spectrometer at 11000 resolution. Thin-layer chromatography was carried out on aluminum oxide N/UV254 or polygram silica gel G/UV254 coated plates (0.2 mm, Analtech, Newark, DE). Chromatography was carried out on preparative TLC alumina GF (1000 microns) or silicagel GF (1500 microns) plates (Analtech). All of the amides/ sulfonamides were purified by preparative TLC chromatography on silicagel GF plates (≥ 95% purity). The results from elemental analysis for C, H and N were within 0.4 of the theoretical value.
The synthesis of compound **1** has been described before.[22]

General synthetic procedure of arylamides/sulfonamides and urea 20

For compounds **2–13** and **21–36:** The appropriate commercially available arylcarbonyl/ arylsulfonyl chloride (1.24 mmol) was dissolved in 2–3 mL dry dichloromethane and added dropwise to a solution of 3-nitro-1H-1,2,4-triazolyl-alkylamine23 (1.24 mmol) and triethylamine (2.48 mmol) in 6–8 mL of dry dichloromethane, at room temperature and under an inert atmosphere. In three cases (**1**, **2, 21**), 3-(2-nitro-1H-imidazolyl)-propylamine23 (1.24 mmol) was used. The reaction mixture was worked up after 12 h of stirring at room temperature. For compounds **3, 5, 22, 26, 30** and **32** the hydrochloride salt of 2-(3-nitro-1H-1,2,4-triazole)ethylamine (instead of the free amine) and 4 eq of triethyl amine were used. In this case, the reaction mixture was a suspension and the yields of the final product were not very good.
For urea **20**, the commercially available 3,5-bis(trifluoromethyl)phenyl isocyanate (1.1 mmol) was added dropwise to a dichloromethane solution of 3-nitro-1H-1,2,4-triazolylpropylamine (1.1 mmol), at room temperature and under an inert atmosphere. The urea was formed immediately at 100% yield, as a white precipitate.
For amides **14–19,** 3-nitro-1,2,4-triazole (0.9–1.0 mmol) was stirred under an inert atmosphere and exclusion of moisture with 1.2 eq of KOH in acetonitrile under mild heating (ca. 40 °C) and then this suspension was slowly added to an acetonitrile solution of the appropriate α-chloroacetamide[24] (**37a–f)**. α-Chloroacetamides **37b–f** were

synthesized at room temperature by adding a dichloromethane solution of an appropriate amine (2.79 mmol) and triethylamine (3.07 mmol) to a dichloromethane solution of α-chloroacetyl chloride (3.07 mmol), according to the literature[24].

N-[3-(2-Nitro-1H-imidazol-1-yl)propyl]-4-(trifluoromethoxy)benzamide (2)—Off white powder (54%): mp 68–70 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 7.84 (d, J=9.0 Hz, 2H), 7.31 (br s, 2H), 7.30 (s, 1H), 7.18 (s, 1H), 6.40 (br s, 1H), 4.54 (t, J=7.0 Hz, 2H), 3.57 (m, 2H), 2.21 (m, 2H). HRESIMS calcd for $C_{14}H_{14}F_3N_4O_4$ and $C_{14}H_{13}F_3N_4NaO_4$ m/z $[M+H]^+$ and $[M+Na]^+$ 359.0962, 381.0781, found 359.0962, 381.0784.

N-[2-(3-Nitro-1H-1,2,4-triazol-1-yl)ethyl]-4-(trifluoromethyl)benzamide (3)— White powder (65%): mp 155–157 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.24 (s, 1H), 7.86 (br s, 1H), 7.72 (d, J=8.0 Hz, 2H), 7.46 (d, J=8.0 Hz, 2H), 4.43 (t, J=5.0 Hz, 2H), 3.76 (m, 2H). HRESIMS calcd for $C_{12}H_{11}F_3N_5O_3$ m/z $[M+H]^+$ 330.0809, found 330.0815.

N-[4-(3-Nitro-1H-1,2,4-triazol-1-yl)butyl]-4-(trifluoromethyl)benzamide (4)— White powder (62%): mp 78–79 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.69 (s, 1H), 8.07 (d, J=8.0 Hz, 2H), 8.04 (br s, 1H), 7.81 (d, J=8.0 Hz, 2H), 4.49 (t, J=7.0 Hz, 2H), 3.49 (m, 2H), 2.06 (m, 2H), 1.69 (m, 2H). HRESIMS calcd for $C_{14}H_{15}F_3N_5O_3$ m/z $[M+H]^+$ 358.1122, found 358.1131.

N-[2-(3-Nitro-1H-1,2,4-triazol-1-yl)ethyl]-4-(trifluoromethoxy)benzamide (5)— White powder (65%): mp 108–109 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.70 (s, 1H), 8.17 (br s, 1H), 7.95 (d, J=8.5 Hz, 2H), 7.41 (d, J=8.5 Hz, 2H), 4.65 (t, J=5.5 Hz, 2H), 3.93 (t, J=5.5 Hz, 2H). HRESIMS calcd for $C_{12}H_{11}F_3N_5O_4$ m/z $[M+H]^+$ 346.0758, found 346.0765.

N-[4-(3-Nitro-1H-1,2,4-triazol-1-yl)butyl]-4-(trifluoromethoxy)benzamide (6)— White powder (72%): mp 64–65 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.26 (s, 1H), 7.82 (d, J=8.5 Hz, 2H), 7.27 (d, J=8.0 Hz, 2H), 6.44 (br s, 1H), 4.39 (t, J=7.0 Hz, 2H), 3.53 (m, 2H), 2.06 (quintet, J=7.0 Hz, 2H), 1.69 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_{14}H_{15}F_3N_5O_4$ m/z $[M+H]^+$ 374.1071, found 374.1075.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-3-(trifluoromethyl)benzamide (7)— White powder (70%): mp 81–83 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.45 (s, 1H), 8.05 (s, 1H), 7.98 (d, J=7.5 Hz, 1H), 7.81 (d, J=8.0 Hz, 1H), 6.63 (t, J= 8.0 Hz, 1H), 6.55 (br s, 1H), 4.43 (t, J=6.5 Hz, 2H), 3.58 (m, 2H), 2.32 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{13}H_{13}F_3N_5O_3$ m/z $[M+H]^+$ 344.0965, found 344.0969.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-3,5-bis(trifluoromethyl)benzamide (8)—White powder (83%): mp 152–153 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.72 (s, 1H), 8.52 (s, 2H), 8.46 (br s, 1H), 8.25 (s, 1H), 4.57 (t, J=7.0 Hz, 2H), 3.59 (q, J=6.5 Hz, 2H), 2.35 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_{14}H_{12}F_6N_5O_3$ m/z $[M+H]^+$ 412.0839 found 412.0844.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-6-(trifluoromethyl)pyridine-3-carboxamide (9)—White powder (71%): mp 92–94 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 9.09 (s, 1H), 8.39 (s, 1H), 8.33 (d, J=8.5 Hz, 1H), 7.82 (d, J=8.0 Hz, 1H), 6.73 (br s, 1H), 4.43 (t, J=6.5 Hz, 2H), 3.60 (q, J=6.5 Hz, 2H), 3.42 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for C12H12F3N6O3 and $C_{12}H_{11}F_3N_6NaO_3$ m/z $[M+H]^+$ and $[M+Na]^+$ 345.0917, 367.0737, found 345.0929, 367.0745. Calculated analysis for $C_{12}H_{11}F_3N_6O_3$: C, 41.87; H, 3.22 ; N, 24.41. Found: C, 41.93; H, 3.38; N, 24.17.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]quinoline-2-carboxamide (10)—Off white powder (70%): mp 135–137 °C; ^{1}H NMR (500 MHz, CD3OD) δ: 8.68 (s, 1H), 8.46 (d, J=8.5 Hz, 1H), 8.17 (t, J=9.0 Hz, 2H), 8.0 (d, J=8.5 Hz, 1H), 7.83 (t, J=8.5 Hz, 1H), 7.69 (t, J=8.0 Hz, 1H), 4.46 (t, J=8.0 Hz, 2H), 3.58 (t, J=6.5 Hz, 2H), 2.34 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{15}H_{15}N_6O_3$ and $C_{15}H_{14}N_6NaO_3$ m/z $[M+H]^+$ and $[M+Na]^+$ 327.1200, 349.1020, found 327.1209, 349.1026.

N-[4-(3-Nitro-1H-1,2,4-triazol-1-yl)butyl]quinoline-2-carboxamide (11)—Off white powder (67%): mp 124–126 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.75 (br s, 1H), 8.70 (s, 1H), 8.52 (d, J=8.5 Hz, 1H), 8.24 (d, J=8.5 Hz, 1H), 8.06 (t, J=9.5 Hz, 2H), 7.84 (t, J=7.0 Hz, 1H), 7.70 (t, J=7.0 Hz, 1H), 4.52 (t, J=7.0 Hz, 2H), 3.58 (q, J=6.5 Hz, 2H), 2.09 (m, 2H),1.76 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_{16}H_{17}N_6O_3$ m/z $[M+H]^+$ 341.1357, found 341.1369.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]quinoxaline-2-carboxamide (12)—Off white powder (66%): mp 143–144 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 9.67 (s, 1H), 8.47 (s, 1H), 8.22 (d, J=8.0 Hz, 1H), 8.21 (br s, 1H), 8.12 (d, J=7.5 Hz, 1H), 7.90 (m, 2H), 4.45 (t, J=6.5 Hz, 2H), 3.66 (q, J=6.5 Hz, 2H), 2.39 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{14}H_{14}N_7O_3$ m/z $[M+H]^+$ 328.1153, found 328.1166. Calculated analysis for $C_{14}H_{13}N_7O_3$: C, 51.38; H, 4.0; N, 29.96. Found: C, 51.29; H, 4.17; N, 29.68.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-4-phenylbenzamide (13)—White powder (96%): mp 177–179 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.48 (s, 1H), 7.85 (d, J=8.5 Hz, 2H), 7.69 (d, J=7.0 Hz, 2H), 7.48 (t, J=7.5 Hz, 2H), 7.40 (t, J=7.5 Hz, 1H), 6.49 (br t, 1H), 4.43 (t, J=6.5 Hz, 2H), 3.57 (q, J=6.5 Hz, 2H), 2.30 (quintet, J=6.5 Hz,

2H). HRESIMS calcd for $C_{18}H_{18}N_5O_3$ and $C_{18}H_{17}N_5NaO_3$ m/z $[M+H]^+$ and $[M+Na]^+$ 352.1404, 374.1224, found 352.1406, 374.1222. Calculated analysis for $C_{18}H_{17}N_5O_3$: C, 61.53; H, 4.88; N, 19.93. Found: C, 61.79; H, 4.96; N, 19.58.

N-Benzyl-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (14)—Off white powder (40%): mp 103–106 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.39 (s, 1H), 7.38-7.28 (m, 5H), 6.26 (br s, 1H), 4.98 (s, 2H), 4.50 (d, J=5.5 Hz, 2H). HRESIMS calcd for $C_{11}H_{12}N_5O_3$ m/z $[M+H]^+$ 262.0935, found 262.0935.

2-(3-Nitro-1H-1,2,4-triazol-1-yl)-N-{[4-(trifluoromethyl)phenyl]methyl}acetamide (15)—White microcrystal (78%): mp 168–170 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.37 (s, 1H), 7.62 (d, J=8.0 Hz, 2H), 7.40 (d, J=8.0 Hz, 2H), 6.35 (br s, 1H), 4.99 (s, 2H), 4.56 (d, J=6.0 Hz, 2H). HRESIMS calcd for $C_{12}H_{11}F_3N_5O3$ and $C_{12}H_{10}F_3N_5NaO_3$ m/z $[M+H]^+$ and $[M+Na]^+$ 330.0809, 352.0628, found 330.0814, 352.0632.

1-(4-Benzylpiperidin-1-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)ethan-1-one (16)—White powder (84%): mp 129–131 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.57 (s, 1H), 7.31-7.18 (m, 5H), 5.47 (dt, J=19.0, 16.5 Hz, 2H), 4.44 (d J=13 Hz, 1H), 3.97 (d, J=13.5 Hz, 1H), 3.16 (t, J=13.5 Hz, 1H), 2.64 (t, J=13.0 Hz, 1H), 2.60 (d, J=7.0 Hz, 2H), 1.88 (m, 1H), 1.76 (d, J=13.0 Hz, 1H), 1.69 (d, J=13.0 Hz, 1H), 1.36-1.32 (dq, J=12.5, 4.5 Hz, 1H), 1.16-1.13 (dq, J=12.0, 4.0 Hz, 1H). HRESIMS calcd for C16H20N5O3 m/z $[M+H]^+$ 330.1561, found 330.1576. Calculated analysis for $C_{16}H_{19}N_5O_3$: C, 58.35; H, 5.82; N, 21.26. Found: C, 58.27; H, 5.83; N, 21.30.

N-(6-methyl-1,3-benzothiazol-2-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (17)—Off white powder (59%): mp 230 °C (dec); ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.81 (s, 1H), 7.74 (s, 1H), 7.63 (d, J=8.0 Hz, 1H), 7.28 (d, J=8.0 Hz, 1H), 5.68 (s, 2H), 2.44 (s, 3H). HRESIMS calcd for $C_{12}H_{11}N_6O_3S$ m/z $[M+H]^+$ 319.0608, found 319.0617.

N-(6-chloro-1,3-benzothiazol-2-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (18)—Off white powder (58%): mp 245–248 °C (dec); 1H NMR (500 MHz, CD3COCD3) δ: 8.81 (s, 1H), 8.06 (s, 1H), 7.74 (d, J=8.5 Hz, 1H), 7.04 (dd J=8.5, 2.0 Hz, 1H), 5.71 (s, 2H). HRESIMS calcd for $C_{11}H_8ClN_6O_3S$ m/z $[M+H]^+$ 339.0062, 341.0034, found 339.0072, 341.0045.

N-(5-chloro-1,3-benzoxazol-2-yl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide (19)—Off white powder (45%): mp 208–210 °C (dec); ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.76 (s, 1H), 7.60 (s, 1H), 7.59 (d, J=9.0 Hz, 1H), 7.34 (dd, J=8.5, 2.0 Hz, 1H), 5.80 (s, 1H). HRESIMS calcd for $C_{11}H_6ClN_6O_4$ m/z $[M-H]^-$ 321.0145, 323.0119, found 321.0147, 323.0143.

1-[3,5-bis(trifluoromethyl)phenyl]-3-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]urea (20)—White powder (95%): mp 151–152 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.71 (s, 1H), 8.68 (br s, 1H), 8.15 (s, 2H), 7.54 (s, 1H), 6.31 (br s, 1H), 4.51 (t, J=6.5 Hz, 2H), 3.35 (m, 2H), 2.21 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{14}H_{13}F_6N_6O_3$ m/z $[M+H]^+$ 427.0948, found 427.0954.

N-[3-(2-Nitro-1H-imidazol-1-yl)propyl]-4-(trifluoromethyl)benzene-1-sulfonamide (21)—White powder (56%): mp 129–131 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 7.99 (d, J=8.0 Hz, 2H), 7.82 (d, J=8.5 Hz, 2H), 7.24 (s, 1H), 7.19 (s, 1H), 4.77 (br t, 1H), 4.57 (t, J=7.0 Hz, 2H), 3.06 (q, J=6.5 Hz, 2H), 2.12 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{13}H_{14}F_3N_4O_4S$ and $C_{13}H_{13}F_3N_4NaO_4S$ m/z $[M+H]^+$, $[M+Na]^+$ 379.0682, 401.0502 found 379.0685, 401.0506.

N-[2-(3-Nitro-1H-1,2,4-triazol-1-yl)ethyl]-4-(trifluoromethyl)benzene-1-sulfonamide (22)—White powder (35%): mp 155–156 °C; ^{1}H NMR (500 MHz, CDCl3 + several drops of CD3COCD3) δ: 8.51 (s, 1H), 7.99 (d, J=8.5 Hz, 2H), 7.82 (d, J=8.5 Hz, 2H), 7.04 (br s, 1H), 4.56 (t, J=6.0 Hz, 2H), 3.56 (m, 2H). HRESIMS calcd for $C_{11}H_{11}F_3N_5O_4S$ m/z $[M+H]^+$ 366.0478, found 366.0481.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-4-(trifluoromethyl) benzene-1-sulfonamide (23)—White powder (88%): mp 67–68 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.36 (s, 1H), 7.97 (d, J=8.5 Hz, 2H), 7.81 (d, J=8.5 Hz, 2H), 5.01 (br t, 1H), 4.51 (t, J=6.5 Hz, 2H), 3.03 (q, J=6.5 Hz, 2H), 2.23 (quintet, J=6.0 Hz, 2H). HRESIMS calcd for $C_{12}H_{13}F_3N_5O_4S$ m/z $[M+H]^+$ 380.0635, found 380.0635.

N-[4-(3-Nitro-1H-1,2,4-triazol-1-yl)butyl]-4-(trifluoromethyl) benzene-1-sulfonamide (24)—White powder (49%): mp 83–85 °C; ^{1}H NMR (500 MHz, CD3OD) δ: 8.59 (s, 1H), 8.03 (d, J=8.0 Hz, 2H), 7.89 (d, J=8.0 Hz, 2H), 4.32 (t, J=7.0 Hz, 2H), 2.94 (t, J=6.5 Hz, 2H), 1.96 (quintet, J=7.5 Hz, 2H), 1.51 (quintet, J=7.5 Hz, 2H). HRESIMS calcd for $C_{13}H_{15}F_3N_5O_4S$ m/z $[M+H]^+$ 394.0791, found 394.0796.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-2,5-bis(trifluoromethyl)benzene-1-sulfonamide (25)—White powder (85%): mp 131–133 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.42 (s, 1H), 8.31 (s, 1H), 8.06 (d, J=8.0 Hz, 1H), 8.00 (d, J=8.0 Hz, 2H), 5.10 (br s, 1H), 4.49 (t, J=6.5 Hz, 2H), 3.07 (m, 2H), 2.25 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{13}H_{12}F_6N_5O_4S$ and $C_{13}H_{11}F_6N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 448.0509, 470.0328, found 448.0496, 470.0310.

N-[2-(3-nitro-1H-1,2,4-triazol-1-yl)ethyl]-3,5-bis(trifluoromethyl)benzene-1-sulfonamide (26)—White powder (40%): mp 164–165 °C; ^{1}H NMR (500 MHz,

CD3COCD3) δ: 8.67 (s, 1H), 8.40 (s, 2H), 8.37 (s, 1H), 4.60 (t, J=5.5 Hz, 2H), 3.67 (t, J=5.5 Hz, 2H). HRESIMS calcd for $C_{12}H_{10}F_6N_5O_4S$ and $C_{12}H_9F_6N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 434.0352, 456.0172, found 434.0358, 456.0178.

N-[3-(3-nitro-1H-1,2,4-triazol-1-yl)propyl]-3,5-bis(trifluoromethyl)benzene-1-sulfonamide (27)—White microcrystals (62%): mp 132–134 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.64 (s, 1H), 8.41 (s, 2H), 8.38 (s, 1H), 7.15 (br s, 1H), 4.53 (t, J=7.0 Hz, 2H), 3.15 (t, J=6.5 Hz, 2H), 2.22 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_{13}H_{12}F_6N_5O_4S$ m/z $[M+H]^+$ 448.0509, found 448,0495.

4-Methyl-N-[3-(3-nitro-1H-1,2,4-triazol-1-yl)propyl]benzene-1-sulfonamide (28)—White microcrystals (81%): mp 122–124 °C; ^{1}H NMR (500 MHz, CD3COD) δ: 8.57 (s, 1H), 7.70 (d, J=8.5 Hz, 2H), 7.37 (d, J=8.5 Hz, 2H), 4.41 (t, J=6.5 Hz, 2H), 3.31 (t, J=6.5 Hz, 2H), 2.42 (s, 3H), 2.08 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{12}H_{16}N_5O_4S$ and $C_{12}H_{15}N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 326.0918, 348.0737, found 326.0917, 348.0734. Calculated analysis for C12H15N5O4S: C, 44.30; H, 4.65; N, 21.53; S, 9.85. Found: C, 44.51; H, 4.81; N, 21.22; S, 9.89.

N-[4-(3-nitro-1H-1,2,4-triazol-1-yl)butyl]-4-(trifluoromethoxy)benzene-1-sulfonamide (29)—White powder (42%): mp 66–68 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.64 (s, 1H), 7.99 (d, J=8.5 Hz, 2H), 7.55 (d, J=8.0 Hz, 2H), 6.68 (br s, 1H), 4.42 (t, J=7.0 Hz, 2H), 3.01 (t, J=6.5 Hz, 2H), 2.01 (m, 2H), 1.59 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{13}H_{15}F_3N_5O_5S$ m/z $[M+H]^+$ 410.0741, found 410.0744.

N-[2-(3-Nitro-1H-1,2,4-triazol-1-yl)ethyl]-1-phenylmethanesulfonamide (30)—White powder (31%): mp 165–166 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.62 (s, 1H), 7.42-7.35 (m, 5H), 6.42 (br s, 1H), 4.50 (t, J=5.5 Hz, 2H), 4.37 (s, 2H), 3.58 (t, J=5.5 Hz, 2H). HRESIMS calcd for $C_{11}H_{14}N_5O_4S$ and $C_{11}H_{13}N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 312.0761, 334.0580, found 312.0773, 334.0594.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-1-phenylmethanesulfonamide (31)—White microcrystals (45%): mp 104–106 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.62 (s, 1H), 7.43-7.35 (m, 5H), 6.25 (br s, 1H), 4.49 (t, J=7.0 Hz, 2H), 4.35 (s, 2H), 3.13 (m, 2H), 2.17 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_{12}H_{16}N_5O_4S$ and $C_{12}H_{15}N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 326.0918, 348.0737, found 326.0923, 348.0737.

1-Methyl-N-[2-(3-nitro-1H-1,2,4-triazol-1-yl)ethyl]-1H-imidazole-2-sulfonamide (32)—White powder (24%): mp 170–172 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.72 (s, 1H), 7.45 (br s, 1H), 7.30 (s, 1H), 7.02 (s, 1H), 4.63 (t, J=6.0 Hz, 2H), 3.90 (s,

3H), 3.79 (t, J=6.0 Hz, 2H). HRESIMS calcd for $C_8H_{12}N_7O_4S$ and $C_8H_{11}N_7NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 302.0666, 324.0485, found 302.0664, 324.0480.

1-Methyl-N-[3-(3-nitro-1H-1,2,4-triazol-1-yl)propyl]-1H-imidazole-2-sulfonamide (33)—White powder (61%): mp 106–109 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.68 (s, 1H), 7.28 (s, 1H), 6.99 (s, 1H), 4.57 (t, J=7.0 Hz, 2H), 3.92 (s, 3H), 3.28 (t, J=6.5 Hz, 2H). 2.25 (quintet, J=7.0 Hz, 2H). HRESIMS calcd for $C_9H_{14}N_7O_4S$ and $C_9H_{13}N_7NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 316.0822, 338.0642, found 316.0832, 338.0649. Calculated analysis for $C_9H_{13}N_7O_4S$: C, 34.28; H, 4.16; N, 31.10; S, 10.17. Found: C, 34.32; H, 4.27; N, 30.83; S, 9.85.

5-Chloro-N-[3-(3-nitro-1H-1,2,4-triazol-1-yl)propyl]thiophene-2-sulfonamide (34)—White powder (75%): mp 104–105 °C; ^{1}H NMR (500 MHz, CD3COCD3) δ: 8.65 (s, 1H), 7.47 (d, J=4.0 Hz, 1H), 7.16 (d, J=4.0 Hz, 1H), 7.01 (br s, 1H), 4.53 (t, J=7.0 Hz, 2H), 3.13 (t, J=6.5 Hz, 2H), 2.23 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_9H_{11}ClN_5O_4S_2$ and $C_9H_{10}ClN_5NaO_4S_2$ m/z $[M+H]^+$ and $[M+Na]^+$ 351.9935, 373.9755, found 351.9930, 353.9899, 373.9751, 375.9721.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]-4-phenylbenzene-1-sulfonamide (35)—White powder (60%): mp 132–133 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 8.36 (s, 1H), 7.88 (d, J=8.5 Hz, 2H), 7.73 (d, J=8.0 Hz, 2H), 7.59 (d, J=7.0 Hz, 2H), 7.49 (t, J=7.5 Hz, 2H), 7.43 (t, J=7.0 Hz, 1H), 4.76 (t, J=6.0 Hz, 1H), 4.51 (t, J=6.5 Hz, 2H), 2.80 (q, J=6.5 Hz, 2H), 2.21 (quintet, J=6.5 Hz, 2H). HRESIMS calcd for $C_{17}H_{18}N_5O_4S$ and $C_{17}H_{17}N_5NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 388.1074, 410.0893, found 388.1070, 410.0887.

N-[3-(3-Nitro-1H-1,2,4-triazol-1-yl)propyl]quinoline-8-sulfonamide (36)—Off white powder (63%): mp 142–143 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 9.04 (d, J=4.0 Hz, 1H), 8.40 (s, 2H), 8.32 (d, J=8.0 Hz, 1H), 8.10 (d, J=8.5 Hz, 1H), 7.68 (t, J=7.5 Hz, 1H), 7.62-7.59 (m, 1H), 6.61 (br t, J=6.0 Hz, 1H), 4.55 (t, J=6.0 Hz, 2H), 2.80 (m, 2H), 2.18 (m, 2H). HRESIMS calcd for $C_{14}H_{15}N_6O_4S$ and $C_{14}H_{14}N_6NaO_4S$ m/z $[M+H]^+$ and $[M+Na]^+$ 363.0870, 385.0689, found 363.0883, 385.0680.

N-Benzyl-2-chloroacetamide (37a)—This was commercially available by Aldrich.

2-Chloro-N-{[4-(trifluoromethyl)pheny]methyl}acetamide (37b)—Pink-white crystallic powder28 (89%): mp 87–88 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 7.62 (d, J=7.5 Hz, 2H), 7.42 (d, J=8.0 Hz, 2H), 6.98 (br s, 1H), 4.57 (d, J=6.0 Hz, 2H), 4.14 (s, 2H). HRESIMS calcd for $C_{10}H_{10}ClF_3NO$ m/z $[M+H]^+$ 252.0398, 254.0370, found 252.0407, 254.0378.

1-(4-benzylpiperidin-1-yl)-2-chloroethan-1-one (37c)—Yellowish oil29 (91%): ^{1}H NMR (500 MHz, CDCl3) δ: 7.32-7.14 (m, 5H), 4.55 (d, J=13.0 Hz, 1H), 4.07 (m, 2H), 3.83 (d, J=13.5 Hz, 1H), 3.05 (t, J=13.0 Hz, 1H), 2.61-2.55 (m, 3H), 1.81-1.74 (m, 3H), 1.20– 1.29 (m, 2H). HRESIMS calcd for $C_{14}H_{19}ClNO$ m/z $[M+H]^+$ 252.1150, 254.1124, found 252.1161, 254.1134.

2-Chloro-N-(6-methyl-1,3-benzothiazol-2-yl)acetamide (37d)—Off white crystallic powder30 (100%): mp 190–191 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 9.74 (br s, 1H), 7.70 (d, J=8.5 Hz, 1H), 7.63 (s, 1H), 7.28 (d, J=8.5 Hz, 1H), 4.31 (s, 2H), 2.49 (s, 3H). HRESIMS calcd for $C_{10}H_{10}ClN_2OS$ m/z $[M+H]^+$ 241.0197, 243.0168, found 241.0194, 243.0163.

2-Chloro-N-(6-chloro-1,3-benzothiazol-2-yl)acetamide (37e)—White microcrystallic powder30,31 (73%): mp 203–204 °C (dec); ^{1}H NMR (500 MHz, CDCl3) δ: 9.71 (br s, 1H), 7.82 (s, 1H), 7.73 (d, J=9.0 Hz, 1H), 7.43 (dd, J=10.5, 6.5 Hz, 1H), 4.33 (s, 2H). HRESIMS calcd for $C_9H_7Cl_2N_2OS$ m/z $[M+H]^+$ 260.9651, 262.9621, found 260.9663, 262.9630.

2-Chloro-N-(5-chloro-1,3-benzoxazol-2-yl)acetamide (37f)—Light brownish powder (70%): mp 168–170 °C; ^{1}H NMR (500 MHz, CDCl3) δ: 9.38 (br s, 1H), 7.80-7.30 (m, 3H), 4.38 (s, 2H). HRESIMS calcd for $C_9H_7Cl_2N_2O_2$ and $C_9H_6C_{l2}N_2NaO_2$ m/z $[M+H]^+$ and $[M+Na]^+$ 244.9879 and 266.9699, found 244.9871 and 266.9700.

In vitro biological evaluation

In vitro activity against *T. cruzi*, *T. b. rhodesiense*, *Leishmania donovani* axenic amastigotes and cytotoxicity assessment using L6 cells (rat skeletal myoblasts) was determined using a 96-well plate format as previously described.[25] Data were analyzed with the graphic program Softmax Pro (Molecular Devices, Sunnyvale, CA, USA), which calculated IC50 values by linear regression from the sigmoidal dose inhibition curves.

In vitro T. brucei brucei antiproliferating assays and susceptibility studies

T. b. brucei bloodstream form parasites were seeded at 1 × 103 ml−1 in 200 μL of growth medium containing different concentrations of a nitrotriazole or nifurtimox. Where appropriate, induction of the TbNTR was carried out by adding tetracycline (1 μg/mL). After incubation for 3 days at 37 °C, 20 μL of Alamar blue was added to each

well and the plates incubated for a further 16 h. The cell density of each culture was determined as described before[11] and the IC50 established.

Enzymatic activity studies

Recombinant TbNTR was prepared and assayed as previously described.[16] The activity of purified his-tagged TbNTR was assessed spectrophotometrically at 340 nm using various nitrotriazole substrates (100 μM) and NADH (100 μM) and expressed as nmol NADH oxidized min−1 mg−1 of enzyme.

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ABBREVIATIONS USED

T. cruzi	*Trypanosoma cruzi*
T. brucei	*Trypanosoma brucei*
HAT	human African trypanosomiasis
Nfx	nifurtimox (4-(5-nitrofurfurylindenamino)-3-methylthio-morpholine-1,1-dioxide)
Bnz	benznidazole (N-benzyl-2(2-nitro-1H-imidazol-1-yl)acetamide)
NTR	type I nitroreductase
TbNTR	*T. brucei* NTR
DNDi	Drugs for Neglected Diseases initiative
SI	selectivity index
SARs	structure-activity relationships
tet	tetracycline

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CHAPTER 7

Novel 3-nitro-1H-1,2,4-triazole-based compounds as potential anti-Chagasic drugs: in vivo studies

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Running Title: Novel nitrotriazole-based compounds as anti-trypanosomal agents in vivo.

Key words: nitrotriazoles, *T. cruzi*, Chagas disease, anti-trypanosomal agents, *in vivo* studies

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ABSTRACT

Background: Chagas disease is caused by the parasite *Trypanosoma cruzi*, is endemic in Latin America and leads to an estimated 14,000 deaths per year and around 100 million people at risk of infection. Drugs currently used in the treatment of Chagas are old, partially effective and have numerous side effects. **Methodology:** We have previously reported that 3-nitro-1*H*-1,2,4-triazole-based compounds demonstrate significant and selective activity against *T. cruzi* amastigotes in infected L6 cells via activation of a type I nitroreductase, specific to trypanosomatids. In the present work we evaluated *in vivo* 13 of these compounds based on their high *in vitro* potency against *T. cruzi* (IC_{50} < 1 μM) and selectivity (SI: toxicity to L6 cells/toxicity against *T. cruzi* amastigotes > 200). Representative compounds of different chemical classes were included. A fast luminescence assay with transgenic parasites that express luciferase, and live imaging techniques were used. A total of 11 out of 13 compounds demonstrated significant antichagasic activity when administered intraperitoneally for 5–10 days at relatively small doses. The best *in vivo* activity was demonstrated by amides and sulfonamide derivatives. ADMET studies were performed for specific compounds. **Conclusion:** At least three compounds were identified as effective, non toxic antichagasic agents suitable for further development.

Executive summary

- American trypanosomiasis or Chagas disease is a neglected disease that is expanding recently in non-endemic countries in North America, Europe and Asia.
- Due to the absence of a vaccine and in view of problems associated with current drugs, there is an urgent need for the development of effective, non-toxic and affordable new drugs.
- We have discovered that 3-nitro-1,2,4-triazole-based amines, amides and sulfonamides demonstrate significant antichagasic activity against Trypanosoma cruzi amastigotes in infected L6 cells with high selectivity for the parasite.
- Such compounds are prodrugs that exert their antiparasitic activity via a type I nitroreductase activation, specific to the trypanosomatids, as has been previously demonstrated.
- At least three such compounds have demonstrated excellent in vivo activity against T. cruzi and are superior to benznidazole, at the acute phase of infection, without systemic or developmental toxicity.
- Limited mutagenicity studies suggest that several of these compounds do not demonstrate mutagenic toxicity, at least at concentrations up to their in vitro toxicity level.

INTRODUCTION

American trypanosomiasis or Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted by blood-sucking insects and remains a major health problem in Latin America. It is estimated that around 100 million people are at risk of infection with *T. cruzi* in endemic areas in Latin America [1]. Despite the fact that in the past two decades the number of incidences has significantly declined, primarily due to vector control initiatives, the epidemiology of the disease has changed due to population migration, illegal drug usage and medical practices. Thus, the number of cases in non-endemic regions such as the USA, Australia, Europe and Japan is on the rise [2,3]. In the absence of successful vaccines, chemotherapy remains the only viable option to fight the parasite in the patient.

Currently, two nitro heterocyclic prodrugs are used to treat Chagas disease: nifurtimox (a nitrofuran; Nfx) and benznidazole (a 2-nitroimidazole; Bnz). Both were introduced over 50 years ago [4], have limited efficacy, can cause various side effects, and some strains are refractory to treatment [5]. Recently, inhibitors of the sterol 14a-demethylase enzyme (CYP51), which is part of the ergosterol biosynthesis, are under development as effective antichagasic agents [6]. Unfortunately, the high cost of these inhibitors prohibits their use in poor countries where the disease is most prevalent [7]. Therefore, we urgently need new, affordable and safer drugs to treat Chagas disease.

Most nitroheterocyclic compounds function as prodrugs and must undergo activation before mediating their cytotoxic effects. It was previously demonstrated that an oxygen-insensitive, type I nitroreductase (NTR), absent from most eukaryotes with trypanosomes being a major exception, is responsible for nitrofuran and Bnz trypanocidal activity [8–10]. This enzyme mediates a series of two-electron reduction reactions that result in the fragmentation of the heterocyclic ring and production of toxic metabolites [11]. The fact that the activation of nitroheterocyclic prodrugs can be catalyzed by a type I NTR specific to trypanosomatids has led to a renewed interest in the use of such compounds as antiparasitic agents [12–17].

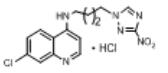
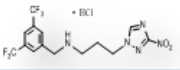
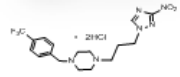
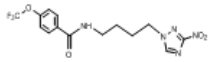
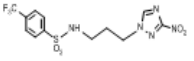
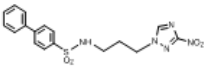
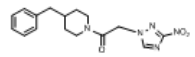
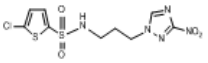
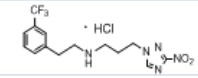
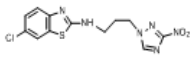
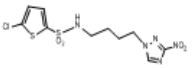
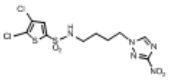
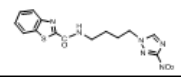
We have recently reported that 3-nitro- 1*H*-1,2,4-triazole-based amines, amides and sulfonamides demonstrate excellent activity against *T. cruzi* amastigotes in infected L6 cells with no toxicity towards the host cells [18,19]. The IC_{50} values of these compounds against the intracellular parasite ranged from low nanomolar to less than 4 μM and have selectivity indices ranging from 66 to 2682. In addition, several of these compounds were up to 56-fold more active than the reference

drug Bnz, tested in parallel [18,19]. We have also demonstrated that nitrotriazole-based compounds are activated by the type I NTR and that when this enzyme is overexpressed in the related *T. brucei*, the recombinant cells displayed hypersensitivity to these compounds [18,19]. However, since there are concerns about the toxicity and potential mutagenicity of nitro-compounds, the ultimate test for any nitro-triazole is their *in vivo* evaluation for efficacy and adverse effects. Interestingly, in preliminary *in vivo* studies, we found out that treatment of *T. cruzi*-infected mice with one nitrotriazole-based aromatic amine, NTLA-1 [20], given at just 2 mg/kg/day × 50 days, resulted in a rapid and persistent drop in peripheral parasite levels and in a fraction of cures [21,22]. More importantly, there was an absolute correlation between treatment efficacy as determined parasitologically and the increase in the fraction of *T. cruzi*-specific CD8+ T cells with a T-central memory phenotype in the peripheral blood of treated mice [21,22].
In the present study we have evaluated *in vivo* 13 nitrotriazole-based compounds based on their high *in vitro* potency against *T. cruzi* (IC_{50} <1 μM) and selectivity index ([SI]: toxicity to L6 cells/toxicity against *T. cruzi* amastigotes >200). Representative compounds of different chemical classes were included. A fast luminescence assay, in which mice are infected with transgenic parasites that express luciferase, and live imaging techniques were used. ADME studies were also performed for specific compounds, to explain discrepancies between *in vitro* and *in vivo* activity. Finally, studies were performed to assess potential toxicity/mutagenicity associated with these compounds.

Results & discussion

As was mentioned earlier, the criteria used for the selection of compounds in the present study were their *in vitro* high potency, selectivity >200 and variability in structure. Thus, compounds **1** and **10** were selected as the most potent *in vitro* aromatic amines, **2** and **3** as potent aliphatic amines, **4**, **7** and **13** as representative potent amides, and **6**, **8**, **11** and **12** as representative potent sulphonamides **(Table 1)**. Compound **1** is a chloroquinoline-based and **13** a chlorobenzothiazole-based aromatic amine. In the class of aliphatic amines, **2** was selected as a benzyl amine whereas **3** as a piperazine derivative. The phenethylamine **9** was added for *in vivo* evaluation later on, although its SI is <200, to test the hypothesis that it may demonstrate better *in vivo* stability than compound **2**. In the class of amides we included the benzylamide **4**, the benzothiazole amide **13** and the amide **7** in which the nitrotriazole ring is connected through the

Table 1. *In vitro* biological data and physical properties of all *in vivo* tested compounds.

ID No	T. cruzi IC-50 (µM)	Selectivity	Cytotox. L6 IC-50 µM	Bnz/Com	clogP	$logD_6$	PSA (Å^2)	Compound Type
Bnz	1.431 µM							Reference
1	0.140	976	136.6	9.6	3.0	1.9	101.5	
2	0.169	816	137.9	8.0	3.5	0.48	88.6	
3	0.340	>562	>191.1	4.0	2.9	0.56	83.0	
4	0.113	>2381	>268	13.8	3.2	3.22	114.9	
5	0.359	656	235.33	4.4	1.9	1.90	122.7	
6	0.028	1764	50	55.8	2.67	2.67	122.70	
7	0.307	468	143.77	5.1	2.2	2.17	96.8	
8	0.438	556	243.5	3.6	1.7	1.74	122.7	
9	0.775	116	90.1	2.0	2.9	-0.27	88.6	
10	0.059	1725	101.9	26.6	3.1	3.10	101.5	
11	0.462	277.5	128.3	4.4	2.3	2.26	122.7	
12	0.373	261.1	97.3	6.07	2.86	2.85	122.7	
13	0.422	364.4	153.8	5.36	2.3	2.30	118.52	

T. cruzi, strain Tulahuen C4 amastigotes; IC_{50} values are means of two independent assays, which varied < ±50%. Selectivity is the ratio: IC_{50} in L6 cells/IC_{50} in *T. cruzi*; Bnz/com: The ratio IC_{50} of Bnz/IC_{50} of each compound against T. cruzi; logD6: the logD at pH 6. All physical properties were predicted using the Marvin Calculator [101]. Bnz: Benznidazole; PSA: Polar surface area; *T. cruzi*: *Trypanosoma cruzi*.

carbonyl rather than the amino functionality. Finally, in the case of sulfonamides, phenyl- **(5)**, biphenyl- **(6)** and thiophene sulfonamides **(8, 11 & 12)** were included. The *in vitro* evaluation of compounds **1–13** against *T. cruzi* intracellular amastigotes in L6 host cells was performed by the Drugs for Neglected Diseases initiative in Switzerland

and the data are presented in **Table 1**. The corresponding data for compounds **1-8** have been presented before [18,19], but are included here for comparison purposes with regard to their *in vivo* activity. A structure–activity relationship discussion, based on the *in vitro* data of compounds **1–13**, is not appropriate since the compounds cover a range of chemical classes with limited number of members in each class. However, comparing compounds in the same chemical class, we can conclude that by increasing lipophilicity (logP) we increase antichagasic potency and toxicity in the host cells (decreasing IC_{50} values in L6 cells); (compare **5** to **6**; **8** to **12**). It is worth mentioning that all compounds apart from **9** demonstrated superior activity against *T. cruzi* amastigotes with IC_{50} values at nM concentrations and selectivity indices (SI = IC_{50} in L6 host cells/IC_{50} in *T. cruzi*) ≥200, namely they fulfilled the criteria set by us for further *in vivo* evaluation. In addition, all compounds in **Table 1** were from 2- to 56-fold more potent than the reference compound Bnz, tested in parallel. In particular, the sulfonamide **6** and the 2-aminobenzothiazole derivative **10** demonstrated exceptional antichagasic activity with IC_{50} values of 28 and 59 nM, respectively, exhibiting excellent selectivity of >1700. Interestingly, compounds with the best antichagasic activity (at low nM concentrations) and selectivity **(1, 2, 4, 6 & 10)** had a clogP value between 3 and 3.5 (with the exception of **6**). No correlation seems to exist between PSA value and antichagasic activity or host cell toxicity **(Table 1)**.

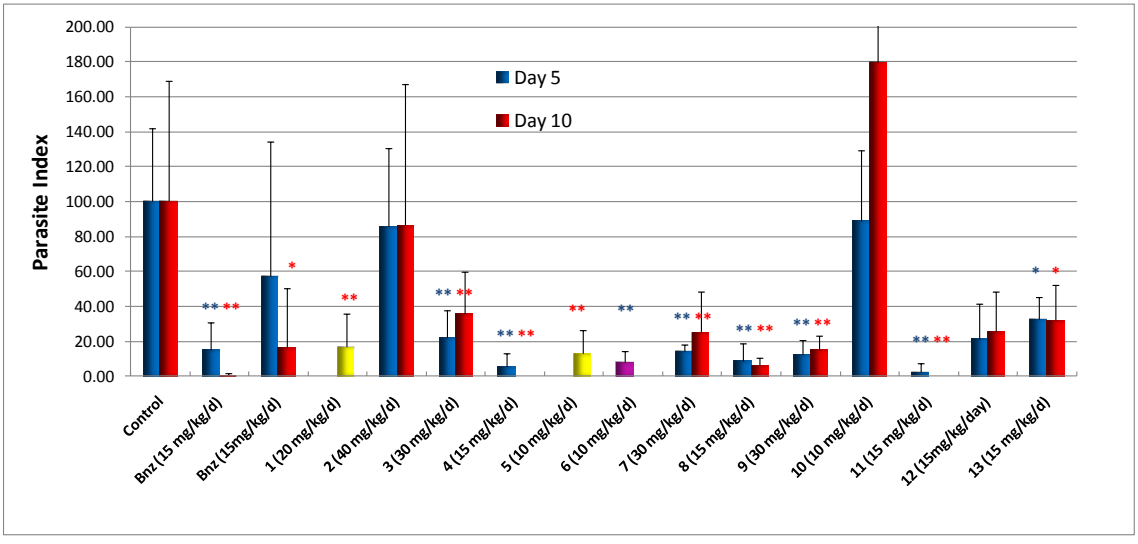


Figure 1. *In vivo* evaluation of compounds in Table 1. Parasite index was determined after 5- and 10-day treatment at the indicated doses. For compounds **1** and **5**, parasite index was determined only after 10-day treatment, whereas for compound **6** after 5-day treatment only. Errors indicate SD. *$p \leq 0.05$; **$p \leq 0.01$.

The *in vivo* antichagasic activity of the compounds in **Table 1** was assessed by using a fast luminescence assay [23] in which mice are infected with transgenic parasites that express luciferase [24]. Animals were treated with each candidate compound for 5–10 days and were imaged as described in detail in the Experimental section. The dose used for each compound was selected based on its *in vitro* activity against *T. cruzi* and toxicity towards host L6 cells. The results of the *in vivo* studies are depicted in **Figure 1**. Mice treatment was continued for up to 10 days and the data were analyzed after 5 (blue bars) and 10 days (red bars) of treatment. For compounds **1** and **5**, the data were analyzed only after 10 days of treatment (yellow bars) whereas treatment with compound **6** was continued only for 5 days (purple bar) due to its high *in vitro* potency **(Table 1)**. Bnz was included in all experiments at an effective low dose of 15 mg/kg/day, since most of the new compounds were tested at this dose. In **Figure 1**, data for Bnz from two individual experiments with the greatest difference are shown. Compounds **1**, **4**, **5**, **6**, **8**, **9 & 11** significantly dropped the parasite index more than 80 and up to 100%. In particular, compounds **4** and **11** demonstrated greater activity than Bnz at 15 mg/kg/day, with no detectable parasite signal after 10 days of treatment.

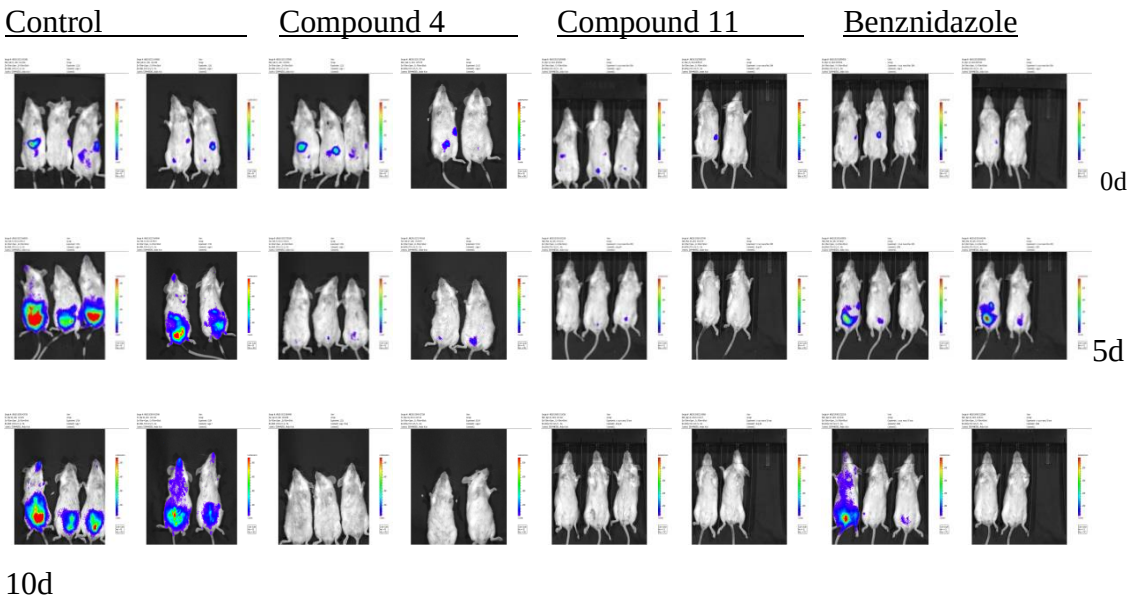


Figure 2. Images of untreated and treated mice with the indicated compounds. Groups of five mice were infected with *T. cruzi* trypomastigotes expressing luciferase and imaged before and after 5- and 10-day treatment. d=day

Images of mice treated with compounds **4** and **11** are shown in **Figure 2**. Compound **8**, at 15 mg/kg/day, also demonstrated significant antichagasic activity, similar to that of

Bnz, dropping the parasite index by 94% after 10-day treatment **(Figure 1)**. Images of mice treated with compound **8** are shown in **Figure 3**. Although compounds **3**, **7**, **12** and **13** demonstrated significant *in vivo* antichagasic activity, they failed to perform better than Bnz after 10-day treatment at 30, 30, 15 and 15 mg/kg/day, respectively **(Figure 1)**. Compounds **2** and **10** failed to demonstrate *in vivo* antichagasic activity after 5- or 10-day treatment at 40 and 10 mg/kg/day, respectively.

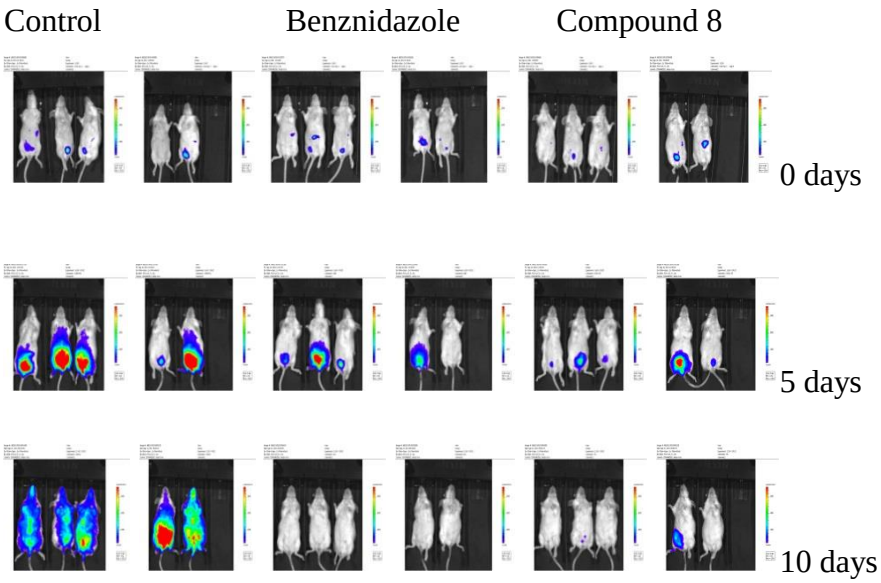


Figure 3. Images of untreated and treated mice with the indicated compounds. Groups of five mice were infected with T. cruzi trypomastigotes expressing luciferase and imaged before and after 5- and 10-day treatment.

Comparing the *in vivo* **(Figure 1)** with the *in vitro* efficacy **(Table 1)** of all tested compounds, we observed no direct correlation. This is expected considering the number of additional parameters that determine drug activity *in vivo* and not modelled in the *in vitro* assay. Thus, compounds **2** and **10** with IC50 values against *T. cruzi* at low nM concentrations, failed to show activity *in vivo*. Moreover, compound ***2*** with an *in vitro* selectivity index of 816 also resulted in some deaths at 40 mg/kg/day, presumably due to its bioactivation to reactive intermediates [25]. Furthermore, it has been reported that although both benzyl- and phenethyl-amines substituted with electron-withdrawing groups are substrates of monoamine oxidase B, several such phenethylamines were acting as inhibitors of the enzyme [26]. Therefore, the phenethylamine compound **9** was tested *in vivo* to compare its activity and toxicity with that of **2**. Indeed, compound **9**, which was 4.6-fold less active *in vitro* than **2** (IC50 775 nM vs 169 nM) dropped the parasite index in mice by about 88 and 85% after 5-day and 10-day treatment,

respectively, at 30 mg/kg/day without any sign of toxicity. Similarly, compounds **8** and **11** with excellent *in vivo* activity were not the most potent antichagasic compounds *in vitro* **(Table 1)**. The lack of a direct correlation between *in vitro* and *in vivo* activity confirms that compounds with *in vitro* IC50 values <2 μM against *T. cruzi* may be also worthy of *in vivo* evaluation.

Table 2. Microsomal stability screen data summary.

Compound	Concentration (μM)	Mean remaining		Comments
		Parent comp (%)	Parent comp (%)	
		(+)NADPH	(-)NADPH	
Verapamil	1	5.6	113	High metabolized control
Warfarin	1	83.9	118	Low metabolized control
1	1	5.1	112	
4	1	90.2	115	
8	1	91.6	120	
10	1	1	108	

To explain some of the discrepancies observed between *in vitro* and *in vivo* activity, we performed some ADME studies for selected compounds **(1, 4, 8 & 10)**. Compounds **4** and **8** were selected because of both their excellent *in vitro* and *in vivo* activity, whereas **10** for its lack of *in vivo* activity despite its excellent *in vitro* activity. Compound **1** was selected for its excellent *in vitro* activity and good *in vivo* activity, despite the fact that it is an aromatic amine similar to the *in vivo* inactive compound **10**. **Table 2** shows microsomal stability data for compounds **1**, **4**, **8** and **10**, using verapamil and warfarin as high metabolized and low metabolized controls, respectively. All compounds were stable in the absence of NADPH. However, in the presence of NADPH, mouse microsomal protein highly metabolized the aromatic amines **1** and **10**, but left the amide **4** and sulphonamide **8** intact. These data are consistent with the lack of *in vivo* activity observed for compound **10**, especially at the relatively low tested dose of 10 mg/kg/day **(Figure 1)**. However, all compounds were relatively stable in mouse plasma **(Table 3)**, and this perhaps partially explains the good *in vivo* activity of compound **1**. Since amide **4** and sulfonamides **8** and **11** were the best compounds in terms of *in vivo* activity, their permeability through Caco-2 monolayers were investigated to evaluate whether such compounds can be administered orally at a sufficient blood concentration [27]. Only compounds **4** and **8** were tested in this system: sulphonamides **8** and **11** are closely

related analogs, therefore data pertaining to one compound should reflect the situation of the other. Both **4** and **8 (Table 4)** demonstrated an excellent permeability since the apparent permeability value (Papp) was more than 5 × 10-6 cm/s [27]. It has also been recently proposed that compounds with a logPapp > -4.96 accurately predict high permeability [28]. In our case the logPapp values of **4** and **8** were -4.61 and -4.55, respectively. Based on the criteria proposed by Chaturvedi *et al.*, it is predicted that compounds **4** and **8** with Papp values >10 × 10-6 cm/s will demonstrate 70–100% oral absorption [29]. Partition coefficients (log *D* and log *P*) and molecular surface area (PSA) are also potential predictors of the intestinal permeability of drugs. According to a recent study [30], the logD value at pH 6 (logD6) can more accurately predict intestinal permeability than the other mentioned parameters and a logD6 > -0.42, (the logD6 value of labetalol) is associated with high permeability [30]. As can be seen in **Table 1**, all compounds **1–13** demonstrate logD6 values > -0.42 and thus they may demonstrate a good intestinal permeability. However, as was shown above, bioavailability is dependent upon a combination of parameters, a crucial one of which is metabolic stability; therefore ADME studies are necessary for reliable predictions.

Table 3. Plasma stability screen data summary.

Compound	Concentration (μM)	Mean remaining Parent comp (%)	Comments
Propantheline	5	30.4	High metabolized control
Warfarin	5	97.8	Low metabolized control
1	5	96.6	
4	5	93.4	
8	5	88.6	
10	5	87.3	

Similarly, in the case of compounds **2** and **9**, ADME studies will confirm whether or not extensive metabolism of **2** is responsible for its inactivity *in vivo*. Such studies are planned in the near future.

All potent *in vivo* compounds did not show apparent toxicity at the doses tested. However, since these compounds are nitro-derivatives, there is a concern for potential mutagenicity/genotoxicity. Therefore, representative 3-nitrotriazoles (some of them not yet tested *in vivo*) were evaluated using the Ames assay [31,32]. Here we present the results for only compound **4**, since this compound demonstrated excellent *in vivo* antichagasic activity. The compound was tested against *Salmonella typhimurium* TA98

strain **(Figure 4)** and against a mixed TA7001–7006 strain (MixS; **Figure 5**), with or without the rat liver metabolic activation system S9, and the mean number of revertants was plotted versus compound concentration [32]. The compound was not mutagenic, with one exception; mutagenicity was seen in mixed strains at the highest tested concentration of 1000 μ g/ml and only in the presence of S9 **(Figure 5)**. This concentration was toxic to the L6 cells **(Table 1)**. Since a linear dose response is not observed in the mutagenicity tests **(Figures 4 & 5)**, we can assume that compound **4** is not mutagenic at non0toxic doses, otherwise a safe threshold presumably exists, as has been suggested for certain compounds [33,34].

Table 4. Caco-2 permeability summary.

Compound	Concentration (μM)	Mean A → B Papp[†] 10^{-6} cm s^{-1}	Comments
Ranitidine	10	0.5	Low permeability control
Warfarin	10	44.2	High permeability control
4	10	24.5	
8	10	27.9	

[†]Permeability ranking: Papp × (10^{-6} cm s^{-1}).

Low: Papp <0.5; Moderate: 0.5 <Papp < 5 ; High: Papp > 5.

Papp: Apparent permeability.

For comparison purposes a 2-nitroimidazolebased compound was also tested in the Ames assay. Mutagenicity was demonstrated against TA98/TA98NR strains in the presence or absence of S9 at doses as low as 20 μg/plate (data not shown). In addition, this compound was highly mutagenic in the TA100/TA100NR strains in the presence or absence of S9 at doses ≥0.8 μg/plate (data not shown). Furthermore, the 2-nitroimidazole-based compound was toxic at doses ≥350 μg/plate to all strains. In general, most 3-nitrotriazole-based compounds that were tested in the Ames test did not show mutagenicity, suggesting that mutagenicity is associated to a greater degree with 2-nitroimidazole rather than 3-nitrotriazole systems, although further compounds should be tested for more accurate conclusions. In addition, the 3-nitrotriazolic compounds that exhibited mutagenicity did so at concentrations significantly higher than their IC50 value in the L6 host cells **(Table 1)**. It should also be mentioned that mutagenicity in *S. typhimurium* strains is not necessarily translated to mutagenicity in humans. For instance, although Bnz shows significant mutagenicity at relatively low concentrations (<62 μg/ml) in *S. typhimurium* strains [35], mutagenicity in humans has never been

reported. A mutagenicity study of serum and urine from guinea pigs treated with Bnz showed that Bnz is not metabolized by the mammalian host into stable mutagenic derivatives detectable by the Ames test, suggesting that the potential cancer risk in humans is minimal [36].

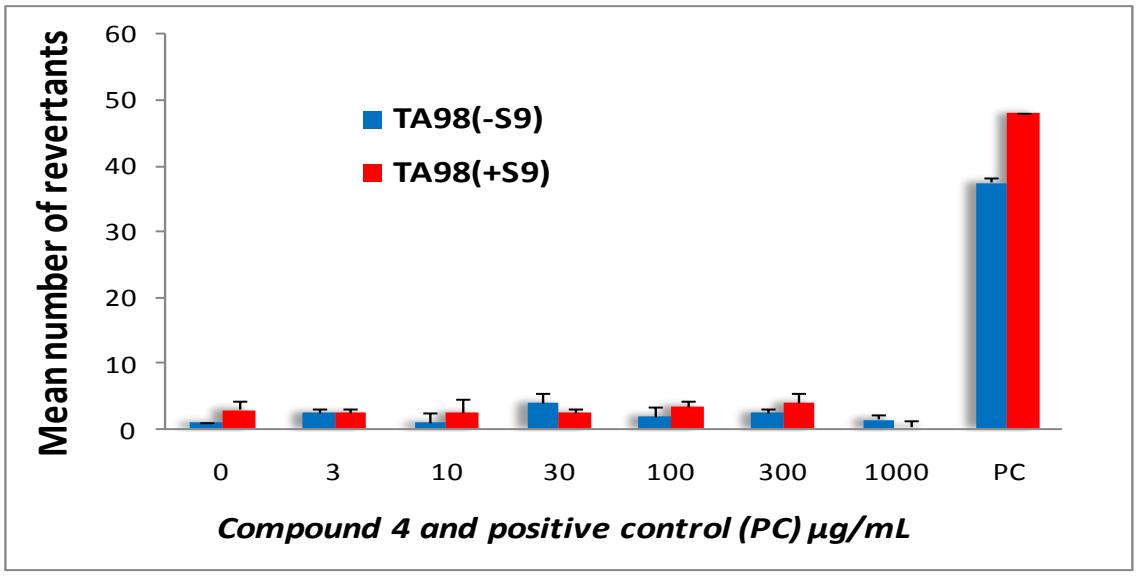


Figure 4. Mutagenicity study (Ames test) for compound 4 in TA98 strains without/with S9. PC was 625/1200ng/mL 4-nitroquinoline-N-oxide / 2-nitrofluorene (-S9) and 2-aminoanthracene (+S9).

We also investigated the effect of compound **1** on zebrafish embryos' development. Drugs were applied to developing zebrafish embryos at 24 h post-fertilization (hpf), at the end of the segmentation stage when the primary stages of organogenesis are complete and the fish have begun to move. Groups of six embryos per dose were examined at three developmental time-intervals (24, 48 and 72 hpf) and each experiment repeated in triplicate. The data are summarized in **Table 5**. No compound-related toxicity or phenotypic changes were observed at all doses and time intervals. Concentrations up to 300 µM were tested. Similar results were obtained with two other 3-nitrotriazole-based amides, analogs of **4** (data not shown). In contrast, incubation of embryos in nifurtimox resulted in weakened heart beat, pericardial oedema or death (data not shown).

The *in vivo* luminescence assay combined with *in vitro* ADMET data provides a rapid method to identify safe, stable compounds with *in vivo* activity and potentially good oral bioavailability before any other expensive pharmacokinetic/ pharmacodynamic evaluation. Thus, this strategy can lead to an accelerated drug discovery process [23].

Compounds with good *in vivo* activity seem to also have good metabolic stability. Through this process we have identified at least three compounds, **4**, **8** and **11** as candidates for further development. However, the question of whether or not a 10-day treatment resulted in the sterile cure of these animals has not been answered yet, since the animals were not kept long after treatment. Experiments in which mice will be kept for an extended period of time post-treatment and treated with an immunosuppressant

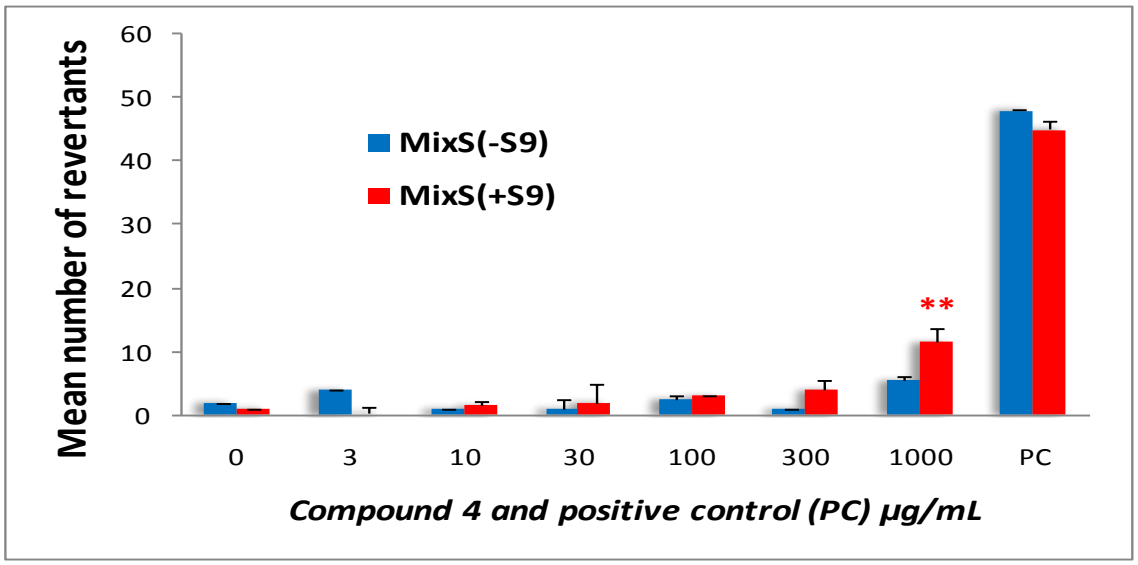


Figure 5. Mutagenicity study (Ames test) for compound 4 in mixed TA7001-7006/ strains without /with S9. PC: -S9-Mix: 625/1200ng/mL 4-nitroquinoline-N-oxide/2-nitrofluorene; +S9 Mix: 10 µg/mL 2-aminoanthracene. * $p \leq 0.05$; ** $p \leq 0.01$.

Table 5. Zebrafish embryos toxicity data for compound 1.

Compound 1 (µM)		Time (hours post-fertilization)[†]		
		24	48	72
3.7	Embryos	18/18	18/18	17/18
11.1	Embryos	18/18	18/18	17/18
33.3	Embryos	18/18	17/18	17/18
100	Embryos	18/18	17/18	17/18
300	Embryos	18/18	18/18	18/18
Control	Embryos	18/18	18/18	16/18
DMSO	Embryos	18/18	17/18	16/18

[†]The data show the ratio of number of surviving zebrafish embryos at different developmental time points (in hours post fertilization) at each compound 1 concentration versus the total number of zebrafish embryos used in the assay.

will be the next step to provide us with an answer [22]. In addition, studies should be done to determine if these compounds can treat the chronic stage of the disease. However, current studies have clearly demonstrated that 3-nitrotriazole-based amides

and sulfonamides have a significant chance to be developed as antichagasic drugs. They can be easily synthesized in high yields and purity with low cost [19], they show very good mouse plasma and metabolic stability **(Tables 2 & 3)** and, in general, are not mutagenic at nontoxic concentrations. However, not all *in vivo* active compounds have been tested yet for mutagenicity. Moreover, additional compounds with IC50 values against *T. cruzi* <2 μ M might be good candidates for ADMET and subsequent *in vivo* evaluation.

Experimental

Chemistry

All starting materials and solvents were purchased from Sigma-Aldrich (WI, USA), were of research-grade quality and were used without further purification. Solvents used were anhydrous and the reactions were carried out under a nitrogen atmosphere and exclusion of moisture. Melting points were determined by using a Mel-Temp II Laboratory Devices apparatus (MA, USA) and are uncorrected. Proton NMR spectra were obtained on a Varian Inova- 500 or a Bruker Avance-III-500 spectrometer at 500 MHz and are referenced to Me_4Si or to the corresponding protonated solvent, if the solvent was not $CDCl_3$. High-resolution electrospray ionization (HRESIMS) MS were obtained on a Agilent 6210 LC–TOF MS at 11000 resolution. Thin-layer chromatography was carried out on aluminum oxide N/UV_{254} or polygram silica gel G/UV_{254}-coated plates (0.2 mm, Analtech, DE, USA). Chromatography was carried out on preparative TLC alumina GF (1000 microns) or silica gel GF (1500 microns) plates (Analtech). All compounds were purified by preparative TLC chromatography on silica gel GF plates (≥95% purity). The synthesis of compounds **1–8** has been described before [18,19]. Similar synthetic procedures were followed to obtain compounds **9–13**. For compound **9**, 3-(trifluoromethyl)phenethyl bromide (1.035 mmol) was added dropwise (15 min) to a solution of 3-nitro-1*H*-1,2,4- triazolyl-propylamine (1.035 mmol) [37] in the presence of potassium carbonate (9.52 mmol) in dry acetonitrile (15 ml), and the reaction mixture was refluxed under a nitrogen atmosphere for 10 h. The reaction mixture was cooled down, filtered, the solids were washed with acetonitrile, the organic filtrate was evaporated and the residue extracted from water-ethyl acetate. The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by preparative TLC on alumina plates with ethyl acetate:MeOH (99:1). A yellowish oil was obtained (R_f = 0.53), which was the

desired monoalkylated product. This was dissolved in ethyl acetate and converted to its HCl salt by treating with HCl gas in dry ether (1 M solution).

[3-(3-nitro-1*H*-1,2,4-triazol-1-yl)propyl] ({2-[3 (trifluoromethyl)phenyl]ethyl})amine hydrochloride **(9)**

Fine white powder (40%): mp 103-104°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 8.66 (s, 1H), 7.64–7.55 (m, 4H), 4.50 (t, *J* = 7.0 Hz, 2H), 3.34 (t, *J* = 7.0 Hz, 2H), 3.17 (t, *J* = 8.0 Hz, 2H), 3.10 (t, *J* = 8.0 Hz, 2H), 2.35 (quintet, *J* = 8.0 Hz, 2H). HRESIMS calculated for $C_{14}H_{17}F_3N_5O_2$ and $C_{14}H_{16}F_3N_5NaO_2$ m/z $[M+H]^+$ and $[M+Na]^+$ 344.1329, 345.1356 and 366.1148, 367.1176, found 344.1336, 345.1363 and 366.1152, 367.1181, respectively.

For compound **10**, the commercially available 2,6-dichloro-1,3-benzothiazole (1.24 mmol) was coupled with 3-nitro-1*H*-1,2,4-triazolyl-propylamine (1.24 mmol) [37], by refluxing in absolute propanol (8 ml) for 16 h, and in the presence of fivefold excess of triethyl amine.

6-chloro-*N*-[3-(3-nitro-1*H*-1,2,4-triazol-1-yl) propyl]-1,3-benzothiazol-2-amine **(10)**

Orange powder (65%): mp 194-195°C (dec.); ^{1}H NMR (500 MHz, CD_3COCD_3) δ: 8.71 (s, 1H), 7.70 (d, *J* = 2.5 Hz, 1H), 7.39 (d, *J* = 8.5 Hz, 1H), 7.37 (br s, 1H), 7.26 (dd *J1* = 8.5, *J2* = 2.5 Hz, 1H), 4.57 (t, *J* = 7.0 Hz, 2H), 3.62 (m, 2H), 2.39 (quintet, *J* = 6.5 Hz, 2H). HRESIMS calculated for $C_{12}H_{12}ClN_6O_2S$ *m/z* $[M+H]^+$ 339.0425, 341.0398, found 339.0427, 341.0395.

General synthetic procedure of arylamides/sulfonamides 11–13

For compounds **11–13**: the appropriate commercially available arylcarbonyl/arylsulfonyl chloride (1.24 mmol) was dissolved in 2–3 ml dry dichloromethane and added dropwise to a solution of 3-nitro-1*H*-1,2,4-triazolyl-butylamine) (1.24 mmol) [37], and triethylamine (2.48 mmol) in 6–8 ml of dry dichloromethane, at room temperature and under an inert atmosphere. The reaction mixture was stirred for 12 h. Consequently, the inorganic salts were filtered off, the filtrate was evaporated and the residue was chromatographed on silica gel.

5-chloro-*N*-[4-(3-nitro-1*H*-1,2,4-triazol-1-yl) butyl]thiophene-2-sulfonamide **(11)**

White crystallic powder (84%): mp 81–83 C; ^{1}H NMR (500 MHz, CD_3COCD_3) δ: 8.65 (s, 1H), 7.46 (d, *J* = 4.0 Hz, 1H), 7.14 (d, *J* = 4.0 Hz, 1H), 6.84 (br t, 1H), 4.44 (t, *J* = 7.0 Hz, 2H), 3.10 (t, *J* = 7.0 Hz, 2H), 2.05 (m, 2H), 1.65 (m, 2H). HRESIMS calculated for $C_{10}H_{13}ClN_5O_4S_2$ and $C_{10}H_{12}ClN_5NaO_4S_2$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 366.0092, 368.0063 and 387.9911, 389.9882, found 366.0091, 368.0063 and 387.9909, 389.9884, respectively.

4,5-dichloro-*N*-[4-(3-nitro-1*H*-1,2,4-triazol-1- yl)butyl]thiophene-2-sulfonamide **(12)** White crystallic powder (75%): mp 104–105oC; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.19 (s, 1H), 7.39 (s, 1H), 4.82 (br t, 1H), 4.34 (t, *J* = 7.0 Hz, 2H), 3.13 (q, *J* = 6.5 Hz, 2H), 2.09 (quintet, *J* = 7.5 Hz, 2H), 1.65 (quintet, *J* = 7.5 Hz, 2H). HRESIMS calculated for $C_{10}H_{12}Cl_2N_5O_4S_2$ and $C_{10}H_{11}Cl_2N_5NaO_4S_2$ *m/z* $[M+H]^+$ and $[M+Na]^+$ 399.9702, 401.9673 and 421.9522, 423.9492 found 399.9704, 401.9671 and 421.9521, 423.9490.

N-[4-(3-nitro-1*H*-1,2,4-triazol-1-yl)butyl]-1,3- benzothiazole-2-carboxamide **(13)** Off-white powder (71%): mp 92–94oC; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.22 (s, 1H), 8.06 (d, *J* = 8.0 Hz, 1H), 7.98 (d, *J* = 8.0 Hz, 1H), 7.57–7.51 (m, 3H), 4.39 (t, *J* = 7.0 Hz, 2H), 3.59 (t, *J* = 7.0 Hz, 2H), 2.10 (quintet, *J* = 7.5 Hz, 2H), 1.75 (quintet, *J* = 7.5 Hz, 2H). HRESIMS calculated for $C_{14}H_{15}N_6O_3S$ *m/z* $[M+H]^+$ 347.0921, found 347.0922.

ADME *in vitro* studies

ADME *in vitro* studies were performed by APREDICA (MA, USA) for several compounds. Samples were analyzed by LC/MS/ MS using an Agilent 6410 MS coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). After separation on a C18 reverse-phase HPLC column (Agilent, Waters or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by MS using ESI ionization in MRM mode.

Microsomal stability screen

Each test compound was dissolved in DMSO and incubated (37°C) at 1 μM final concentration with 0.3 mg/ml of mouse microsomal protein in 100 mM potassium phosphate, 3 mM MgCl2, pH 7.4, in the presence or absence of 2 mM NADPH (to

detect NADPH-free degradation) for up to 60 min. At indicated times (0 and 60 min), an aliquot was removed from each experimental and control reaction then mixed with an equal volume of ice-cold Stop Solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard). Stopped reactions were incubated for at least 10 min at -20°C, and an additional volume of water was added. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC/MS/MS to quantitate the remaining parent compound. Data were reported as % remaining by dividing by the time zero concentration value [38].

Plasma stability screen

Each test compound (in a DMSO stock solution) was incubated at 5 μM final concentration with mouse plasma and 2% DMSO at 37°C in duplicate. At indicated times (0 and 60 min), an aliquot was removed from each experimental and control reaction and mixed with three volumes of ice-cold Stop Solution (methanol containing haloperidol, diclofenac or other internal standard). Stopped reactions were incubated at least for 10 min at -20°C. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC/MS/MS to quantitate the remaining parent compound. Data were converted to % remaining by dividing by the time zero concentration value [39].

Caco-2 monolayer permeability studies

Caco-2 cells grown in tissue culture flasks were trypsinized, suspended in medium, and the suspensions were applied to wells of a collagencoated BioCoat Cell Environment in 96-well format. The cells were allowed to grow and differentiate for 3 weeks, feeding at 2-day intervals. For apical to basolateral (A→B) permeability, the test agent was added to the apical (A) side at 10 μ M final concentration and amount of permeation was determined on the basolateral (B) side. The A-side buffer contained 100 μM Lucifer yellow dye, in Transport Buffer (1.98 g/l glucose in 10 mM HEPES, 1×Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer was Transport Buffer, pH 7.4. Caco-2 cells were incubated with these buffers for 2 h and the receiver side buffer was removed for analysis by LC/MS/ MS. To verify that the Caco-2 cell monolayers were properly formed, aliquots of the cell buffers were analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow. Data were expressed as

permeability (Papp): P_{app} = (dQ/dt)/C0A, where dQ/dt is the rate of permeation, C_0 is the initial concentration of test agent, and A is the area of the monolayer [27].

In vitro evaluation against *T. cruzi*

In vitro activity against *T. cruzi* intracellular amastigotes and cytotoxicity assessment in the host L6 cells (rat skeletal myoblasts) was determined using a 96-well plate format as previously described [40]. Data were analyzed with the graphic program Softmax Pro (Molecular Devices, CA, USA), which calculated IC_{50} values by linear regression from the sigmoidal dose inhibition curves.

In vivo anti-T. cruzi activity assessment

Trypomastigote forms from transgenic *T. cruzi* Y strain expressing firefly luciferase [24] were purified, diluted in PBS and injected intraperitoneally in Balb/c mice (10^5 trypomastigotes per mouse). 3 days after infection the mice were anesthetized by inhalation of isofluorane (controlled flow of 1.5% isofluorane in air was administered through a nose cone via a gas anesthesia system). Mice were injected with 150 mg/kg of d-luciferin potassium-salt (Goldbio) dissolved in PBS. Mice were imaged 5–10 min after injection of luciferin with an IVIS 100 (Xenogen, CA, USA) and the data acquisition and analysis were performed with the software LivingImage (Xenogen) as described before [23]. 1 day later (4 days after infection) treatment with compounds at a specific concentration (usually 15 mg/kg/day) or vehicle control (2% methylcellulose + 0.5% Tween 80) was started by intraperitoneal injection in groups of five mice and continued daily for 5–10 days. On the days indicated, mice were imaged again after anesthesia and injection of luciferin as described above. Parasite index is calculated as the ratio of parasite levels in treated mice compared with the control group and is multiplied by 100. The ratio of parasite levels is calculated for each animal dividing the luciferase signal after treatment by the luciferase signal on the first imaging (before treatment). Mean values of all animals in each group ± SD were then used to calculate the parasite index [23].

Toxicity studies in zebrafish embryos

Wildtype (WT) zebrafish strains (*Tubingen* and *Tupfel long fin*) were bred and raised in-house at the zebrafish facility of Queen Mary College, University of London, UK. Embryos were collected by natural spawning and staged according to Kimmel and

colleagues [41] – given in the text as standard developmental time at 28.5°C (hpf). Work on zebrafish embryos (prior to independent feeding) is exempt under the UK Animals (Scientific Procedures) Act 1986 and does not require ethical approval. For each experiment six zebrafish embryos (three embryos per well in a 24-well plate) were treated per compound concentration (concentration was varied from 3.7 to 300 μM) and the viability of the developing embryos assessed with respect to time (hpf) as the ratio:number of live zebrafish at the time indicated/number of zebrafish at time 0 per each concentration. Each experiment was conducted in triplicate using a total of 18 embryos being analyzed per each concentration.

Mutagenicity studies

The Ames mutagenicity test was performed with *S. typhimurium* TA98, TA100, TA98NR (nitroredutase deficient) and TA100NR (nitroreductase deficient) strains according to a method described before [31]. Concurrently, nitrofurantoin (NFT), 2-nitrofluorene, 4-nitroquinoline- *N*-oxide and benzo[a]-pyrene were included in the assays with TA98/TA98NR strains or sodium azide, nitrofurantoin, metronidazole and 2-aminoanthracene in the assays with TA100/ TA100NR strains. In one case, mixed TA7001–7006 series of *Salmonella*, his^{-} mutant strains were used [32]. The assays were performed in the presence (for metabolic activation) or absence of the liver S9 mix [31,32]. All tested compounds were dissolved in DMSO and the same amount of DMSO was delivered to each plate. Prior to starting the assay, the concentrations to be tested were selected in terms of solubility and toxicity results in the test system. Concentrations up to 1000 μg/plate were tested. Triplicate (in one case duplicate) plates were used for each dose and mean values of His^{+} revertants per plate are indicated as the results.

Statistical analysis

Data were analyzed by using the t-test (Prism vs 4.0c, GraphPad). Statistics were considered significant if p was ≤ 0.05 (*) or $p \leq 0.01$ (**).

Future perspective

Recently Chagas disease was characterized as '*the new AIDS of the Americas*' because its spread resembles the early dissemination of HIV [42]. Although this characterization is an exaggeration, there are similarities in the sense that both HIV and *T. cruzi* cause

life-long infections and, like all blood-borne pathogens, are potentially transmitted by blood transfusion and congenitally from mother to newborn. As a result of reactivation among immigrant populations, an increase in Chagas disease infections has been reported in non-endemic settings and this 'globalization' will be of concern [2,3]. Currently, around 8–10 million individuals are infected with *T. cruzi* in endemic areas, while it has been estimated there are around 325,000 cases in the USA and about 100,000 cases in Europe, 87,000 of which are in Spain [1]. In addition, Chagas disease creates financial and social burdens to individuals, their households and countries. The early mortality and substantial disability caused by this disease, which often occurs in the most productive population, young adults, results in a devastating economic loss in the Americas.

As was mentioned earlier, with no immediate prospect for vaccines, chemotherapy is the only way to fight the parasite in the patient. One way to develop effective drugs is by targeting enzymes specific to the parasite, for example, cruzipain or CYP51. However, such approaches may lead to drug-resistant phenotypes that will create additional searches for new targets. Another approach is to utilize an enzyme specific to the parasite that activates a prodrug. We have followed the latter strategy. We have shown that 3-nitrotriazole-based compounds can be very effective *in vitro* against *T. cruzi* amastigotes via NTR-activation, without showing toxicity to the host cells [18,19]. *In vitro* active compounds demonstrating good metabolic and plasma stability also showed *in vivo* effectiveness against the parasite. Furthermore, our data have shown that 3-nitrotriazole-based compounds do not cause developmental toxicity, they are not mutagenic at non-toxic doses and are significantly less mutagenic than 2-nitroimidazoles. Therefore, further *in vivo* evaluation of these compounds is necessary to determine whether or not we can obtain cures without long-term toxicity and whether or not such compounds have an effect against the chronic phase of the disease. In addition, studies in combination with target-specific or even currently used antichagasic drugs may reveal a synergistic interaction, which could result in lowering of doses and shortening of the treatment-period in humans. Therefore, there is a considerable future in drug development research against Chagas disease. Moreover, the treatment for Chagas disease is currently expensive and effective agents with low cost are desperately needed. Nitrotriazole-based compounds could be a potential future solution. However, additional studies are necessary to determine the efficacy of these compounds in the chronic stage of the disease and under oral administration.

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Ethical conduct of research

Animal studies were approved by the Institutional Animal Care and Use Committee of New York University School of Medicine (protocol #81213). This protocol adheres to the guidelines of the Association For Assessment and Accreditation Of Laboratory Animal Care International (AAALAC).

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CHAPTER 8

Agrochemicals against Malaria, Sleeping Sickness, Leishmaniasis and Chagas Disease

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Abstract

In tropical regions, protozoan parasites can cause severe diseases with malaria, leishmaniasis, sleeping sickness, and Chagas disease standing in the forefront. Many of the drugs currently being used to treat these diseases have been developed more than 50 years ago and can cause severe adverse effects. Above all, resistance to existing drugs is widespread and has become a serious problem threatening the success of control measures. In order to identify new antiprotozoal agents, more than 600 commercial agrochemicals have been tested on the pathogens causing the above mentioned diseases. For all of the pathogens, compounds were identified with similar or even higher activities than the currently used drugs in applied *in vitro* assays. Furthermore, *in vivo* activity was observed for the fungicide/oomyceticide azoxystrobin, and the insecticide hydramethylnon in the *Plasmodium berghei* mouse model, and for the oomyceticide zoxamide in the *Trypanosoma brucei rhodesiense* STIB900 mouse model, respectively.

Author Summary

Even though agrochemistry and infectious disease control have the same principle goal - the suppression of harmful organisms without harming human health and the environment - there have been only very limited activities to exploit this overlap for the development of new anti-infectious drugs so far. In this study and for the first time, over 600 commercial agrochemicals were systematically screened against the infectious pathogens causing malaria, sleeping sickness, Chagas disease and leishmaniasis. Many highly active compounds with known low mammalian toxicity were identified in cell based assays, and the activity of some of them could even be confirmed in first animal model studies. Further expansion of this concept to other pathogens and the examination of analogues of the identified hits, potentially available from agrochemical companies, would allow for a very efficient source of novel drug candidates.

Introduction

The Protozoan parasites of the genera *Plasmodium spp.*, *Leishmania spp*., *Trypanosoma brucei spp*. and *Trypanosoma cruzi*, are the disease causative agents threatening entire populations in mainly resource poor countries around the world.

Malaria, due to infection with *Plasmodium spp*., is one of the most devastating diseases in developing countries, with 216 million cases in 2010, causing an estimated 655,000 deaths per year [1]. Other recent estimates assume up to 1.2 million deaths per year [2]. For the treatment of malaria several highly active drugs are available, like chloroquine, quinine, mefloquine, atovaquone, artesunate, and their analogs. Thus, malaria is often not included in the list of the neglected tropical diseases. Unfortunately, significant resistance to almost all of these drugs has developed; even to the ‘‘last resort’’ artemisinin-derivatives, first cases of delayed clinical efficacy have been reported [3]. Recently, large libraries from pharma companies have been screened against protozoan parasites and some interesting hits [4,5,6,7] have been found, especially against malaria with the spiroindolones currently undergoing clinical evaluation [8,9].

Most of the promising compounds in the development pipeline are in a rather early clinical stage, so that a high failure rate is expected [10]. Considering the rapid development of resistance, and the challenges seen with the development of malaria vaccines [11], a continuous refilling of research pipelines with compounds in preclinical/clinical evaluation will be necessary, for the long term perspective. Therefore new compounds for resistance management would be highly desirable, even if they might not show the same remarkably high activity levels as the recently promoted peroxide candidates like OZ439 [12]. In addition, the global malaria agenda has shifted from the mere control of clinical cases to malaria elimination and eventually eradication urgently requiring transmission blocking agents [13].

Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by infections of *T. b. rhodesiense* and *T. b. gambiense*. Populations living in remote rural areas of sub-Saharan Africa are at risk of acquiring HAT. The disease burden in 2000 was estimated at 1.3 Mio DALYs (Disability-Adjusted Life Years) and the estimated number of cases up to 70,000 in 2006 [14]. In recent years the public health situation has improved due to increased monitoring and chemotherapy, resulting in the decrease of reported HAT cases to approximately 10,000 [15]. Only 4 drugs are currently registered as HAT treatment. Pentamidine and suramin are used to treat the

hemolymphatic stage (stage 1) of the disease, while melarsoprol and eflornithine (DFMO) are used in stage 2 of the disease when the parasites have invaded the central nervous system (CNS) and which is lethal if untreated. The available drugs are unsatisfactory due to cost, toxicity, poor oral bioavailability, long treatment and lack of efficacy. Melarsoprol is highly toxic, and up to 5% of the second stage patients treated with melarsoprol die of a reactive encephalopathy. Eflornithine treatment is expensive and logistically difficult; it requires four daily intravenous infusions over fourteen days. Recently the eflornithine-nifurtimox combination therapy (NECT) was introduced [16]. The requirement of intravenous administration although reduced to a quarter of injections as compared to monotherapy is still a limitation, with a need for new and more easily administrable drugs.

Trypanosoma cruzi infection elicts Chagas disease and is an important public health problem causing approximately 14,000 deaths and 0.7 Mio DALY annually [17]. Treatment options are limited due to toxicity of available drugs, parasite resistance, and poor drug activity during the chronic phase of the disease. Currently there are two medications being used to treat Chagas disease, nifurtimox and benznidazole [18]. Severe toxicity and long treatment requirements are associated with both drugs [19]. Therefore new medications are badly needed for treating this disease especially in its chronic phase.

Leishmaniasis causes approximately 50,000 deaths and 2.1 Mio DALY annually [20]. It threatens about 350 million people around the world and 12 million people are believed to be infected, with 1–2 million estimated new cases every year [21]. Widely used medications are still based on i.v. application of antimony compounds like stilbogluconate, resulting in severe side effects. More modern, but also more expensive medications are liposomal amphotericin B, miltefosine, and paromomycin [22].

Thus new affordable and effective therapies are urgently needed to combat these disastrous diseases. Registration requirements for agrochemicals are in some aspects even more stringent than for pharmaceuticals, as side effects that are tolerated for drugs against many life threatening diseases, are not acceptable for agrochemicals that potentially could enter the food chain [23,24,25]. As a consequence, all commercialized agrochemicals must go through broad toxicological profiles including e.g. chronic and reprotoxicological studies in different mammalian species, covering at least part of the preclinical studies required for drug development. Furthermore, agrochemicals are highly optimized on agrochemical pest targets with often good selectivities in mammals

and excellent temperature and storage stability. Another interesting feature of commercial agrochemicals is the very low production cost of only a few cent/g, as the compounds are produced in highly optimized processes on the multi-ton scale. Surprisingly, these aspects have not led to a systematic evaluation of agrochemicals for pharmacological use so far [26].

Here we present data of over 600 commercial agrochemicals which have been systematically tested for the first time for their antiparasitic activity.

Materials and Methods

Chemical library

A library of over 600 compounds (for a list of CAS-numbers and common names of the tested agrochemicals see Supporting Information S1), that are or have been active ingredients in commercial agrochemical products, has been compiled from the

BASF compound depository and was dissolved in DMSO stock solutions in a concentration of 10 mg/ml. These samples were then further diluted according to the requirements of the assays. The structural integrity of the dissolved samples has been confirmed subsequently by LCMS-analysis.

Bioassays

Plasmodium falciparum (Pf). P. falciparum drug-sensitive strain NF54 was cultivated in a variation of the medium previously described, consisting of RPMI 1640 supplemented with 0.5% ALBUMAX II, 25 mM Hepes, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine, and 100 μg mL^{-1} neomycin. Human erythrocytes served as host cells. Cultures were maintained in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers at 37°C. Compounds were dissolved in

$(CH_3)_2SO$ (10 mg mL^{-1}), diluted in hypoxanthine-free culture medium and titrated in duplicates over a 64-fold range in 96-well plates. Infected erythrocytes (1.25% final hematocrit and 0.3% final parasitemia) were added into the wells. After 48 h incubation, 0.5 μCi of [^{3}H]hypoxanthine per well was added and the plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded and expressed as a percentage of the untreated controls. Fifty percent inhibitory concentrations (IC_{50}) were estimated by linear interpolation. Assays were run

in duplicate and at least repeated once. Artesunate and chloroquine were used as positive controls.

Trypanosoma brucei rhodesiense (Tb). This stock was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions [27] Minimum Essential Medium (50 µL) supplemented with 25 mM HEPES, 1g L^{-1} additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg mL^{-1} were prepared. Then $4x10^3$ bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µL was added to each well and the plate incubated at 37 °C under a 5 % CO_2 atmosphere for 70 h. 10 µL resazurin solution (resazurin, 12.5 mg in 100 ml double-distilled water) was then added to each well and incubation continued for a further 2–4 h [28]. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC_{50} values were calculated by linear regression [29] from the sigmoidal dose inhibition curves. Melarsoprol was used as positive control.

Trypanosoma cruzi (Tc). Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h, the medium was replaced by 100 µL per well medium containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene (Buckner et al. 1996) [30]. After 48 h the medium was removed from the wells and replaced by 100 µL fresh medium with or without a serial drug dilution of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg mL^{-1}. After 96 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG / Nonidet (50 µL) was added to all wells. A color reaction developed within 2-6 h and could be measured photometrically at 540 nm with a VersaMax microplate reader (Molecular Devices Cooperation, Sunnyvale, CA, USA). The IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves. Benznidazole was used as positive control.

Leishmania donovani (Ld). Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium [31] at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. One hundred microlitres of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtitre plates. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 μg mL^{-1} were prepared. After 70 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 μL resazurin solution (resazurin, 12.5 mg in 100 ml double-distilled water) [32] were then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves. Miltefosine was used as positive control.

P. berghei in vivo **model.** From a donor mouse with approximately 30% parasitaemia (PbANKA-GFP_{CON}) [33], heparinized blood (containing 50 μL of 200 u mL^{-1} Heparin) was taken and diluted in physiological saline to 10^8 parasitized erythrocytes per mL. Of this suspension, 0.2 mL were injected intravenously (*i.v.*) into experimental groups of 3 mice, and a control group of 5 mice. 6, 24, 48 and 72 hours after infection (6 hour time point omitted during 3 times treatment), the experimental groups were treated with a single daily dose (*p.o.* or *s.c.*). 24 hours after the last drug treatment (96 hours after infection), 1 μL tail blood was taken and the parasitaemia determined with a FACScan. The difference between the mean value of the control group and those of the experimental groups was calculated and expressed as a percent relative to the control group (= activity). The survival of the animals was monitored up to 30 days. Mice surviving for 30 days were checked for parasitaemia by slide reading. A compound was considered curative if the animal survived to day 30 post-infection with no detectable parasites. All protocols and procedures were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

T. b. rhodesiense in vivo **model.** The STIB900 acute mouse model mimics the first stage of the disease [34,35].Four female NMRI mice were used per experimental group.

Each mouse was inoculated i.p. with 10^4 bloodstream forms of STIB900. Heparinized blood from a donor mouse with approximately $5x10^6$ mL^{-1} parasitaemia was suspended in PSG to obtain a trypanosome suspension of 4×10^4 mL^{-1}. Each mouse was injected with 0.25 ml. Compound treatment was initiated 3 days post-infection on four consecutive days for all administration routes (i.p., p.o.) in a volume of 10 mL kg^{-1}. Three mice served as infected-untreated controls. They were not injected with the vehicle alone since we have established in our labs that these vehicles do not affect parasitaemia nor the mice. Parasitaemia was monitored using smears of tail-snip blood twice a week after treatment for two weeks followed by once a week until 60 days post-infection. Mice were considered cured when there was no parasitaemia relapse detected in the tail blood over the 60-day observation period. Mean relapse days were determined as day of relapse post-infection of mice. All protocols and procedures were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

Ethics Statement

All work was conducted in accordance to relevant national and international guidelines. The *in vivo* efficacy studies were approved by the veterinary authorities of the Canton Basel-Stadt. The *in vivo* studies were carried out under license No. 1731 and license No. 739 of the Kantonales Veterinäramt, CH-4025 Basel, Switzerland adhering to the Tierschutzverordnung from 23.04.2008 (based on the Tierschutzgesetz from 26.12.2005).

Results and Discussion

Starting with the analysis of the phylogenetic relationship of the pests combated with agrochemicals, and the most important tropical infectious disease pathogens as defined by WHO [36], the close relationship of oomycetes, to which important agricultural pathogens like potato blight or downy mildew belong, with protozoan parasites was realized [37]. As a result, a first set of oomyceticidal agrochemicals was tested, resulting in a number of interesting hits. Based on this finding, over 600 commercially available agrochemicals were selected and their activity against the tropical disease pathogens *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei rhodensiense* tested in cell based screens.

Activity against *Plasmodium falciparum*

In vitro* Activity against *Plasmodium falciparum. For 24 commercial agrochemicals sub-μM activity on P. falciparum could be shown (Figure 1), therefore only the most active compounds will be discussed in more detail. The standards Artesunate (LD_{50} rat i.p. 352 mg/kg; LD_{50} p.o. not available) [38,39] and Chloroquine in the same assay (LD_{50} rat p.o. 330 mg/kg) [40] exhibited an activity of 5.7 and 17.1 nM, respectively.

Fluacrypyrim, (LD_{50} rat p.o. >2000 mg/kg) [41] demonstrated the best activity against *P. falciparum* of all agrochemicals with an IC50 of 8.3 nM. Fluacrypyrim is an acaricide from the group known as the strobilurins, which is mainly used in Japan against mites in orchards. The acaricidal mode of action is the inhibition of respiration by binding to the Qo-site of the bc1-complex [42]. This target is also addressed by the antimalarial drug Atovaquone. Other strobilurin-analogues have been examined before as antimalarials [43].

Azoxystrobin (LD_{50} rat p.o. >5000 mg/kg), also a strobilurin, showed activity at 15 nM. Azoxystrobin is a broadspectrum fungicide and oomyceticide with annual sales of >1 bn€, and production volumes of several 1000 tons/year. It is one of the predominant agrochemicals in the market. Azoxystrobin has also been identified in a high throughput screening campaign of Glaxo- SmithKline, where it showed an IC_{50} value of 41 nM against P. falciparum [44]. This result has surprisingly not been mentioned in the analysis and no follow up has been published.

Hydramethylnon (LD_{50} rat p.o. 1131 mg/kg), an insecticide used in baits against ants, termites and cockroaches, showed 53 nM activity. It is also inhibiting the respiration chain, but probably not at the Qo binding site [45].

Iminoctadine (LD_{50} rat p.o. 300 mg/kg), a broad spectrum fungicide, showed 68 nM activity, with the mode of action presumed to be interaction with cell membranes and lipid biosynthesis. Related bisguanidines have also been examined extensively as antiprotozoal drugs before [46].

Acequinocyl (LD_{50} rat p.o. >5000 mg/kg), an acaricide used predominantly against mites in ornamentals, exhibited an IC50 value of 76 nM. It is also inhibiting the Qo-site in the bc1-complex like atovaquone, to which it also shows some structural similarities.

Additional strobilurins with broadspectrum fugicidal and oomyceticidal activity were tested including trifloxystrobin (LD_{50} rat p.o. >5000 mg/kg), dimoxystrobin (LD50 rat p.o. >5000 mg/ kg), picoxystrobin (LD_{50} rat p.o. >5000 mg/kg), and pyraoxystrobin

(LD_{50} not available), resulting in IC50 values of 84, 148, 305 and 859 nM activity, respectively.

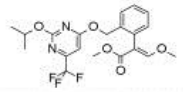
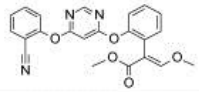
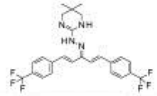
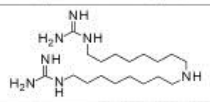
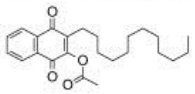
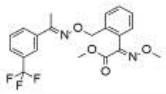
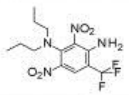
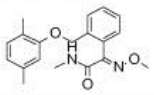
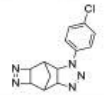
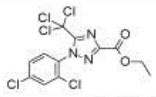
Structure	IC 50 Pf NF54 ng/ml	IC 50 Pf NF54 nM	CAS-Nr.	common name	LD50 rat p.o. mg/kg[32]	indication
	3,6	8,3	229977–93–9	Fluacrypyrim	>5000	acaricide
	6,2	15	131860–33–8	Azoxystrobin	>5000	fungicide/oomyceticide
	26	53	67485-29-4	Hydramethylnon	1131	insecticide
	36	68	115044-19-4	Iminoctadine	360	fungicide
	29	76	57960-19-7	Acequinocyl	>5000	acaricide
	34	84	141517–21–7	Trifloxystrobin	>5000	fungicide/oomyceticide
	41	118	29091-21-2	Prodiamine	>5000	herbicide
	48	148	149961–52–4	Dimoxystrobin	>5000	fungicide/oomyceticide
	47	171	65245-23-0	Tetcyclacis	260	fungicide/growthregulator
	90	223	103112-35-2	Fenchlorazole-ethyl	>5000	herbicide safener

Figure 1. ***In vitro*** **activity of the top 10 most active commercial agrochemicals on** ***P. falciparum*** **NF54 strain**. The IC50 values are the means of two independent assays; the individual values vary by less than a factor of 2.

The pre-emergence herbicides from the dinitroaniline-type including prodiamine (LD_{50} rat p.o. >5000 mg/kg), dinitramine (LD_{50} rat p.o. 3000 mg/kg), and fluchloralin (LD_{50} rat p.o. 1550 mg/kg), showed values of 118, 253 and 816 nM activity, respectively. Their mode of action is the inhibition of mitosis. Other herbicidal dinitroanilines have been shown before to have antiplasmodial activity, but on a significantly weaker level [47].

The plant growth regulator tetcyclacis (LD_{50} rat p.o. 261 mg/kg) inhibits P450 enzymes [48] and exhibits an IC_{50} value of 194 nM.

Fenchlorazol-ethyl (LD50 rat p.o. >5000 mg/kg), an herbicide safener used in cereals, showed 223 nM activity. Furthermore, the corresponding acid, which is potentially the first metabolite of fenchlorazol-ethyl, showed no activity in the assay. Other submM agrochemicals are fluazinam (IC_{50} = 258 nM), cafenstrole (493 nM), difenthiuron (560 nM), fenamidone (641 nM) and butamifos (816 nM).

The biocides fentin acetate (33 nM) (LD_{50} rat p.o. 140–278 mg/ kg), berberine (83 nM) (LD_{50} rat i.v. 60 mg/kg), cycloheximide (101 nM) (LD_{50} rat p.o. 2 mg/kg), fentin hydroxide (408 nM) (LD_{50} rat p.o. 150–165 mg/kg) and thiocyclam (525 nM) (LD_{50} rat p.o. 370 mg/kg) which are used in agrochemistry e.g. as seed dressing, also showed high activity against P. falciparum, but were not further followed up due to their published high toxicity in mammalian species.

***In vivo* antimalarial activity.** In the *P. berghei* mouse model azoxystrobin showed after 4x100 mg/kg p.o. application no significant activity using the Tween-formulated a.i.; but using the aqueous suspension of the commercial fungicidal formulation (200 g/l suspension concentrate) in p.o. application, an extension of survival time from 6–7 to 10.7 days compared to untreated control animals was achieved. With s.c. application of the aqueous formulation a reduction of parasitemia by 98% compared to the untreated mice 24 hrs after last compound application (or 96 hrs after infection) and an extension of the survival time from 6–7 to 13.3 days was observed. This suggests some potential for further optimization of the delivery system.

Hydramethylnon showed with 4x100 mg/kg s.c. application a reduction of parasitemia of 87% and an extension of survival time from 6–7 to 14 days. Furthermore, with a 4x100 mg/kg p.o. application, the parasitemia was reduced by 96% and the survival time was increased to up to 16 days. Considering the challenging physicochemical properties, moderate transfer factor and the nonoptimized dosing regime and formulation of hydramethylnon there might still be some potential to reach a sufficient activity level especially in combination therapies. This warrants further follow up and is currently under examination

Activity on *Trypanosoma cruzi* (Chagas disease)

38 agrochemicals with sub-mM activity on T. cruzi were identified, many of which being azoles with P450-inhibiting activity (Figure 2). P450-monoxygenases have been discussed before as targets against *T. cruzi*, especially the sterol 14αdemethylase [49].

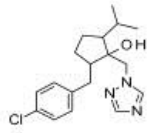
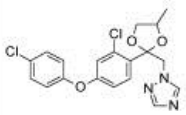
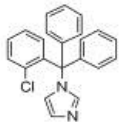
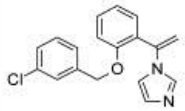
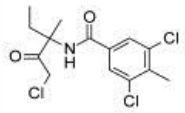
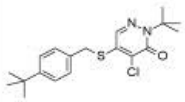
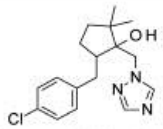
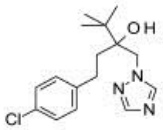
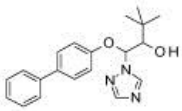
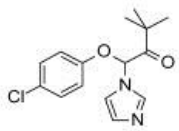
Structures	Tc IC50 ng/ml	Tc IC50 nM	CAS	common name	LD50 rat p.o. mg/kg[32]	indication
	1,0	3,0	125225–28–7	Ipconazole	1338	fungicide
	3,0	7,4	119446-68-3	Difenoconazole	1453	fungicide
	5,0	14	23593-75-1	Clotrimazole	n.a.	fungicide drug
	8,0	26	77175-51-0	Viniconazole	n.a.	fungicide drug
	9,0	27	156052-68-5	Zoxamide	>5000	oomyceticide
	11	30	96489-71-3	Pyridaben	1350	insecticide
	10	31	125116-23-6	Metconazole	660	fungicide
	11	36	80443-41-0	Tebuconazole	4000	fungicide
	18	53	55179-31-2	Bitertanol	>5000	fungicide
	16	55	38083-17-9	Climbazole	400	fungicide

Figure 2. Top 10 most active commercial agrochemicals on *T. cruzi*. The IC50 values are the means of two independent assays; the individual values vary by less than a factor of 2.

The standard drug benznidazole (LD_{50} rat p.o. not available) [50,51] has an IC_{50} of 1871 nM in this assay.

Ipconazole (LD_{50} rat p.o. 888 mg/kg), has an IC_{50} of 3.0 nM, the most active agrochemical against *T. cruzi*. It is a fungicide used predominantly in seed dressing. The

tested material is, like the commercial material, racemic and a mixture of diastereomers, therefore an enantiopure isomer could potentially have even higher activity.

Difenoconazole (LD_{50} rat p.o. 1453 mg/kg), a broad spectrum and systemic fungicide, showed an IC_{50} value of 7.4 nM. This commercial agrochemical is again a racemic diasteromeric mixture and could therefore also have intrinsically higher activity as a pure isomer.

Clotrimazole (14 nM), and viniconazole [52] (26 nM), are two azole drugs used against fungal skin infections, that have also been discussed as agro fungicides and therefore have been tested in this screen. As they have a complete pharmacological dossier they might also be interesting drug candidates.

Zoxamide (LD_{50} rat p.o. >5000 mg/kg), a broadspectrum oomyceticide used in fruits and vegetables, showed 27 nM activity. It is sold and was tested as a racemate. Its mode of action against oomycetes is the inhibition of microtubule formation.

Pyridaben (30 nM), and tolfenpyrad (55 nM), are insecticides/ acaricides inhibiting the complex 1 in the mitochondrial electron transport chain.

A number of further azole fungicides showed activities below 100 nM including metconazole 31 nM, tebuconazole 36 nM, bitertanol 35 nM, climbazole 55 nM, prochloraz 69 nM, hexaconazole 73 nM, and fenapanil 99 nM. Further agrochemicals with high activity in this assay were penconazole (130 nM), epoxyconazole (136 nM), imazalil (148 nM), propiconazole (160 nM), fenarimol (193 nM), fluquinconazole (199 nM), picoxystrobin (248 nM), cyproconazole (257 nM), myclobutanil (374 nM), tetraconazole (478 nM), and pyrifenox (491 nM).

In spite of the excellent *in vitro* activity initial experiments in a *T. cruzi* mouse model did so far not show *in vivo* efficacy for selected hits (personal communication Nazare´ Soiro).

Activity on *Leishmania donovani* (Leishmaniasis)

Against L. donovani only two agrochemicals showed sub-μM activity (Figure 3). The standard miltefosine (LD_{50} rat p.o. 246 mg/kg) showed in this assay an IC_{50} value of 250 nM.

Zoxamide (LD_{50} rat p.o. >5000 mg/kg) showed an IC_{50} of 250 nM. The oomyceticidal compound has been discussed in the T. cruzi section.

Tolylfluanid (LD_{50} rat p.o. >5000 mg/kg) resulted in an IC_{50} value of 861 nM. It is a protective fungicide and oomyceticide with presumed thiol conjugating activity.

Other agrochemicals with moderate activity against L. donovani were flocumafen (2451 nM), dimoxystrobin (3248 nM), bromofenoxin (3839 nM), cyhexatin (4517 nM), and cyazofamid (4988 nM).

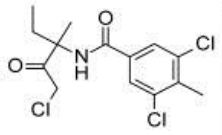
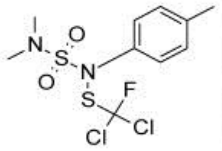
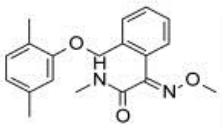
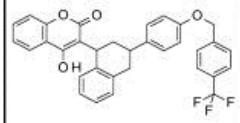
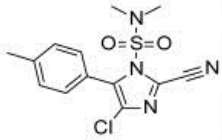
Structure	Ld IC50 ng/ml	Ld IC50 nM	common name	CAS	LD50 rat p.o. mg/kg[32]	indication
	84	250	Zoxamide	156052-68-5	5000	oomyceticide
	299	861	Tolylfluanide	731-27-1	>5000	fungicide/oomyceticide
	1060	3248	Dimoxystrobin	145451-07-6	>5000	fungicide/oomyceticide
	1330	2451	Flocumafen	90035-08-8	0,25	rhodenticide
	1620	4988	Cyazofamide	120116-88-3	>5000	fungicide

Figure 3. Most active commercial agrochemicals on *L. donovani*. The IC50 values are the means of two independent assays; the individual values vary by less than a factor of 2.

Activity on *Trypanosoma brucei rhodensiense*

In vitro* activity against *T. b. rhodensiense. The standard melarsoprol, an arsenate derivative (LD_{50} in mouse *i.v.* 44 mg/kg), showed an IC_{50} value of 5 nM in this assay.

Seven sub-mM active agrochemicals could be identified in the T. b. rhodensiense assay (Figure 4). The two agrochemicals thiram (IC_{50} 12 nM), and thiolutin (IC_{50} 9 nM) [53] are known to have rather high cytotoxicity in cell systems, which likely interferes with this assay.

Zoxamide (LD_{50} rat p.o. >5000 mg/kg) showed the highest activity with an IC_{50} value of 6 nM. This oomyceticidal compound has been discussed above. Toylfluanid

(LD_{50} rat p.o. >5000 mg/kg), showed an IC_{50} value of 52 nM, in addition to its activity against *L. donovani*.

In addition to the above described antimalarial activity, hydramethylnon (LD_{50} rat p.o. 1131 mg/kg), showed 663 nM activity.

Chlorothalonil (LD_{50} rat p.o. >5000 mg/kg), a protective fungicide with thiol conjugating activity, showed 688 nM activity.

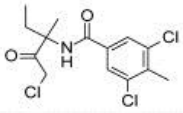
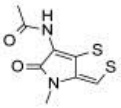
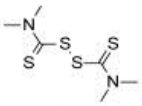
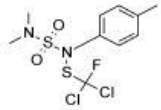
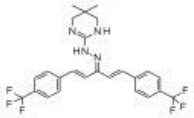
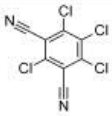
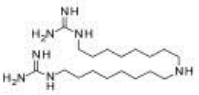
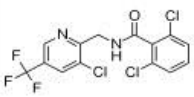
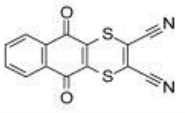
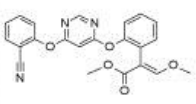
Structure	Tb IC50 ng/ml	Tb IC50 nM	CAS No	common name	LD50 rat p.o. mg/kg[32]	indication
	2,0	5,9	156052-68-5	Zoxamide	5000	oomyceticide
	2,0	8,8	87-11-6	Thiolutin	n.a.	biocide
	3,0	12	137-26-8	Thiram	1800	fungicide/oomyceticide
	18	52	731-27-1	Tolylfluanid	5000	fungicide/oomyceticide
	328	663	67485-29-4	Hydramethylnon	1131	insecticide
	183	688	1897-45-6	Chlorothalonil	>5000	fungicide
	398	743	39202-40-9	Iminoctadine	300	fungicide
	616	1605	239110-15-7	Fluopicolide	5000	fungicide
	543	1832	3347-22-6	Dithianon	300	fungicide
	900	2231	131860–33–8	Azoxystrobin	>5000	fungicide/oomyceticide

Figure 4. Top 10 most active commercial agrochemicals on *T. b. rhodesiense*. The IC50 values are the means of two independent assays; the individual values vary by less than a factor of 2.

Iminoctadin/guacetin (LD_{50} rat p.o. 360 mg/kg), showed 743 nM activity and is discussed in the *P. falciparum* chapter.

In vivo* activity against *T. b. rhodensiense. Zoxamide has been tested in the *T. b. rhodesiense* mouse model for the acute phase of human African trypanosomiasis. Zoxamide showed with 4x200 mg/kg i.p. a weak activity. On day 7 post infection, 24 hours after the last treatment, no *T. b. rhodesiense* could be detected; on day 10 all mice showed a relapse.

Conclusion

Due to the split of most life science companies into their agro- and pharma branches in the 1990s, the companies active in agrochemistry have not been involved in the recent screening activities to identify new drugs against infectious tropical diseases, even though agrochemicals might have a high potential to yield interesting hits for these applications.

In this cooperation between industrial and public partners, it was shown for several commercial agrochemicals that they are highly active against some of the most important pathogens of infectious tropical diseases. Interestingly as anticipated, several of the oomyceticides (strobilurins against *P. falciparum*, zoxamide against *T. b. rhodesiense* and *L. donovani*) were active against these protozoans, but also other agrochemicals (e.g. hydramethylnon against *P. falciparum*; azoles like iproconazole against *T. cruzi*) showed very interesting activities. Exemplified by one of the major commercial agrochemicals, the fungicide azoxystrobin, as well as for the insecticide hydramethylnone, the reduction of parasitemia, and significant life extension for *P. berghei* infected mice was achieved. For zoxamide, an effect against *T. brucei* in the mouse model was also demonstrated. This successful *in vitro– in vivo* transfer without galenic optimization could not be taken for granted, as these agrochemicals have not been optimized for mammalian pharmacokinetics.

There is still a high probability that the identified hits in the end might not be suitable for human use, as there are still several hurdles to overcome. However, the results of this highly focussed and relatively low input approach are more promising than could have been hoped for. It is especially noteworthy, that the screen of less than 700 agrochemical resulted in e.g. 24 new sub-μM hits against *P. falciparum*, compared

to 4 new sub-μM hit in over 2687 recently tested commercial drugs (excluding known antimicrobial and anticancer a.i.) [54,55]. This clearly demonstrates that agrochemistry can be a very interesting and so far untapped source of new leads, and maybe even drug candidates, against protozoal diseases. It would also be very interesting to screen commercial agrochemicals against the pathogens of other neglected diseases, like schistosomes, nematodes, food borne trematodes, diarrhoeal amoebas and also tropical bacterial pathogens, for which good antibiotic cures are missing. These studies are still to be done.

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Supporting Information

Supporting Information S1 CAS-numbers and common names of the tested agrochemicals. (DOC)

Author Contributions

Conceived and designed the experiments: MW MR MK RB. Performed the experiments: MR MK. Analyzed the data: MW MR MK RB. Wrote the paper: MW MR MK RB.

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CHAPTER 9

Antiprotozoal activity profiling of approved drugs: a starting point toward drug repositioning

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Abstract

Neglected tropical diseases cause significant morbidity and mortality and are a source of poverty in endemic countries. Only a few drugs are available to treat diseases such as leishmaniasis, Chagas' disease, human African trypanosomiasis and malaria. Since drug development is lengthy and expensive, a drug repurposing strategy offers an attractive fast-track approach to speed up the process. A set of 100 registered drugs with drug repositioning potential for neglected diseases was assembled and tested in vitro against four protozoan parasites associated with the aforementioned diseases. Several drugs and drug classes showed in vitro activity in those screening assays. The results are critically reviewed and discussed in the perspective of a follow-up drug repositioning strategy where R&D has to be addressed with limited resources.

Author Summary

Neglected tropical diseases affect the poorest people in developing countries and cause significant morbidity and mortality. There are only few drugs available for the treatment of these diseases. For combating these diseases, new and better drugs are needed. Drug development is a lengthy and expensive process. In this study we were looking for low-hanging fruits and followed a drug repurposing strategy. A set of 100 registered drugs with drug repositioning potential for neglected diseases was assembled. The compound collection was systematically screened against protozoan parasites, T. b. rhodesiense, L. donovani, T. cruzi and P. falciparum. This low-hanging fruit approach using a relatively small collection of compounds was certainly worth the effort. Several drugs and drug classes exhibited in vitro activity and selectivity against one of the protozoan parasites. The results offer opportunities for drug repurposing but the identified compound classes could also be starting point for new drug discovery projects.

Introduction

Neglected tropical diseases (NTDs) such as leishmaniasis, human African trypanosomiasis and Chagas' disease affect the poorest people in developing countries. NTDs are responsible for substantial global morbidity, mortality and economic losses [1]. Leishmaniasis is endemic in 88 countries around the globe with 350 million people

living at risk, and there are an estimated 1.5 to 2 million new cases per year [2]. Human African trypanosomiasis is transmitted by tsetse flies and the disease threatens millions of people in over 20 countries in sub-Saharan Africa. Due to reinforced surveillance and vector control the prevalence has come down in the last 15 years from approximately 40,000 to less than 8,000 cases [3]. Chagas' disease is endemic in 18 countries of Central and South America. It is estimated that 120 million people are at risk of infection and that 8 million are already infected [4]. Malaria, caused by Plasmodium spp., is one of the most devastating diseases in developing countries, with 207 million reported cases in 2012, causing 627,000 deaths in that year [5]. There are only a few drugs available for the treatment of these diseases. These drugs have associated liabilities including lack of efficacy, severity of side effects, high costs, or lack of practicality for field use, all of which constitute hurdles in terms of access to treatments for the patients. To combat these neglected diseases, new and better drugs are needed. The next generation of drugs will need to be very effective and safe, orally-available, and with a long shelf-life in tropical field conditions. Those drugs should provide simple, short-course drug administration regimens (maximum 10 days, ideally 1-3 days for malaria) amenable for drug combinations, to prevent the emergence of resistance. The latter demand applies to all diseases but is especially important for malaria due to the global spread of drug resistance to existing antimalarials including artemisinin-based derivatives, for which the first cases of delayed clinical efficacy have already been reported [6].

There are several strategies to develop new drugs against NTDs. De novo drug discovery and drug development is a highly rational approach but it is a lengthy and expensive process [7, 8]. Alternatively, a drug repurposing strategy can be used as a fast-track approach guided by the established Target Product Profiles (TPP) [9, 10]. However, this attractive approach can only be considered with drugs achieving in vitro activity in relevant assays in the first place. Here, we report the in vitro activity against Trypanosoma brucei rhodesiense, Leishmania donovani, Trypanosoma cruzi and Plasmodium falciparum - of 100 registered drugs selected for their potential to be repurposed for the antiprotozoal diseases based on their respective TPPs.

Methods

Chemicals

Antiviral compounds were received from the NIH AIDS Reagent Program (USA) Other compounds were purchased from Sigma-Aldrich

Bioassays

The *in vitro* activities against the protozoan parasites *T. b. rhodesiense*, *T. cruzi*, *L. donovani* axenic amastigotes, and *P. falciparum*, and cytotoxicity assessment against L6 cells were determined as reported in Orhan et al 2010 [11] . Selectivity index (SI) was calculated as IC_{50} L6 cells/IC_{50} parasite.

Activity against Leishmania donovani intracellular amastigotes in macrophage assay: Mouse peritoneal macrophages (4 x 10^4 in 100 µl RPMI 1640 medium containing 10% heat-inactivated FBS) were seeded into wells of Lab-tek 16-chamber slides. After 24 h 1.2 x 10^5 amastigote *L. donovani* in 100 µl were added. The amastigotes were taken from an axenic culture grown at pH 5.4. Four hours later, the medium containing free amastigote forms was removed and replaced by fresh medium. The next day the medium was replaced by medium containing different compound dilutions. Parasite growth in the presence of the drug was compared to control wells. After 96 hours of incubation, the medium was removed and the slides fixed with methanol for 10 min followed by staining with a 10% Giemsa solution. Infected and non-infected macrophages were counted for the control cultures and the ones exposed to the serial drug dilutions. The infection rates were determined. The results were expressed as percent reduction in parasite burden compared to control wells, and the IC_{50} calculated by linear regression analysis.

In vitro cytotoxicity with mouse peritoneal macrophages. Mouse peritoneal macrophages were seeded in 96-well microtitre plates at 10^4 cells/well in 100 µL RPMI 1640 medium containing 10% FBS and 2 mM l-glutamine. After 48 h 100 µl fresh medium was added with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 100 to 0.14 µg/ml. After 96 h of incubation, the plates were inspected under an inverted microscope to assure sterility. Alamar Blue (20 µl) was added to each well and the plates incubated for a further 4 hours. The plates were then read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave-length of 536 nm and an

emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, Sunnyvale, CA, USA). Podophyllotoxin was used as control.

Cluster analysis

The drugs selected in this study were clustered according to certain criteria including a) main indication(s) for which they are registered, b) chemical class and c) mechanisms of action(s). Whenever possible, the DrugBank classification (http://www.drugbank.ca) was followed to assign indication as well as mechanism of action labels to the selected drugs. These labels do not intend to be exhaustive as additional indications as well as mechanisms of action are known for several of the drugs. Chemical classes were arbitrarily defined according the chemical scaffolds of the molecules under consideration, with the exception of protease inhibitors that are better captured under this appellation due to structural variety.

Results and Discussion

A set of 100 registered drugs were collected (Table S1) in the framework of DND*i* exploratory activities and submitted systematically to a panel of *in vitro* assays to be profiled for their antiprotozoal activities. These drugs and drug classes were primarily selected for their potential to be repurposed provided that *in vitro* activity could be demonstrated. The inclusion criteria comprised favorable bioavailability profile, moderate cost of goods and a good safety profile. The selection is heavily biased for anti-infectious indications (66 compounds) including antibiotics (26), antifungals (14), antivirals/antiretrovirals (16) as well as antiparasitic compounds (10) and 15 psychoactive compounds. Another 19 drugs are related to other indications (Table S1). In some instances, drugs were selected based on literature reports of antiprotozoal activity in relation with one specific molecule or class of compounds. A panel of well-known antiprotozoal drugs such as artesunate, mefloquine, pentamidine, nifurtimox and amphotericin B (not an exhaustive list) were included as benchmarks as well as to cross-profile these drugs in the entire screening assay panel.

The results (Table S2) are ranked in agreement with the *in vitro* activity cutoffs defined at the hit stage for kinetoplastids [12] and for *P. falciparum* [13].

Human African trypanosomiasis

Pentamidine and nifurtimox were, unsurprisingly, identified as active against *T. b. rhodesiense*; both drugs are used for the treatment of human African trypanosomiasis (HAT) (Table 1, Figure S1). The mode of action of pentamidine - an aromatic diamidine, a chemical class well-known for its antitrypanosomal activity - is not fully understood. There is evidence that impairment of mitochondrial function is involved [14] and that this family of compounds can rapidly accumulate within trypanosomes as demonstrated with DB75 and DB820 [15]. Nifurtimox is a well-known antitrypanosomal nitrofurane, only partially understood in terms of its mechanism of action as being related to the induction of oxidative stress in the target cell [16]. More recently, the activation of nifurtimox by trypanosomal type I nitroreductases leading to the generation of cytotoxic nitrile metabolites has been described [17].

Two 5-nitrofuran antibiotics chemically related to nifurtimox (Figure S1), namely nifuroxazide (IC_{50} = 0.03 μM, SI: 410) and nitrofurantoin (IC_{50} = 0.5 μM, SI: 180) were identified as being remarkably potent against *T. b. rhodesiense* (Table 1). These compounds are reduced by nitrofuran reductase to reactive intermediates that cause oxidative stress [16]. The nitrophenylbenzamine niclosamide showed a lower *in vitro* activity (IC_{50} = 1.67 μM), whereas the 5-nitroimidazole derivatives metronidazole and tinidazole were shown to be inactive in the same assay, presumably as they are not activated *via* enzymatic reduction under the experimental conditions. Overall, the potential for drug repurposing of any nitroheterocycles for HAT heavily depends on their toxicity – and notably genotoxicity/mutagenicity - profile in respect to their efficacy in relevant rodent models, as demonstrated by the successful development of fexinidazole currently in Phase II/III clinical trials [18,19].

Rifamycin SV (IC_{50} =0.99 μM, SI: 16) exhibited a selective activity profile against *T. b. rhodesiense* (Table 1), whereas other members of the rifamycin family (rifabutin, rifampicin and rifaximin) were devoid of antitrypanosomal activity. Rifamycins have been used for the treatment of several diseases, the most important one being HIV-related tuberculosis. They are particularly active against mycobacteria *via* the inhibition of bacterial DNA-dependent RNA polymerase [20]. Rifamycin SV is a semi-synthetic broad-spectrum antibiotic with activity against Gram-positive and Gram-negative bacteria and mycobacteria. It belongs to the class of ansamycins obtained from rifamycin B, which is produced by fermentation of *Streptomyces mediterranei n. sp.*

Rifamycin SV is poorly bioavailable and is used parenterally or topically in the treatment of cutaneous and soft tissue infections such as osteomyelitis, bronchopulmonary and biliary tract infections, traveler's diarrhea, infectious colitis and staphylococcal septicemias. Rifamycin SV accumulates especially in the bile but - as other rifamycins - has rather limited penetration into the brain due to its high molecular weight (800 Da) as well as a high protein binding capacity (around 80%). These features and its lack of bioavailability are clear liabilities for the repositioning of this drug for HAT.

Auranofin showed good and selective activity against *T. b. rhodesiense* (IC_{50} =0.01 μM, SI: 479) (Figure S1). Auranofin is a gold complex used to treat rheumatoid arthritis. It putatively acts as an inhibitor of kappa B kinase and thioredoxin reductase which would lead to a decreased immune response and decreased free radical production, respectively [21]. It is a compound that targets selenoproteins in the bloodstream form and procyclics of *T. brucei* [22]. In a recent high-throughput drug screen, high activity against *Entamoeba* was discovered [23]. Auranofin showed 10 times better activity against *Entamoeba histolytica* than the standard drug metronidazole. The very high potency of auranofin *in vitro* constitutes an excellent starting point towards repurposing. In addition, auranofin suppressed *T. brucei* parasitemia *in vitro* within a 6-hour time period at a concentration of 0.92 μM. It also presents a favorable tissue permeability profile based on data obtained from an *in vitro* MDR1-MDCK assay (data not presented). Given the relatively good bioavailability (17-23%) of auranofin as well as favorable drug exposure in various tissues in rats (terminal half-life of 29 and 43 hours based on blood and serum levels, respectively) following oral administration of a single dose (6.7 mg /kg) [24], we performed an *in vivo* efficacy study in an acutely infected *T. brucei* murine model. However after daily oral administration of up to 25 mg/kg auranofin over a 4 day period no *in vivo* efficacy was observed in comparison to an untreated control group regarding reduction of parasitemia or increased survival time (data not presented). This negative outcome could be explained by the lack of a cidal-mechanism of action, which needs to be further investigated through an *in vitro* time-kill assay using a drug wash-out step, or a sub-optimal drug exposure *in vivo* in mice. The latter might be due to degradation or metabolism of auranofin as the analytical method that had been used was based on the detection of gold by atomic absorption spectrometry [24]. Alternative explanations might be a significantly different

pharmacokinetic profile in mouse versus rat, from where the published data came from, or high protein binding of auranofin.

Two adamantane derivatives were tested, one of which rimantadine, was selectively acting (IC_{50} = 13.83 μM SI: 23) against *T. b. rhodesiense*, albeit at a moderate level. The activity of rimantadine and of other adamantane derivatives against *T.brucei* had already been reported by Kelly et al. in 1999 and 2001 [25, 26] and Zoidis et al. 2008 [27]. Ademantanes presumably target essential *T. brucei* membrane-localized ion channels or transporters [28, 29]. They are known to act on viral matrix protein 2 with respect to their primary antiviral indication [30]. Adamantanes are inexpensive, orally active drugs [31]. They exhibit steady-state levels in serum of 2.5 to 5.0 μM and plasma half-lives of 24 to 36 hours in humans [32, 33]. Furthermore, adamantanes readily cross the blood-brain barrier [34]. As such adamantanes, and more particularly the *T. brucei* active rimantadine, seem to offer promising potential in terms of drug repurposing for HAT, although the moderate *in vitro* potency of rimantadine might be insufficient to demonstrate efficacy *in vivo* given the aforementioned serum levels. Adamantanes may therefore be preferably pursued as part of a lead optimization program to increase potency against *T. brucei* while keeping, and possibly improving, the favorable pharmacokinetic properties of the series. A limited evaluation of 17 adamantanes supported this approach as the most active derivative (1-adamantyl-4-amino-cyclohexane) was about 20 to 25 times more effective than rimantadine [26]. The same study delivered the first proof of principle of efficacy of adamantanes *in vivo*, with a transient 98% suppression of parasitemia in mice with an acute *T. brucei* infection. These encouraging results seem to indicate that lead optimization might be more promising than a repurposing strategy for this class of compounds.

A key feature of the TPP to cure the second stage of HAT is CNS penetration [9]. Psychoactive compounds, by definition cross the blood-brain barrier. All anti-depressant and antipsychotic drugs – including tricyclics, and selective serotonin reuptake inhibitors - displayed IC_{50} values in the range of 0.5 – 2 μM (Table 1) against *T. b. rhodesiense*, as well as a limited selectivity window with respect to the L-6 rat myoblast cell line apart from nortriptyline (SI> 20) (Figure S2). The only exception was spirenone – a butyrophenone derivative known to act via serotonin and dopamine receptor inhibition – that was reported to be inactive in this assay. These drugs act in various ways and levels on dopaminergic and serotoninergic central receptors indicating

that they all have the potential of crossing the blood brain barrier. The related drugs thioridazine, trifluprомazine, promazine and chlorpromazine are D2 dopamine receptor antagonists and Ca^{2+} channel blockers. Nortriptyline inhibits reuptake of norepinephrine and is a strong antagonist of the H_1 receptor. It is also known as a Na^{+} channel blocker. There were earlier attempts to develop tricyclic compounds as trypanothione reductase inhibitors *via* lead optimization efforts [35, 36]. However no clear relationship between the activities measured on trypanothione reductase and the *T. brucei* whole cell assay could be drawn from a series of 22 inhibitors [36]. It is, to our knowledge, the first time that selective serotonin reuptake inhibitors (including sertraline and paroxetine) are reported to show activity against *T. b. rhodesiense*. Demonstration of a cidal profile of these drugs in a time-kill non-reversible *T. brucei* assay can be proposed as the next experimental study towards a repurposing strategy. The poly-pharmacology profile of these drugs, notably in respect to associated central effects and toxicity will have to be carefully considered with respect to dose finding in mouse models.

Chagas' disease

Not surprisingly nitroheterocycles, in particular nitrofurane derivatives including nifurtimox, nifuroxazide and nitrofurantoin, exhibited the highest antichagasic activity (Table 2, Figure S3). Nitrofuranes are well known for their antichagasic activity: Nifurtimox - as well as benznidazole, the second treatment available for Chagas' disease- has been shown to be activated by a NADH-dependent, mitochondrially localized type I nitroreductase [37]. A repurposing strategy for any nitrofurans or nitroimidazole analogues including nifuroxazide and nitrofurantoin, must be based primarily on the safety profile compared to currently used drugs. This notably includes genotoxicity/mutagenicity as previously mentioned in the case of human African trypanosomiasis. In addition, the compound should demonstrate equivalent or better *in vitro* activity and *in vivo* efficacy than the current drugs. Interestingly, another compound from the nitroimidazole class – fexinidazole - has recently also been reported for its oral efficacy in acute and chronic experimental models of benznidazole-susceptible, partially resistant, or resistant *T. cruzi* isolates [38] and could therefore be considered as a good candidate for drug repositioning.

Azoles were identified as the most potent class of inhibitors: six representatives with IC_{50} values in the range of 0.003 – 0.3 μM and SI: >100 (bifonazole, clotrimazole, econazole nitrate, miconazole and tioconazole as imidazoles as well as itraconazole and

ketoconazole as triazoles) while other compounds from this class displayed lower activity and/or selectivity against *T. cruzi* (Table 2). These well-known antifungal drugs are already known for their activities against *T. cruzi* and for acting *via* inhibition of 14-alpha-sterol demethylase, an enzyme of the sterol biosynthesis pathway [39]. Two triazole antifungals, posaconazole and E1224 (a prodrug of ravuconazole), have recently been reported as failing to demonstrate sustained clearance of *T. cruzi* parasitemia in chronically infected patients in phase II clinical trials, putting azoles as a therapeutic class at stake for the treatment of Chagas'disease, at least in monotherapy [40]. This outcome might well be correlated with the inability of azoles and of non-azole CYP51 inhibitors to achieve parasite clearance *in vitro* in various *T. cruzi* lineages [41].

Two other compounds that popped up showing moderate micromolar *in vitro* activity against *T. cruzi* are tadalafil (IC_{50} = 8.6 μM SI: >26) and mebeverine (IC_{50} = 3.89 μM SI: 18) (Table 2, Figure S3). Tadalafil is a phosphodiesterase type 5 (PDE5) inhibitor used in treating erectile dysfunction. PDEs are cAMP-specific hydrolases and play a major role in cyclic nucleotide signaling [42]. One of the main challenges to be considered in terms of drug repurposing of PDE inhibitors relates to the safety profile associated to the structural similarity between the human and protozoan PDE.

The antispasmodic mebeverine is used for the treatment of irritable bowel syndrome (IBS) and the associated abdominal cramping. It works by relaxing the muscles in and around the gut. It is a musculotropic antispasmodic drug acting directly on the gut muscles at the cellular level to relax them. Mebeverine is also a functional inhibitor of acid sphingomyelinase (FIASMA) [43] as well as a serotonin 5-HT3 receptor antagonist. To our knowledge this is the first time that tadalafil and mebeverine are reported to have antichagasic properties. Even if the antitrypanosomal activity is moderate, a more careful evaluation of their activity needs to be conducted to better understand their potential of drug repositioning for Chagas' disease, notably their ability to exert a cidal, irreversible and total clearance of *T. cruzi* infection *in vitro* as well as *in vivo* in relevant models. If successful, the dose regimens of tadalafil and mebeverine required to cure a *T. cruzi* infection *in vivo* will have to be compared with the maximum tolerated doses identified from preclinical *in vivo* models, to ensure that the safety profiles of the two drugs are compatible with this new indication.

Leishmaniasis

All selected candidates were tested in two different assays, involving axenic amastigotes and intracellular amastigotes of *L. donovani*, respectively. The latter assay used peritoneal mouse macrophages as host cells. Amastigotes in macrophages are currently considered as more relevant for the visceral disease pathology than axenic amastigotes [44]. For cytotoxicity the compounds were counter-screened against non-infected peritoneal mouse macrophages. Apart from amphotericin B and sitamaquine that can be considered as control drugs in this screening, clofazimine was the only compound exhibiting activity in the *Leishmania donovani* intracellular assay as well as an acceptable level of selectivity (SI ~10) (Table 3, Figure S4). Amphotericin B is a polyene antifungal drug displaying either fungistatic or fungicidal activity depending on the drug concentration in body fluids with respect to the susceptibility of the investigated fungal microorganism. The liposomal formulation of amphotericin B (marketed as AmBisome) is currently used as monotherapy for the treatment of visceral leishmaniasis. Amphotericin B binds irreversibly to ergosterol, resulting in disruption of membrane integrity and leakage of intracellular components leading to cell death [45].

Sitamaquine, a known antileishmanial drug, displayed only moderate activity against both axenic and intracellular amastigotes (Table 3). The drug development of sitamaquine was discontinued in Phase II clinical trials by GlaxoSmithKline due to safety concerns related to methemoglobinemia, a known feature of 8-aminoquinolines [46].

Clofazimine is a lipophilic riminophenazine derivative possessing both antimycobacterial and anti-inflammatory properties. Its efficacy has been demonstrated only in the treatment of leprosy in combination with rifampicin and dapsone, but not in human tuberculosis, despite the fact that it is impressively active *in vitro* against multidrug-resistant strains of *Mycobacterium tuberculosis* [47]. Interestingly, clofazimine is more active against intracellular than axenic *Leishmania donovani*, putatively due to the accumulation of clofazimine in the macrophages, a known feature of riminophenazines [48]. The antileishmanial properties of clofazimine have previously been reported both *in vitro* and in animal models for three different *Leishmania* species including *L. donovani* [49]. Clofazimine binds to guanine bases leading to an inhibition of cell proliferation [50, 51]. Additionally, clofazimine inhibits acid sphingomyelinase (FIASMA) and increases the activity of phospholipase A2 [43]. Cell membrane

destabilization and subsequent dysfunction as well as intracellular redox cycling involving oxidation of reduced clofazimine leading to the generation of reactive oxygen species were proposed as mechanisms contributing to the antimycobacterial activity of clofazimine. These putative mechanisms of action have recently been reviewed by Cholo et al. 2012 [47]. The safety profile of clofazimine related to the tendency of this drug to concentrate in fatty tissues and in cells of the mononuclear phagocyte system is certainly a factor that needs to be carefully considered in view of drug repurposing. Considering the very good pharmacokinetic, distribution and safety profiles of clofazimine in the mouse [48] it seems quite reasonable to envisage an *in vivo* efficacy study of this drug in a relevant mouse model infected with *Leishmania donovani*.

Auranofin, tipranavir (a non-peptidic protease inhibitor [52], the antimalarial artesunate and other antibacterials like nitrofurantoine, nifuroxazide, rifampicin and rifamycin SV were all active (IC_{50}: < 3μM) against axenic amastigotes of *L. donovani*, but inactive against the intracellular amastigotes (Table 3). The hydroxypyridinone antifungal ciclopirox olamine showed activity against axenic amastigotes and activity against intracellular amastigotes of *L. donovani* (IC_{50} = 0.1 μM, SI: 9) with moderate selectivity (Table 3, Figure S4). The mode of action of cicloporix is not well understood, a loss of function of certain catalase and peroxidase enzymes, and various other components of cellular metabolism are involved [53, 54]. The two azoles clotrimazole and tioconazole were active with low selectivity against intracellular *L. donovani* too (Table S1). Niclosamide used as anthelmintic, in addition to auranofin, showed the best activity of all tested compounds against *L. donovani* axenic amastigotes but it was inactive against intracellular amastigotes at a concentration of 0.1 μg/ml, and toxic at higher concentrations (>0.3μg/ml) to mouse macrophages (Table S1). The repurposing potential of these few drugs seems rather low as they were not able to demonstrate any significant activity in the intracellular *L. donovani* assay or alternatively lacked selectivity.

Malaria

The *in vitro* activity of all of the tested standard animalarials (artesunate, mefloquine, tafenoquine, chloroquine and sitamaquine) was confirmed against *P. falciparum* as shown in Table 4. Interestingly, four of the tested azoles (clotrimazole, econazole, miconazole and tioconazole) were active against *P. falciparum* (Table 4, Figure S5)

confirming the finding of Penna Coutinho et al. 2011 [55] who described the antimalarial activity of posaconazole and itraconazole.

Rifamycins, especially rifampicin (IC_{50}= 0.1 μM, SI: >100), showed remarkably selective activity in the antiplasmodial assay (Table 4). The anti-tuberculosis drug rifampicin is a RNA polymerase inhibitor of bacterial transcription and was previously described for its *in vitro* and *in vivo* antimalarial activities [56, 57]. To our knowledge, other compounds from this class have not been reported to have antimalarial activity.

The antiplasmodial activity associated with tricyclic antidepressants (Table 4, Figure S5) is certainly one of the most striking observations of this screen. Promazine and nortriptyline displayed the highest selective activity against *P. falciparum*. Promazine is a phenothiazine compound D2 dopamine receptor antagonist and showed an IC_{50} value of 0.49 μM with a selectivity index of 61. Nortriptyline, a tricyclic antidepressant and potent inhibitor of the norepinephrine transporter exhibited an IC_{50} value of 0.58 μM against *P. falciparum*, and a selectivity index of 48. Tricyclic antidepressant drugs have previously been shown to reverse chloroquine resistance in *P. falciparum in vitro* and in monkey studies [58]. The reversal of resistance is probably mediated by the inhibition of a chloroquine efflux pump. Tricyclic compounds were additionally described in a recent publication as blocking agents for *Plasmodium* oocyst development and transmission [59]. Transmission blocking is an important feature for the elimination of malaria. It is worth noting that further tricyclics (including fluphenazine and amitriptyline) as well as selective serotonin reuptake inhibitors (sertraline and fluoxetine) also displayed antiplasmodial activities in the micromolar range as well as reasonable selectivity profiles against the L-6 cell line. Additionally, *in vitro* selective activities against *P. falciparum* were identified for the antiviral rimantadine, the anti-thrombotic dipyridamole, the anti-tussive clopersatine, and the anti-histamine ketotifen. All of these activities have already been reported elsewhere [60, 61, 62]. Providing a cidal mechanism of action can be confirmed for these drugs, the next step will consist of an evaluation of their potential to suppress parasitemia in a mouse malaria model, ideally following single oral dose. If successful, the repurposing potential of these drugs will need to be carefully assessed considering the safety profile at the defined curative dose, notably in relation to the pharmacological effects of these drugs at the used dosing regimen. This constitutes a major challenge, especially for the drugs for which there is a dramatic discrepancy in terms of *in vitro* activities between their primary indication

(generally 1-10 nM range) and malaria (100 nM-1μM range). The compatibility of these drugs with a short (1-3 day) oral treatment, their low susceptibility to generate resistance, and their amenability to be used in combination with existing antimalarial drugs will similarly need to be considered.

Conclusion

Several drugs and drug classes were confirmed to have *in vitro* activity against protozoan parasites including *T. brucei rhodesiense, L. donovani*, *T. cruzi* and *P. falciparum,* offering various opportunities for drug repurposing. Several of these antiparasitic activities – but not all- have already been reported. To our knowledge it is indeed the first time that tadalafil and mebeverine have been described for their antichagasic activity. The confirmation of an activity in state-of-the-art *in vitro* assays and eventually in animal models is obviously just the beginning of a long journey. Several of these candidates might indeed be discarded at a relatively early stage for various reasons including safety, inadequate mode of drug administration, or lack of efficacy *in vivo* to mention just a few obstacles down the road of development. On a more specific basis, any *in vitro* cytotoxicity - and therefore derived selectivity - data generated in the frame of this work should be put in perspective with the existing toxicity profiles of these drugs and should not be considered *per se* as a rule-out filter unless the results clearly compromise the antiprotozoal readout of intracellular assays. For these drugs a wealth of preclinical and clinical data can be used to determine whether their safety profiles are compatible with the anticipated dose of drug to be used in animal models and eventually in patients. The candidates for further development should ideally be associated with a favorable bioavailability profile, as oral drug administration is preferable for the next generation of drugs used to treat kinetoplastid diseases. The reasons for a lack of or insufficient *in vi*vo efficacy in relevant preclinical animal models shall be further investigated to assist in the decision to drop or further pursue an existing drug for repurposing. Indeed, one of the reasons might be a clear "no-go'" such as an inappropriate mechanism of action (e.g. drug working *via* a static mechanism, CYP51 inhibition for Chagas' disease), while other characteristics might be surmountable (e.g. lack of drug exposure due to a suboptimal drug dosing regimen). There is a clear need to carefully define the types of preclinical experiments that need to

be run to progress the candidates identified from screening in the framework of a defined drug discovery cascade supported by DMPK and toxicity assays.

Drug repurposing - also called drug repositioning - has proven to be an attractive way to address drug development in a cost-effective and less risky manner when compared to *de novo* drug discovery and development [63]. This discovery strategy aims at making the best use of pre-existing preclinical and clinical knowledge accumulated on registered drugs and drug candidates for a new indication, and is nowadays actively pursued by the pharmaceutical companies [63] and currently accounts for approximately 30% of the newly approved drugs and vaccines by the US Food and Drug Administration - in recent years [64]. Drug repurposing led to a number of success stories that have been reviewed and documented elsewhere [63, 65] including the famous revival of thalidomide – a sedative drug which was banned in the early 60s for causing severe skeletal birth defects in new born children – for the treatment of erythema nodosum laprosum, an agonizing inflammatory condition of leprosy. The repositioning of sildenafil (Viagra) in the 1980s from a cardiac related indication (angina) to erectile dysfunction is certainly a striking example of an opportunistic exploitation of an observed side-effect that led to a switch of therapeutic use for this PDE-5 inhibitor. Other examples include the repurposing of the antiparkisonian atomoxetine for the treatment of attention deficit hyperactivity disorder, and the anti-emetic and antihistamine chlorpromazine as a non-sedative tranquilizer. The area of neglected diseases has similarly accounted a few drug repositioning successes such as the antibacterial sulfonamides (dapsone, sulfadoxine), tetracyclines (doxycycline) and combination of trimethoprim/sulfamethoxazole in the area of malaria [66], fluoroquinolones for tuberculosis [66], the anticancer agent miltefosine as well as the antifungal amphotericin B for the treatment of visceral leishmaniasis [65].

Several approaches can be used to address the identification of novel drug candidates at an early discovery stage using a drug repositioning approach. They notably include target-based screening, phenotypic ("target unbiased or blinded") screening, knowledge-based methods (e.g. chemoinformatics and bioinformatics), signature-based methods, pathway or network methods and targeted mechanism-based methods, reviewed and illustrated elsewhere [64]. From a recent comparative analysis based on 259 approved agents [67], 50 were shown to be first-in-class small molecules associated with a new molecular mechanism of action, of which 28 and 17 of these drugs were identified from

phenotypic screening and target-based approaches, respectively. These results illustrate the impressive potential of phenotypic screening in the area of drug discovery. The screening of a library of drugs and drug candidates in a phenotypic assay is therefore seen as an attractive way to identify new potential candidates with a modest work load. This can be illustrated by the discovery of the antimalarial properties of astemizole from the screening of 2687 approved drugs or drug candidates using a *P. falciparum* whole cell *in vitro* screening assay [68].

In summary, this low-hanging fruit approach is certainly worth the effort as related to a "low risk, high return on investment" drug discovery process, especially in the field of Neglected Diseases where R&D has to be addressed with limited resources. The availability of a significant amount of data and expertise, notably related to preclinical and clinical toxicity as well as pharmacokinetics for all of these drugs can indeed lead to significant savings in terms of time and money related to drug development. This information can be used to support decision-making related to the progression of the early candidates in a rather fast-track mode, using existing drugs from the same class for benchmarking, if this option is available. Some of the approved marketed drugs will have the additional advantage of being off-patent, facilitating the drug repurposing process from an intellectual property management standpoint. A drawback related to the progression of old drugs might however be the lack or the paucity of recently generated data reports (e.g. lack of quality of pharmacokinetic measurements based on LC/MS, and toxicity assays performed in obsolete *in vitro* and *in vivo* predictive models).

Supporting Information

Table S1. Table 1. Set of 100 registered drugs tested for their antiparasitic activity.

Table S2. Table 2. *In vitro* activity profile in IC_{50} (μM) of all tested compounds.

Figure S1. HAT cluster of all tested compounds. Chemical class vs log (IC_{50} in μM)

Figure S2. HAT cluster of antidepressant and antipsychotics. Chemical class vs log(IC_{50} in μM)

Figure S3. Chagas disease cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)

Figure S4. Leishmaniasis cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)

Figure S5. Malaria cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)

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Author Contributions

Conceived and designed the experiments: MK JRI RB. Performed the experiments: MK. Analyzed the data: MK PM LPT JRI. Wrote the paper: MK PM LPT JRI RB.

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Tables

Table 1. *In vitro* activity against *T .b. rhodesiense* in IC_{50} (μM) of compounds fulfilling hit criteria

Drug ID	[a]*T. b. rhod.*	[b]Cytotox. L6	[c]SI	Indication	Chemical Class	Mode of Action
Pentamidine	0.01	8.87	887	Antibacterial/Antiprotozoal	Dibenzimides	Interferes with nuclear synthesis/ interfering agent/ DNA, RNA, phospholipids and protein synthesis inhibitor
Auranofin	0.01	4.79	479	Antirheumatic	Gold agent	kappaB kinase and thioredoxin reductase inhibitor
Nifuroxazide	0.03	12.31	410	Antibacterial	Nitroheterocycles	Lipoamide dehydrogenase inhibition
Nitrofurantoin	0.5	90.31	181	Antibacterial	Nitroheterocycles	Oxygen-insensitive NADPH nitroreductase
Thioridazine	0.53	5.39	10	Antipsychotic/Antidepressant	Tricyclics	Dopamine D1 and D2 inhibitor
Amphotericin B	0.76	10.27	14	Antifungal/Antiprotozoal	Polyenes	Membrane cell sterol binder
Sertraline	0.77	8.10	11	Antipsychotic/Antidepressant	Tetrahydro-napthalenamines	Selective serotonin-reuptake inhibitors
Rifamycin SV	0.99	15.68	16	Antibacterial/Antituberculotic	Rifamycins	bacterial DNA-dependent RNA synthesis inhibitor
Paroxetine	1.13	13.84	12	Antipsychotic/Antidepressant	Dehydrophenyl-piperidines	Selective serotonin-reuptake inhibitors
Nortryptyline	1.17	27.87	24	Antipsychotic/Antidepressant	Tricyclics	Serotonin reuptake inhibitor
Triflupromazine	1.42	18.5	13	Antipsychotic/Antiemetic	Tricyclics	Dopamine D1 and D2 receptor inhibitors
Nifurtimox	1.44	87.02	60	Antibacterial/Antiprotozoal	Nitroheterocycles	Induction of oxidative stress in target cell
Clomipramine	2.06	19.79	10	Antipsychotic/Antidepressant	Tricyclics	Serotonin reuptake inhibitor
Promazine	2.16	30.06	14	Antipsychotic/Antidepressant	Tricyclics	Dopamine, serotonine, alpha1 and histamine receptor inhibitor
Amitriptyline	3.03	42.18	14	Antipsychotic/Antidepressant	Tricyclics	Norepinephrine and serotonin reuptake inhibitor
Chloroquine	3.81	50.61	13	Antimalarial	Quinolines	Heme polymerase inhibitor
Pizotifen	3.99	45.02	11	Antimigraine	Tricyclics	serotonin receptor antagonist

[a]*T. b. rhod.:T. b. rhodesiense* strain STIB 900, trypomastigotes. [b]Cytotoxicity on L6 cells. [c]Selectivity index: IC_{50} Cytotoxicity L6/ IC_{50} *T. b. rhodesiense*

IC_{50} values are means of two independent assays, which varied < ±50%.

Table 2. *In vitro* activity against *T. cruzi* in IC_{50} (μM) of compounds fulfilling hit criteria

Drug ID	[a]*T. cruzi*	[b]Cytotox. L6	[c]SI	Indication	Chemical Class	Mode of Action
Bifonazole	0.003	39.30	>1000	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Itraconazole	0.004	1.11	278	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Clotrimazole	0.006	2.99	498	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Miconazole	0.04	15.44	383	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Econazole	0.04	15.60	390	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Tioconazole	0.064	19.47	304	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Ketoconazole	0.27	50.99	189	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Fluconazole	9.96	>294	>30	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Nifurtimox	0.19	87.02	458	Antibacterial/Antiprotozoal	Nitroheterocycles	Induction of oxidative stress in target cell
Nifuroxazide	0.23	12.31	54	Antibacterial	Nitroheterocycles	Lipoamide dehydrogenase inhibition
Nitrofurantoine	4.35	90.31	21	Antibacterial	Nitroheterocycles	Oxygen-insensitive NADPH nitroreductase
Mebeverine	3.89	70.77	18	Antispasmotic	Phenylbenzoates	serotonin 5-HT3 receptor antagonist
Fluconazole	8.60	221.1	26	Erectile dysfunction	Pyridoiindolediones	cGMP-specific 3',5'-cyclic phosphodiesterase inhibitor

[a]*T. cruzi*, strain Tulahuen C4, intracellular amastigotes. [b]Cytotoxicity on L6 cells. [c]Selectivity index: IC_{50} Cytotoxicity L6/ IC_{50} *T. cruzi*. IC_{50} values are means of two independent assays, which varied < ±50%.

Table 3. *In vitro* activity against *L. donovani* in IC_{50} (μM) of compounds fulfilling hit criteria

Drug ID	[a]*L.don.* axen.	[b]*L. don.* intracell	[c]Cytotox. mac.inf.	[d]Cytotox. PMM	[e]SI	Indication	Chemical Class	Mode of Action
Auranofin	0.11	>1.47	4.42	N/A	40	Antirheumatic	Gold agent	kappaB kinase and thioredoxin reductase inhibitor
Amphotericin B	0.34	0.31	32.4	22.39	95	Antifungal/ Antiprotozoal	Polyenes	Membrane cell sterol binder
Ciclopirox olamine	1.64	9.09	20.3	20.27	12	Antifungal	Pyridinones	Polyvalent metal cation chelator
Tolnaftate	4.33	50.1	97.6	N/A	>23	Antifungal	Thiocarbamates	Squalene epoxidase inhibitor
Artesunate	0.35	>7.8	7.8	N/A	>22	Antimalarial	Endoperoxides	Unknown, acting *via* reactive oxygen radical species
Rifamycin SV	1.5	>13.87	41.62	N/A	28	Antibacterial/ Antituberculotic	Rifamycins	bacterial DNA-dependent RNA synthesis inhibitor
Rifampicin	1.53	>36.45	36.5	N/A	>24	Antibacterial/ Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Nitrofurantoine	2.12	>41.81	125.44	N/A	59	Antibacterial	Nitroheterocycles	Oxygen-insensitive NADPH nitroreductase
Nifurtimox	2.76	20.68	34.8	15.7	13	Antibacterial/ Antiprotozoal	Nitroheterocycles	Induction of oxidative stress in target cells
Troglitazone	4.26	>67.94	68	N/A	>16	Antidiabetic/ Antinflammatory	Thiazolidinediones	Nuclear receptor (PPAR) binder
Clofazimine	22.39	0.95	6.34	10.65	10	Antibacterial/ Antituberculotic	Riminophenazines	Mycobacterial DNA binder, Redox cycling, Cell membrane destabilizer, Acid sphingomyelinase inhibitor
Nifuroxazide	2.83	>10.86	36.2	N/A	13	Antibacterial	Nitroheterocycles	Lipoamide dehydrogenase inhibition
Tipranavir	1.64	>49.78	50	N/A	>30	Antiviral/ Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Lonidamine	8.66	>93.41	93.4	N/A	>11	Anticancer	Indazoles	Glycolysis inhibition *via* hexokinase activation

[a]*L. don.* axen.: axenic amastigotes of *L. donovani*, strain MHOM-ET-67/L82. [b]*L. don.* intracell: intracellular amastigotes of *L. donovani* strain MHOM-ET-67/L82. [c]Cytotoxicity on macrophages infected with *L. donovani*. [d]Cytotoxicity on peritoneal mouse macrophages. [e]Selectivity index: IC_{50} Cytotoxicity macrophages/ IC_{50} *L. donovani*. IC_{50} values are means of two independent assays, which varied < ±50%.

Table 4. *In vitro* activity against *P. falciparum* in IC_{50} (μM) of compounds fulfilling hit criteria

Drug ID	[a]*P. falc.* K1	[b]Cytotox. L6	[c]SI	Indication	Chemical Class	Mode of Action
Mefloquine	0.002	3.25	1354	Antimalarial	Quinolines	Unknown, putative heme polymerase inhibitor
Artesunate	0.003	0.78	260	Antimalarial	Endoperoxides	Unknown, acting *via* reactive oxygen radicals
Chloroquine	0.17	50.61	298	Antimalarial	Quinolines	Heme polymerase inhibitor
Tafenoquine	0.27	5.52	20	Antimalarial	Quinolines	Unknown, putative heme polymerase inhibitor
Sitamaquine	0.08	32.31	404	Antileishmanial	Quinolines	Unknown
Rifampicin	0.1	75.22	752	Antibacterial/Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Rifamycin SV	0.55	15.68	29	Antibacterial/Antituberculotic	Rifamycins	bacterial DNA-dependent RNA synthesis inhibitor
Rifaximin	0.92	88.05	96	Antibacterial/Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Amphotericin B	0.8	10.27	13	Antifungal/Antiprotozoal	Polyenes	Membrane cell sterol binder
Clotrimazole	0.11	2.99	27	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Econazole	0.32	15.6	49	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Miconazole	0.49	15.44	32	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Tioconazole	0.63	19.47	31	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Promazine	0.49	30.06	61	Antipsychotic/Antidepressant	Tricyclics	Dopamine, serotonin, alpha1 and histamine receptor inhibitor
Fluphenazine	0.50	11.54	23	Antipsychotic/Antidepressant	Tricyclics	Dopamine receptor inhibitor
Sertraline	0.51	8.10	16	Antipsychotic/Antidepressant	Tetrahydro-napthalenamines	Selective serotonin-reuptake inhibitors
Nortryptyline	0.58	27.87	48	Antipsychotic/Antidepressant	Tricyclics	Serotonin reuptake inhibitor
Ketotifen	0.75	147.04	196	Antihistamine	Cycloheptathio-phenones	H1-Histamine receptor antagonist
Cloperastine	0.87	43.35	50	Cough Suppressant	Phenylmethoxy-piperidines	Unknown
Rimantadine	0.97	311.2	321	Antiviral/Antiretroviral	Adamantanes	Matrix protein 2 inhibitor

[a]*P. falc.*: *P. falciparum* strain K1. [b]Cytotoxicity on L6 cells. [c]Selectivity index: IC_{50} Cytotoxicity L6/ IC_{50} *P. falciparum*.
IC_{50} values are means of two independent assays, which varied < ±50%.

Supporting Information

Table S1. Table 1. Set of 100 registered drugs tested for their antiparasitic activity.

Drug ID	Indication	Chemical Class	Mode of Action
Rimantadine	Antiviral/Antiretroviral	Adamantanes	Matrix protein 2 inhibitor
Amantadine	Antiviral/Antiretroviral	Adamantanes	Matrix protein 2 inhibitor
Terbinafine (Hydrochloride)	Antifungal	Allylamines	Squalene epoxidase inhibitor
Tioconazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Ketoconazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Bifonazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Satranidazole	Antibacterial/Antiprotozoal	Azoles	14alpha-sterol demethylase inhibitor
Secnidazole	Antibacterial/Antiprotozoal	Azoles	14alpha-sterol demethylase inhibitor
Ornidazole	Antibacterial/Antiprotozoal	Azoles	14alpha-sterol demethylase inhibitor
Itraconazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Clotrimazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Albendazole	Antihelmintic	Azoles	Tubulin polymerization inhibitor
Econazole (Nitrate salt)	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Voriconazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Miconazole (Nitrate salt)	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Fluconazole	Antifungal	Azoles	14alpha-sterol demethylase inhibitor
Omeprazole	Antiulcer agent	Azoles	Proton pump inihibitor
Spiperone	Antipsychotic/Antidepressant	Butyrophenones	Serotonin and dopamine receptor inhibitor
Bacitracine	Antibacterial	Cyclic polypeptides	Insulin-degrading enzym
Ketotifen	Antihistamine	Cycloheptathiophenones	H1-Histamine receptor antagonist
Paroxetine	Antipsychotic/Antidepressant	Dehydrophenylpiperidines	Selective serotonin-reuptake inhibitors
Pentamidine	Antibacterial/Antiprotozoal	Dibenzimides	Interfer nuclear synthesis interfering agent/ DNA, RNA, phospholipids and protein synthesis inhibitor

Table S1 continued

Drug ID	Indication	Chemical Class	Mode of Action
Dapsone	Antibacterial/Antileprotic	Diphenylsulfones	Dihydofolic acid synthesis inhibitor
Artesunate	Antimalarial	Endoperoxides	Unknown, acting via reactive oxygen radical species
Benfluorex	Anorectic/Hypolipidemic	Fenfluramines	Lipase stimulator; PPAR agonist
Auranofin	Antirheumatic	Gold agent	kappaB kinase and thioredoxin reductase inhibitor
Cimetidine	Antihistamine	Imidazoles	Histamine H2-receptor antagonist
Lonidamine	Anticancer	Indazoles	Glycolysis inhibition via hexokinase activation
Leflunomide	Antirheumatic	Isoxazoles	Unknown
Lincomycin	Antibacterial	Lincosamides	Ribosomal protein synthesis inhibitor
Erythromycine (Hydrate)	Antibacterial	Macrolides	Ribosomal protein synthesis inhibitor
Nitrofurantoine	Antibacterial	Nitroheterocycles	Oxygen-insensitive NADPH nitroreductase
Nifuroxazide	Antibacterial	Nitroheterocycles	Lipoamide dehydrogenase inhibition
Metronidazole	Antibacterial/Antiparasitic	Nitroheterocycles	Oxygen-insensitive NADPH nitroreductase
Nifurtimox	Antibacterial/Antiprotozoal	Nitroheterocycles	Induction of oxidative stress in target cell
Tinidazole	Antibacterial/Antiprotozoal	Nitroheterocycles	DNA damaging via reactive intermediates
Niclosamide	Antiparasitic/Anthelmintic	Nitroheterocycles	oxidative phosphorylation uncoupler
Zidovudine	Antiviral/Antiretroviral	Nucleosides	Nucleoside reverse transcriptase inhibitor
Stavudine	Antiviral/Antiretroviral	Nucleosides	Nucleoside reverse transcriptase inhibitor
Fluoxetine	Antipsychotic/Antidepressant	Phenlyphenoxypropanamine	Selective serotonin-reuptake inhibitors
Mebeverine	Antispasmotic	Phenylbenzoates	serotonin 5-HT3 receptor antagonist
Cloperastine	Cough Suppressant	Phenylmethoxypiperidines	Unknown
Triamterene	Diuretic	Phenylpteridines	Epithelial sodium channel inhibitor
Cetirizine (Hydrochloride)	Antihistamine	Phenylpyperazinylaceticacids	Histamine H1-receptor inhibitor
Amphotericin B	Antifungal/Antiprotozoal	Polyenes	Membrane cell sterol binder
Indinavir (Sulfate)	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor

Table S1 continued

Drug ID	Indication	Chemical Class	Mode of Action
Ritonavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Amprenavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Tipranavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Ganciclovir	Antiviral/Antiretroviral	Protease Inhibitors	Thymidine kinase activator, DNA polymerase inhibitor
Atazanavir (Sulfate)	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Saquinavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Darunavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Lopinavir	Antiviral/Antiretroviral	Protease Inhibitors	HIV protease inhibitor
Nelfinavir	Antiviral/Antiretroviral	Protease inhibitors	HIV protease inhibitor
Famciclovir	Antiviral/Antiretroviral	Protease inhibitors	Thymidine kinase activator, DNA polymerase inhibitor
Penciclovir	Antiviral/Antiretroviral	Protease inhibitors	Thymidine kinase activator, DNA polymerase inhibitor
Pyrazinamide	Antibacterial/Antituberculotic	Pyrazines	Fatty acid synthetase I inhibitor
Izoniazide	Antibacterial/Antituberculotic	Pyridines	Micolic acid synthesis inhibition
Isoniazide	Antibacterial/Antituberculotic	Pyridines	Miclic acid synthesis inhibition
Nicotinamide	Vitamin	Pyridines	(N/A)
Ciclopirox olamine	Antifungal	Pyridinones	Polyvalent metal cations chelator
Tadalafil	Erectile dysfunction	Pyridoiindolediones	cGMP-specific 3',5'-cyclic phosphodiesterase inhibitor
Pyrimethamine	Antimalarial	Pyrimidines	Dihydrofolate reductase inhibitor
Pirenperone	Antipsychotic/Antidepressant	Pyrimidinones	Serotonin 5-HT 1a receptor agonist
Dipyridamole	Antithrombotic	Pyrimidopyrimidines	cGMP-specific 3',5'-cyclic phosphodiesterase

Table S1 continued

Drug ID	Indication	Chemical Class	Mode of Action
Tafenoquine	Antimalarial	Quinolines	Unknown, putatively heme polymerase inhibitor
Mefloquine (Hydrochloride)	Antimalarial	Quinolines	Unknown, putatively heme polymerase inhibitor
Primaquine	Antimalarial/Antiprotozoal	Quinolines	Unknown, putatively reactive oxygen species or electron transport interference
Sitamaquine	Antileishmanial	Quinolines	Unknown
Chloroquine (Diphosphate)	Antimalarial	Quinolines	Heme polymerase inhibitor
Ciprofloxacin	Antibacterial	Quinolones	DNA gyrase subunit A inhibitor
Enoxacin	Antibacterial	Quinolones	DNA gyrase subunit A inhibitor
Rifabutin	Antibacterial/Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Rifampicin	Antibacterial/Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Rifaximin	Antibacterial/Antituberculotic	Rifamycins	Bacterial DNA-dependent RNA synthesis inhibitor
Rifamycin SV (Sodium salt)	Antibacterial/Antituberculotic	Rifamycins	bacterial DNA-dependent RNA synthesis inhibitor
Clofazimine	Antibacterial/Antituberculotic	Riminophenazines	Mycobacterial DNA binder, Redox cycling, Cell membrane destabilizer, Acid sphingomyelinase inhibitor
Silver sulfadiazine	Antibacterial	Silver agent	Cell membane interfering agent
Griseofulvin	Antifungal	Spirobenzofuranediones	Tubulin binder
Danazol	Endomitriosis	Steroids	Gonadotropin inhibitor
Ganaxolone	Anesthetic	Steroids	GABAA receptor modulator
Glybenclamide	Antidiabetic	Sulfonylureas	Sulfonylurea receptor 1 activator
Doxycycline	Antibacterial	Tetracyclines	Ribosomal protein synthesis inhibitor

Table S1 continued

Drug ID	Indication	Chemical Class	Mode of Action
Minocycline	Antibacterial	Tetracyclines	Ribosomal protein sythesis inhibitor
Sertraline	Antipsychotic/Antidepressant	Tetrahydronapthaleneamines	Selective serotonin-reuptake inhibitors
Troglitazone	Antidiabetic/ Antinflammatory	Thiazolidinediones	Nuclear receptors (PPAR) binder
Tolnaftate	Antifungal	Thiocarbamates	Squalene epoxidase inhibitor
Clomiphene	Fertility agent	Triarylethylenes	Estrogen receptor inhibitor
Thioridazine	Antipsychotic/Antidepressant	Tricyclics	Dopamine D1 and D2 inhibitor
Triflupromazine	Antipsychotic/Antiemetic	Tricyclics	Dopamine D1 and D2 receptor inhibitors
Amoxapine	Antipsychotic/Antidepressant	Tricyclics	Selective serotonin-reuptake inhibitors
Fluphenazine	Antipsychotic/Antidepressant	Tricyclics	Dopamine receptor inibitor
Clomipramine	Antipsychotic/Antidepressant	Tricyclics	Serotonin reuptake inhibitor
Nortryptyline	Antipsychotic/Antidepressant	Tricyclics	Serotonin reuptake inhibitor
Promazine	Antipsychotic/Antidepressant	Tricyclics	Dopamine, serotonine, alpha1 and histamine receptor inhibitor
Chlorpromazine	Antipsychotic/Antidepressant	Tricyclics	Dopamine, serotonin alpha1/2 and histamine receptor antagonist
Amitriptyline	Antipsychotic/Antidepressant	Tricyclics	Norepinephrine and serotonin reuptake inhibitor
Trifluoperazine	Antipsychotic/Antiemetic	Tricyclics	Dopamine D1 and D2 receptor inhibitors
Pizotifen	Antimigraine	Tricyclics	serotonin receptor antagonist

Table S2. Table 2. *In vitro* activity profile in IC_{50} (μM) of all tested compounds.

Drug ID	[a]T. b. rhod.	[b]T. cruzi	[c]L. don. axen.	[d]L. don. intracell	[e]P. falc.	[f]Cytotox. L6	[g]Cytotox. mac.inf.	[h]Cytotox. PMM
Melarsoprol	**0.01**							
Benznidazole		**1.99**						
Miltefosine			**0.44**	**1.21**				
Chloroquine					**0.13**			
Podophyllotoxin						**0.017**		
Rimantadine	13.83	320.68	427.2	>167.31	0.97	311.2	>167.31	N/A
Amantadine	52.76	371.3	>595.03	>198.34	5.67	214.87	>198.34	N/A
Terbinafine (Hydrochloride)	95.15	40.87	23	69.54	7.72	46.97	>91.49	N/A
Tioconazole	29.92	0.064	9.26	13.1	0.63	19.47	77.38	51.07
Ketoconazole	33.12	0.27	38.76	>18.82	N/A	50.99	56.45	N/A
Bifonazole	26.8	0.003	5.48	>32.22	3.18	39.3	>32.22	N/A
Satranidazole	18.7	19.08	10.41	>103.71	N/A	>311.13	>103.71	N/A
Secnidazole	28.68	>161.12	>161.12	>161.12	N/A	>483.37	>161.12	N/A
Ornidazole	25.93	130.08	>135.97	>135.97	N/A	>407.91	>135.97	N/A
Itraconazole	8.53	0.004	2.42	>1.42	1.37	1.11	4.25	N/A
Clotrimazole	23.69	0.006	2.23	14.67	0.11	2.99	87	32.48
Omeprazole	51.82	68.32	90.9	>86.85	13.23	125.64	>86.85	N/A
Albendazole	14.7	12.32	>113.06	>3.77	N/A	0.41	11.31	N/A
Econazole (Nitrate salt)	41.7	0.04	6.15	13.97	0.32	15.6	67.62	22.82
Voriconazole	>286.27	11.25	>286.27	>85.88	>143.14	194.95	>85.88	N/A
Miconazole (Nitrate salt)	36.6	0.04	4.39	10.79	0.49	15.44	62.74	23.89
Fluconazole	273.28	9.96	210.59	>97.95	N/A	>293.85	>97.95	N/A
Spiperone	22.35	64.58	>227.57	>75.86	1.76	54.36	>75.86	N/A
Bacitracine	>70.29	47.09	>70.29	>21.09	19.75	42.24	>21.09	N/A

Table S2 continued

Drug ID	[a]T. b. rhod.	[b]T. cruzi	[c]L. don. axen.	[d]L. don. intracell	[e]P. falc.	[f]Cytotox. L6	[g]Cytotox. mac.inf.	[h]Cytotox. PMM
Ketotifen	15.19	141	>290.86	>96.95	0.75	147.04	>96.95	N/A
Paroxetine	1.13	14.36	>273.25	>9.11	9.23	13.84	30.36	N/A
Pentamidine	0.01	4.44	19.77	2.63	N/A	8.87	>29.37	7.78
Dapsone	352.79	219.89	>402.73	>120.82	167.54	203.78	>120.82	N/A
Artesunate	16.78	8.97	0.35	>7.8	0.003	0.78	>7.8	N/A
Benfluorex	21.06	73.71	243.05	54.36	6.52	49.15	>85.38	N/A
Auranofin	0.01	2.27	0.11	>1.47	1.67	4.79	4.42	N/A
Cimetidine	>396.28	351.11	>396.28	>118.89	29.17	301.57	>118.89	N/A
Lonidamine	134.82	177.48	8.66	>93.41	>15.57	273.07	>93.41	N/A
Leflunomide	212.8	152.1	21.76	>111.02	>18.5	123.98	>111.02	N/A
Lincomycin	>221.38	>221.38	>221.38	>73.79	7.08	>221.38	>73.79	N/A
Erythromycine (Hydrate)	72.08	113.97	>132.99	>39.9	9.91	92.16	>39.9	N/A
Nitrofurantoine	0.5	4.35	2.12	>41.81	N/A	90.31	125.44	N/A
Nifuroxazide	0.03	0.23	2.83	>10.86	7.82	12.31	36.2	N/A
Metronidazole	337.7	278.69	491.36	>175.28	276.94	338.87	>175.28	N/A
Nifurtimox	1.44	0.19	2.76	20.68	N/A	87.02	>34.81	15.7
Tinidazole	16.57	>80.95	>80.95	>80.95	N/A	>242.86	>80.95	N/A
Niclosamide	1.67	2.49	0.15	>0.31	1.14	2.3	1.01	N/A
Zidovudine	139.57	155.66	>374.19	>112.26	174.75	206.93	>112.26	N/A
Stavudine	119.53	340.74	>446	>133.8	186.43	266.26	>133.8	N/A
Fluoxetine	2.01	19.14	>290.95	>9.7	1.21	14.97	32.33	N/A
Mebeverine	54.24	3.89	>209.52	54.94	1.74	70.77	>69.84	N/A
Cloperastine	4.91	9.46	265.26	>30.31	0.87	43.35	90.94	N/A
Triamterene	21.68	22.74	306.79	>11.85	17.21	8.53	>11.85	N/A

Table S2 continued

Drug ID	[a]T. b. rhod.	[b]T. cruzi	[c]L. don. axen.	[d]L. don. intracell	[e]P. falc.	[f]Cytotox. L6	[g]Cytotox. mac.inf.	[h]Cytotox. PMM
Cetirizine (Hydrochloride)	99.45	119.9	186.43	>70.53	19.98	148.82	>70.53	N/A
Amphotericin B	0.76	56.69	0.34	0.31	0.8	10.27	32.4	22.39
Indinavir (Sulfate)	48.19	93.55	>140.89	>42.27	6.03	>140.89	>42.27	N/A
Ritonavir	4.3	20.67	8.97	>41.61	16.23	37.73	>41.61	N/A
Amprenavir	28.48	105.21	112.53	>59.33	15.17	159.8	>59.33	N/A
Tipranavir	26.38	28.21	1.64	>49.78	44.63	53.43	>49.78	N/A
Ganciclovir	251.53	240.17	>391.8	>117.54	>195.9	179.44	>117.54	N/A
Atazanavir (Sulfate)	12.74	33.34	24.1	>37.46	7.95	46.45	>37.46	N/A
Saquinavir	12.27	17.14	77.36	>44.72	11.94	18.19	>44.72	N/A
Darunavir	31.41	92.21	125.26	>54.78	46.93	155.57	>54.78	N/A
Lopinavir	10.81	15.38	11.83	>47.71	1.92	18.92	>47.71	N/A
Nelfinavir	20.61	10.23	9.62	20.25	10.44	12.05	>52.84	N/A
Famciclovir	225.31	206.95	>311.2	>93.36	>155.6	179.87	>93.36	N/A
Penciclovir	>394.85	254.28	>394.85	>118.45	169.39	219.54	>118.45	N/A
Pyrazinamide	>812.28	564.54	>812.28	>243.68	178.7	458.13	>243.68	N/A
Nicotinamide	>736.94	>736.94	>736.94	>245.65	>40.94	>736.94	>245.65	N/A
Izoniazide	>656.26	>656.26	>656.26	>218.75	>36.46	>656.26	>218.75	N/A
Isoniazide	>729.18	382.82	>729.18	>218.75	246.46	602.3	>218.75	N/A
Ciclopirox olamine	0.8	2.49	1.64	9.09	3.75	1.04	>11.18	20.27
Tadalafil	97.58	8.6	40.06	>77.04	>12.84	221.1	>77.04	N/A
Pyrimethamine	6.75	5.71	277.83	>12.06	9.85	2.51	>12.06	N/A
Pirenperone	29.99	128.35	161.57	>76.25	4.35	232.3	>76.25	N/A
Dipyridamole	7.45	29.33	62.02	>59.45	1.3	38.64	>59.45	N/A
Tafenoquine	1.42	6.11	17.91	2.16	0.27	5.52	6.47	N/A

Table S2 continued

Drug ID	[a]T. b. rhod.	[b]T. cruzi	[c]L. don. axen.	[d]L. don. intracell	[e]P. falc.	[f]Cytotox. L6	[g]Cytotox. mac.inf.	[h]Cytotox. PMM
Mefloquine (Hydrochloride)	0.53	4.48	38.58	2.41	0.0024	3.25	7.23	N/A
Primaquine	4.78	17.85	48.58	28.19	N/A	43.18	>38.56	39.72
Sitamaquine	7.39	21.98	13.65	19.48	0.08	32.31	>29.11	21.37
Chloroquine (Diphosphate)	3.81	99.25	>196.15	>58.85	0.17	50.61	>58.85	N/A
Ciprofloxacin	35.91	92.05	>301.8	>90.54	13.19	118.01	>90.54	N/A
Enoxacin	113.01	162.65	>312.18	>93.66	76.8	25.01	>93.66	N/A
Rifabutin	12.61	27.51	56.43	>35.42	1.59	60.92	>35.42	N/A
Rifampicin	16.74	>109.36	1.53	>36.45	0.1	75.22	>36.45	N/A
Rifaximin	17.14	40.21	15.01	>38.17	0.92	88.05	>38.17	N/A
Rifamycin SV (Sodium salt)	0.99	49.95	1.5	>13.87	0.55	15.68	41.62	N/A
Clofazimine	7.63	38.23	22.39	0.95	4.1	9.97	6.34	10.65
Silver sulfadiazine	18.45	24.87	125.57	>12.04	1.44	20.62	40.12	N/A
Griseofulvin	39.97	87.31	87.03	>85.04	75.12	20.52	>85.04	N/A
Danazol	45.93	6.25	10.16	>29.63	14.13	32	>29.63	N/A
Ganaxolone	5.47	64.96	42.46	>90.22	>15.04	23.49	>90.22	N/A
Glybenclamide	87.65	93.93	9.8	>60.73	38.26	116.19	>60.73	N/A
Doxycycline	63.68	39.15	24.08	>22.5	4.48	14.49	67.5	N/A
Minocycline	36.55	22.3	45.68	>21.86	3.58	9.42	>21.86	N/A
Sertraline	0.77	6.76	86.4	>9.8	0.51	8.1	32.65	N/A
Troglitazone	59.34	32.61	4.26	>67.94	7.27	80.63	>67.94	N/A
Tolnaftate	69.29	19.97	4.33	50.1	18.99	84.58	>97.59	N/A
Clomiphene	6.21	11.45	21.6	>7.39	1.37	13.06	24.63	N/A
Thioridazine	0.53	5.83	22.41	>7.74	1.07	5.39	25.79	N/A
Triflupromazine	1.42	15.01	79.79	>8.51	2.72	18.5	28.38	N/A

Table S2 continued

Drug ID	[a]T. b. rhod.	[b]T. cruzi	[c]L. don. axen.	[d]L. don. intracell	[e]P. falc.	[f]Cytotox. L6	[g]Cytotox. mac.inf.	[h]Cytotox. PMM
Amoxapine	1.87	17.94	>286.82	>9.56	1.14	16.16	31.87	N/A
Fluphenazine	2.03	12.98	46.69	>6.86	0.5	11.54	22.86	N/A
Clomipramine	2.06	20.29	75.68	>9.53	1.3	19.79	31.76	N/A
Nortryptyline	1.17	22.89	>341.71	>11.39	0.58	27.87	37.97	N/A
Promazine	2.16	49.15	>316.43	>35.16	0.49	30.06	>35.16	N/A
Chlorpromazine	1.25	17.15	48.45	>9.41	3.03	12.32	31.36	N/A
Amitriptyline	3.03	35.11	131.25	>10.81	1.16	42.18	36.05	N/A
Trifluoperazine	1.23	10.13	52.93	>7.36	1.48	10.99	24.54	N/A
Pizotifen	3.99	30.63	215.03	>33.85	1.86	45.02	>101.54	N/A

[a]*T. b. rhodesiense* strain STIB 900, trypomastigotes. [b]*T. cruzi*, strain Tulahuen C4, intracellular amastigotes. [c]*L. don*. axen.: axenic amastigotes of *L. donovani*, strain MHOM-ET-67/L82. [d]*L. don*. intracell: intracellular amastigotes of *L. donovani* strain MHOM-ET-67/L82. . [e]*P. falc*.: *P. falciparum* strain K1. [f]Cytotoxicity on L6 cells. [g]Cytotoxicity on macrophages infected with *L. donovani*. [h]Cytotoxicity on peritoneal mouse macrophages
IC_{50} values are means of two independent assays, which varied < ±50%.

Figure S1. HAT cluster of all tested compounds. Chemical class vs log (IC_{50} in μM)

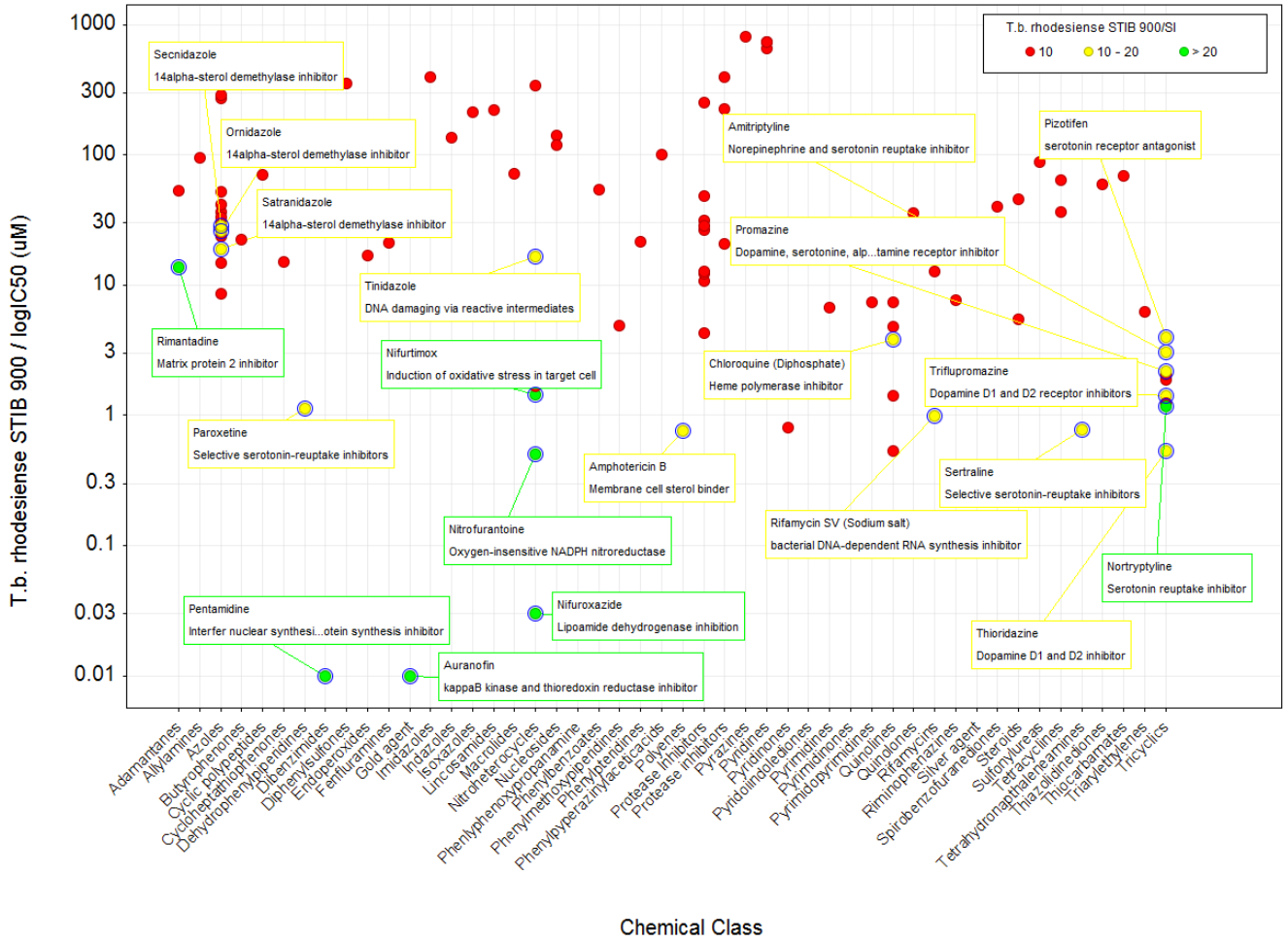


Figure S2. HAT cluster of antidepressant and antipsychotics. Chemical class vs log(IC_{50} in μM)

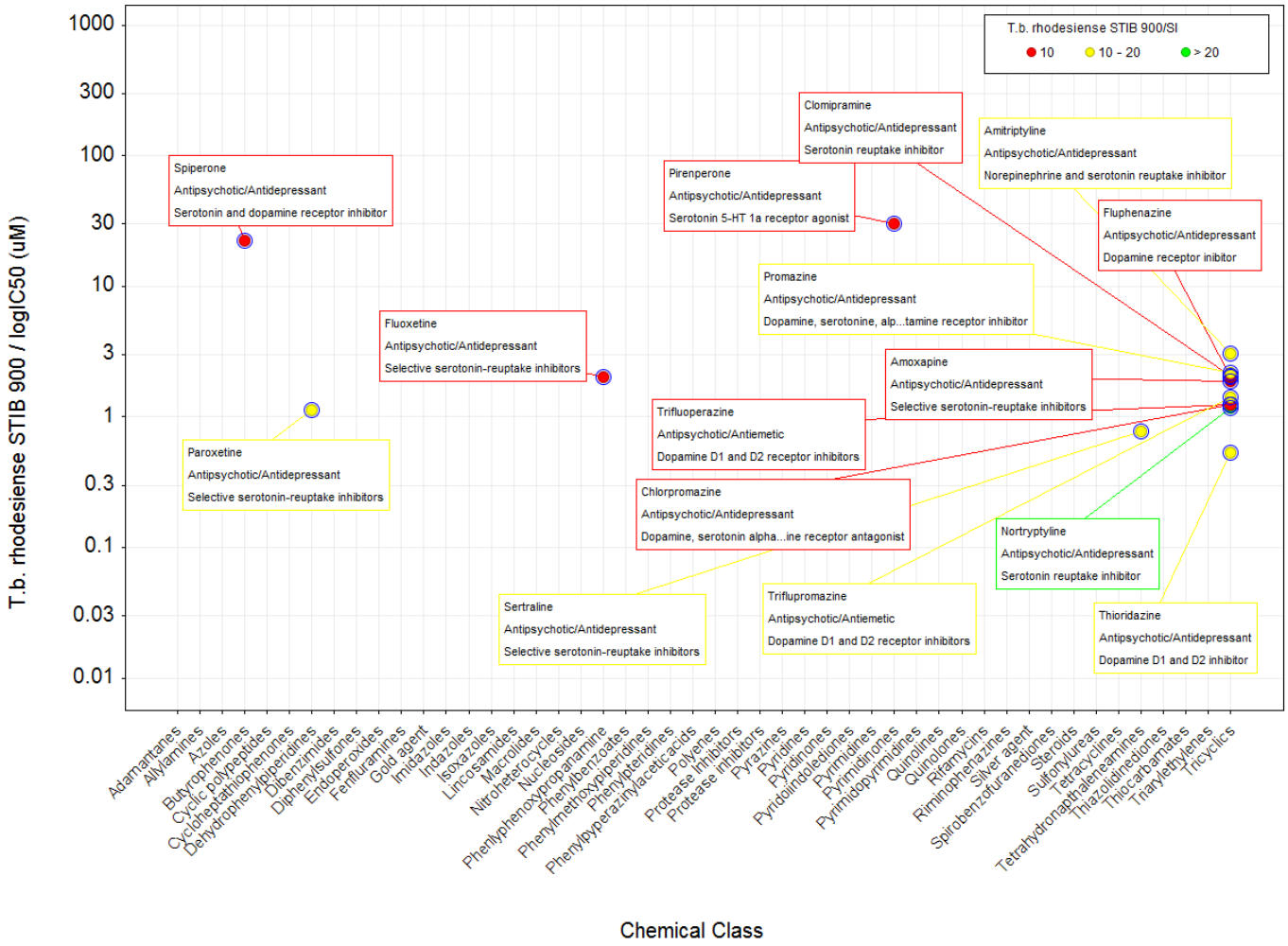


Figure S3. Chagas disease cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)

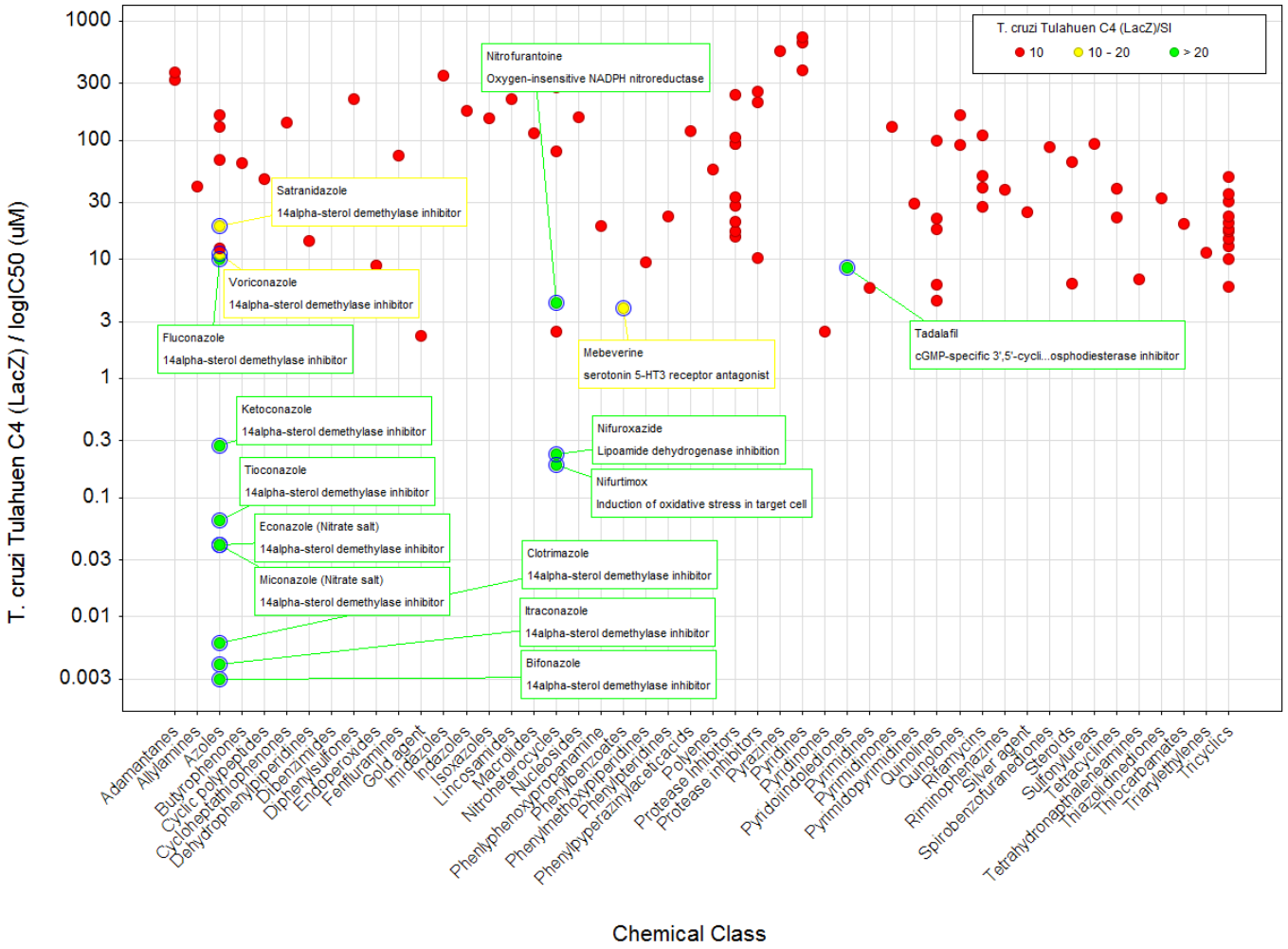


Figure S4. Leishmaniasis cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)

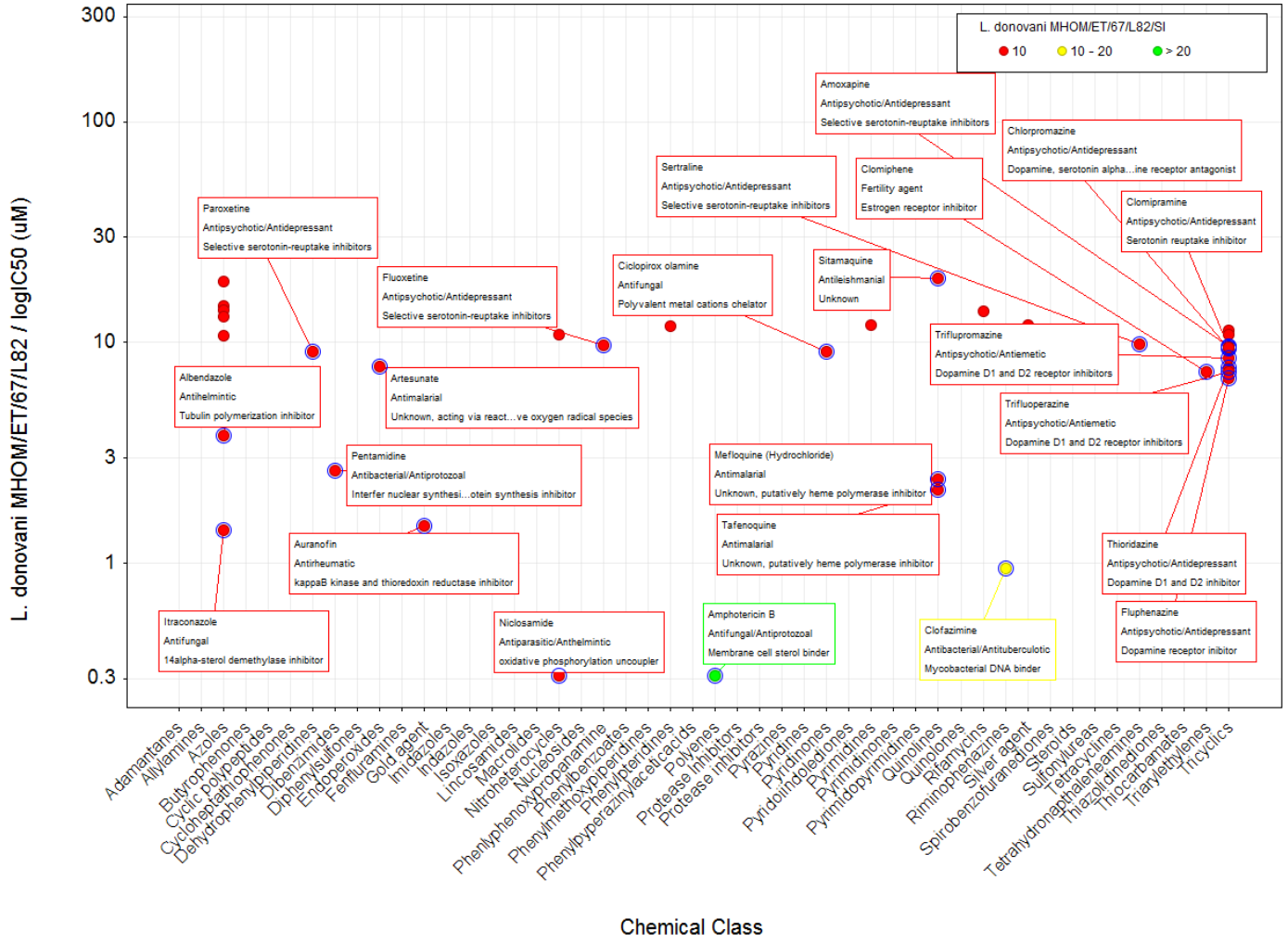
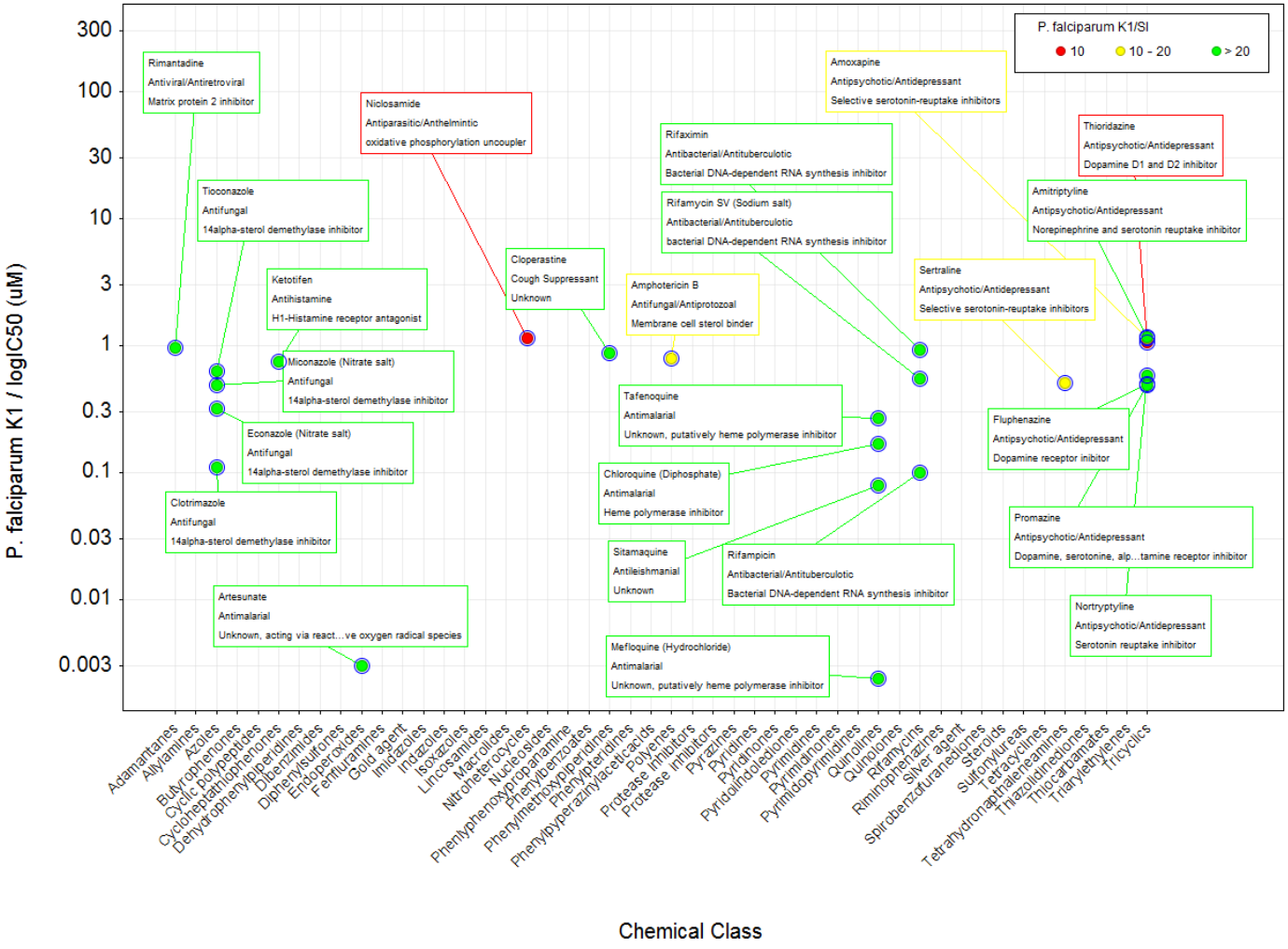


Figure S5. Malaria cluster of all tested compounds. Chemical class vs log(IC_{50} in μM)



CHAPTER 10

General Discussion

Drug discovery for neglected diseases: Why and How?

The neglected tropical diseases (NTDs) [1,2,3,4] are a group of communicable diseases which are highly endemic in low income countries of Africa, Asia and America. The NTDs include more than 10 infectious diseases, most of which parasitic infections [2,5]. The NTDs cause a large part of the global morbidity, mortality and poverty [2]. More than 1 billion are affected and hundred thousands of people die by an NTD every year. The burden of a disease is measured in DALYs (disability-adjusted life years). The number of DALYs gives an estimate of the sum of years of potential life lost due to mortality and the years of productivity life lost. It is estimated that NTDs cause 20 million DALYs. [2,6].

There is no doubt that the welfare of people in the developing world can only be improved if the NTDs are controlled. WHO recommends five strategies for the prevention and control of NTDs: (i) preventive chemotherapy; (ii) intensified case-management; (iii) vector control; (iv) provision of safe water, sanitation and hygiene; and (v) veterinary public health [7]. Chemotherapies of most NTDs are unsatisfactory, due to limited number of drugs, lack of efficacy, significant side effects or the affordability of the drugs. This fact is especially true for the protozoan diseases, Chagas' disease, leishmaniasis and human African trypanosomiasis (HAT) [8,9,10,11]. There is a broad consensus that new, more efficient, non-toxic, easy to be administered and cost-effective drugs are urgently needed.

Essential in drug discovery is the identification of starting points. Paul Ehrlich (1854 – 1915) was one of the first chemists who systematically tested compounds for their antibacterial and antiprotozoal effect. With phenotypic screening he detected the antitrypanosomal activity of trypan red [12] and the antimalarial activity of methylene blue [13]. It can be said that the modern drug discovery starts with Paul Ehrlich [14]. Phenotypic screening was for a long time the only approach to discover new drugs.

Only in 1995, with the whole genome sequencing of bacteria [15], a new era was launched in drug discovery. The molecular approach, also called target-based approach, was heralded. The target-based approach is hypothesis-driven with the initial step of identification and validation of a potential target. The first rationally developed drugs are HIV protease inhibitors (Saquinavir). In the meantime several parasite genomes have been sequenced and a number of targets were identified and proved to be essential e.g. dehydrofolate reductase (DHFR) [16,17], cyclin-dependent kinase [18] or sterol synthesis [19,20]. Despite all the identified targets, the target based screening approach to drug discovery for neglected diseases is still in the early discovery phase. Parasites are perfectly adapted to the host and obtain nutrients and other vital substances from their host. Putative targets that are essential in the in vitro cultured parasites need not be absolutely essential for the parasite in the host; there could be redundancy between biosynthesis and import. The more proteins a pathogen has, the lower is the percentage of essential ones and the harder it is to identify suitable targets. These aspects could be explanations why an effect on a target does not translate into an effect on the parasite. It seems that the target-based approaches are not the most successful strategy for drug discovery against NTDs [21]. The pragmatic way of phenotypic screening is still a very valuable alternative. In this thesis not only was the approach of phenotypic testing followed, it was also tried to implement the piggyback [22] approach and the advice of Sir Black "...to start with an old drug" [23]. Three different series of compounds (agrochemicals, marketed drugs and nitroimidazoles) were screened to identify new lead compounds or even clinical candidates against three neglected diseases Chagas' disease, leishmaniasis, and human African trypanosomiasis.

Agrochemicals against neglected tropical diseases

Agrochemicals are used worldwide on a large scale in food production. For registration the agrochemicals must fulfill stringent requirements, e.g. it is not accepted that the chemicals get into the food chain either in the diet or in the drinking water [24]. That means that all agrochemicals on the market were subjected to rigorous toxicological screening. Agrochemicals are highly optimized for selectivity and the mode of action is well understood. For economic reasons the production of agrochemicals must be very cheap. All these aspects are also important for drugs against neglected tropical diseases. Nevertheless agrochemicals have so far not been systematically screened for their anti-parasitic activity. Only few studies can be found in the literature in which agrochemicals

were tested against parasites. Bajsa et al (2007) [25] tested herbicides against *P. falciparum* and describe some compounds which could be lead structure for optimization.

Recently the plant fungicide fenarimol belonging to the pyrimidinylcarbinols was described to show activity against *T. cruzi* [26]. This compound was identified by testing a set of diverse agrochemicals against *T. cruzi*. Fenarimol inhibits ergosterol formation by inhibiting 14α-demethylase [27]. Feranimol was taken as a lead structure and new analogues were synthesized to successfully improve the in vitro and in vivo activity [23].

Within a PPP we tested a series of over 600 agrochemicals against the 4 protozoan parasites *P. falciparum*, *T. b. rhodesiense*, *T. cruzi*, and *L. donovani* (Chapter 8) This screening campaign resulted in a number of new hits with submicromolar activity (Fig 1): 24 hits against *P. falciparum*, 7 hits against *T. b. rhodesiense*, 2 hits against *L. donovani* and 38 hits against *T. cruzi*. The two strobilurins, fluacryprym and azostrobin, showed, with IC50's of 8.2 nM and 15 nM, respectively, the best in vitro activity against *P. falciparum*. Strobilurins inhibit cell respiration by the interruption of electron transport in the mitochondrial respiratory chain at the cytochrome bc1 complex [29]. Azostrobin is not toxic (LD50 rat p.o. >5000mg/kg); it is a broad-spectrum fungicide and oomyceticide with annual sales of >1bn Euro and a production volume of several 1000 tons per year. Azoxystrobin has also been identified in a high throughput screening campaign of GlaxoSmithKline, where it showed an IC50 value of 41 nM against *P. falciparum* (ChEMBL website). In the *P. berghei* mouse model azostrobilin showed moderate activity. The trifluoromethyl aminohydrazone compound hydramethylnon is an insecticide and showed good in vitro activity against *P. falciparum*. This insecticide reduced the parasitemia of *P. berghei* infected mice and significantly prolonged their survival. Hydramethylnon is an inhibitor of the respiration chain too, but probably not of the bc1 complex [30].

Zoxamide, a broadspectrum oomyceticide, showed in vitro activity against all three trypanosomatid parasites. It had an IC50 of 6 nM against *T. b. rhodesiense*, 27 nM against *T. cruzi* and an 250 nM against *L. donovani*. The mode of action of zoxamide is the inhibition of nuclear division and the destruction of the microtubule cytoskeleton [31]. Zoxamide showed only little activity in the acute *T. b. rhodesiense*

mouse model. The fungicide tolylfluanid was active against *T. b. rhodesiense* (IC50 of 52 nM) and against *L. donovani* (IC50 of 861 nM).

38 compounds showed sub-μM activity against *T. cruzi*. Most of these compounds belong to the compound class of azoles inhibiting sterol 14a-demethylase, a target of *T. cruzi* [32]. Ipconazole, clotrimazole and viniconazole showed IC50's <30 nM against *T. cruzi*. Non-azoles such as pyridaben and tolfenpyrad inhibitors of the complex I in the mitochondrial electron transport chain showed also in vitro activity. In spite of this excellent in vitro activity, the selected hits did not show efficacy in a *T. cruzi* mouse model.

Agrochemicals are not optimized for use in mammals. Nevertheless there was a considerable number of molecules with good and selective in vitro activity and some were also active in the corresponding rodent model. These results demonstrate that agrochemicals can be very interesting new leads, and maybe even drug candidates against protozoan diseases.

The results also suggest that the agrochemicals should be tested against other neglected pathogens like schistosomes, nematodes, food-born trematodes, amoebae and bacteria for which good drugs are missing.

Approved Drugs

One strategy to identify new drug candidates for NTDs is the screening of libraries of diverse compounds for the identification of novel scaffolds. However, the restriction to drug or drug-like compounds is an ideal starting point because substantial pharmacokinetic and toxicological data are available for approved drugs. The success of this approach has been confirmed several times [33,34,35,36], but these screening campaigns focused on a single parasite. Here, a series of approved drugs including antibiotic, antiviral, antifungal and antipsychotic drugs was evaluated in an integrated screening against the three trypanosomatid parasites (Chapter 9).

From the 23 tested antibiotics nifuroxazide and nitrofurantoin showed activity against *T . b. rhodesiense* and *T. cruzi*. Both belong to the class of nitro-compounds. Clofazimine, used in leprosy therapy, was active against axenic amastigotes and intracellular amastigotes of *L. donovani*. It also exhibited some cytotoxicity against L6 cells and mouse macrophages. Clofazimine inhibits cell proliferation [37] and it is also

an inhibitor of acid sphingomyelinase [38]. Ryfamacins showed remarkable selective activity in the antiplasmodial assay. Especially the antituberculosis drug rifampicin was highly active.

Psychoactive compounds are an interesting class of compounds because they cross the blood-brain barrier. This feature is important for curing the second stage of HAT. Thioridazine, trifluprom azine, chlorpromazine, nortriptylene, paroxetine and sertraline showed moderate activity and selectivity against *T. b. rhodesiense*. Another interesting result is the antimalarial activity of the tricyclic antidepressants. Thioridazine is a piperidine compound that inhibits CYP1A2 and CYP3A2 [39]. The three related drugs thioridazine, trifluprom azine and chlorpromazine are D2 dopamine receptor antagonists and Ca2+ channel blockers. Nortriptylene inhibits reuptake of norepinephrine and sertraline is a tricyclic serotonin reuptake inhibitor [40].

Of the tested anti-fungal drugs, all which showed activity against *T. b. rhodesiense*, *T. cruzi* and *P. falciparum* belonged to the class of azoles; these interfere with the biosynthesis of ergosterol by inhibiting cytochrome P450-dependent enzymes [41]. The hydroxypyridinone antifungal ciclopirox olamine showed activity against axenic amastigotes and intracellular amastigotes of *L. donovani* with a limited selectivity.

Two other compounds, tadalafil and meberverine, were in vitro activity against T. cruzi. Tadalafil is a phosphodiesterase type 5 (PDE5) inhibitor used in treating erectile dysfunction. The antispasmodic mebeverine is used as treatment of the irritable bowel syndrome (IBS) and the associated abdominal cramping. It works by relaxing the muscles in and around the gut.

Thus a number of the tested approved drugs showed selective antiparasitic activity. Some of these activities have already been reported through literature, but not the activity of tadalafil and mebeverine against T. cruzi and the antimalarial activity of the rifamycins. The results confirm that this low hanging-fruit approach is worthwhile to follow up, especially when R&D has to be addressed with limited resources. These active compounds and compound classes respectively should now be further characterized and tested in the corresponding rodent models. The novel scaffolds could also be a starting point for medicinal chemistry programs.

Nitrotriazoles

The current treatments for Chagas disease are two nitroheterocyclic compounds, benznidazole and nifurtimox [8]. Both drugs have side effects, limited efficacy, and a long treatment is needed and some *T. cruzi* strains are refractory to treatment. Benznidazole and nifurtimox were introduced over 40 year ago. Currently the most promising antichagasic drug candidates are ergosterol biosynthesis inhibitors. *T. cruzi* requires endogenous sterols for its survival and proliferation because it is not able to use the cholesterol available in mammalian hosts [42]. In the past years several azole derivatives blocking ergosterol biosynthesis were described [42,43]. These compounds are inhibitors of the cytochrome P-450-dependent C14α sterol demethylase (CYP51). Two of these azoles, posaconazole and E1224 (a pro-drug of ravuconazole), are in Phase II clinical trials for the treatment of chronic Chagas disease [44,45]. Unfortunately the production costs of both compounds are very high, and maybe prohibit their use in poor countries. New cheap and efficacious drugs are urgently needed.

The initial series of compounds tested were originally designed as a DNA-targeting anticancer drug [46,4]. The first tested compounds showed activity against *T. cruzi* without overt cytotoxicity. One of the initially screened compounds showed also some activity in an acute *T. cruzi* mouse model [48]. This topoisomerase I and II inhibitor demonstrated also in vivo toxicity in mice. Due to the promising first results the development of less toxic and more efficacious nitrotriazole- and nitroimidazole-based compounds was initiated. A first set of compounds was evaluated against *T. b. rhodesiense*, *T. cruzi* and *L. donovani* (Chapter 5). Six compounds were active against *T. b. rhodesiense*, one compound was active against *L. donovani*, and 18 compounds showed selective activity against *T. cruzi*. With the obtained data set a structure-activity relationship (SAR) was conducted. 2-nitroimidazole derivatives were active against *T. cruzi* (IC50's < 4 μM) but also to some extent cytotoxic. In contrast, 3-nitrotriazoles demonstrated in vitro anti-*T. cruzi* activity coupled with excellent selectivity (SI>100). The nitro group is essential for the anti-trypanosomal activity. Nine nitrotriazole-based compounds were identified as potential in vivo candidates for the acute *T. cruzi* rodent model. All of them demonstrated activity at low to intermediate nanomolar concentrations, SI values > 200, and satisfied the Lipinski's rule of 5. Based on these promising results, the synthesis of a second set of compounds was restricted to new 3-nitrotriazole-based amids and sulfonamids. In this second set 36 compounds were tested

against the three trypanosomatid parasites (Chapter 6). Nine compounds showed activity against *T. b. rhodesiense* (IC50 of 0.35 – 2.5 μM, SI>100) and 2 compounds showed moderate activity against axenic amastigotes of *L. donovani*. Most of the tested compounds, 30 out of 36 compounds, were active and selective against *T.cruzi* (IC50 of 0.05 - 3.5 μM, SI>100). In the second set of compounds a further 19 in vivo candidates for the *T.cruzi* mouse model were identified. All these in vivo candidates fulfilled Lipinski's rule of 5, demonstrated activity in submicromolar concentrations and selectivity >200.

Nifurtimox and benznidazole are both prodrugs, as most nitroheterocycles are. The mechanism of action of these drugs was for a long time not well understood. It was believed that the cytotoxic effect of nifurtimox is based on oxidative stress, through the reduction of its nitro group and the subsequent formation of superoxide anions and nitro radicals [49]. The cytotoxic mechanism of benznidazole was not understood. Recent studies have shown that the trypanocidal activity of nitroheterocyclic compounds on parasites depends on type I nitroreductase (NTR) [50,51]. This enzyme is absent in most eukaryotes including mammalian cells. The NTR enzyme catalyzes a series of reduction reactions, whereby the heterocyclic ring is fragmented and toxic metabolites are formed. The process produces no significant amounts of superoxide. Type I NTR is NADH-dependent, oxygen intensive, and localized in the mitochondria. The down-regulation of the enzyme leads to resistance to benznidazole and nifurtimox [49,50,51,52,53]. Compounds showing IC50 < 5 μM against *T. b. brucei* were tested in a transgenic *T. b. brucei* line overexpressing tertracycline-inducible TbNTR. Parasites with tetracycline induced overexpressing of TbNTR were more susceptible to the test compounds than wildtype parasites or parasites without induced overexpression of TbNTR. These results suggest that the type I NTR-mediated activation of the compounds is essential for their antitrypanosomal activity. The enzyme assays also indicated that the compounds are substrates of type I NTR and that enzyme inhibition correlates with the whole cell activity.

Based on the high in vitro potency against *T.cruzi*, 13 nitrotriazole compounds with IC50's < 1 μM and SI >200 were selected for in vivo testing in an acute rodent model (Chaper 7). The compounds were tested in a fast luminescence assay [54] in which mice are infected with transgenic *T.cruzi* Y strain expressing firefly luciferase [55]. The mice were treated on day 4 post infection for 5-10 consecutive days and were

imaged after treatment. The dose was between 10 mg/kg/day and 40 mg/kg/day depending on the in vitro activity and selectivity. Seven compounds lowered the parasite index (ratio of parasite level in treated mice compared with the control group multiplied by 100) by more than 80%. Two compounds demonstrated better activity than benznidazole. As expected the in vivo results did not correlate with the in vitro IC50 values because additional parameters such as bioavailability, blood levels and metabolic stability have a great influence on in vivo activity. These parameters are not modeled in the vitro assays. The two most potent compounds in vitro were inactive in the mouse model. In order to explain the discrepancies observed between the in vitro and in vivo activity some ADME studies for selected compounds were performed. All tested compounds showed microsomal stability in the absence of NADPH. However, the aromatic amines were metabolized in the presence of NADPH by mouse microsomal protein, but not the amide and sulfonamide compound. All compounds were relatively stable in mouse plasma. The two most active compounds in vivo showed excellent permeability when tested in the Caco-2 monolayer system. This indicates that the compounds are orally bioavailable [56].

For all nitro compounds there are concerns about genotoxicity and mutagenicity. Therefore, some compounds were tested in the Ames test [57,58]. Most 3-nitrotriazole-based compounds did not show mutagenicity in the Ames test. Although not all compounds were tested for mutagenicity, it seems that 3-nitrotriazoles are not generally mutagenic. 3-nitrotriazoles showed good in vitro and in vivo antichagasic potency, they were non-toxic and metabolically stable. However, further investigations of this class of compounds are required to determine the efficacy in the chronic stage of the disease.

Nitroimidazoles

Nitroimidazoles are a well-known class of compounds showing antibacterial and antiprotozoal activity [59,60]. Megazol was a drug candidate to treat HAT [61], but it turned out to be mutagenic [62,63]. Metronidazole, another nitroimidazole, was introduced as an antibiotic more than 50 years ago. There are other nitro-compounds on the market such as tinidazole, nimorazole, nitrofurantoin, nifurtimox ,or benznidazole. This class of compounds is often associated with genotoxicity [64] and not seen as the ideal new drugs. Nevertheless, PA-825 [65] and OPC-67683 [66], two nitroimidazoles, were recently described as drug candidates against tuberculosis. Both are in clinical development. The genotoxicity of both drugs was investigated and it was concluded that

the compounds do not pose a genotoxic risk to humans. It is believed that the genotoxicity problems depend on the position of the nitro group in the azole ring, increasing in the following order: 4-NO2 <5-NO2 <2-NO2 [67,68].

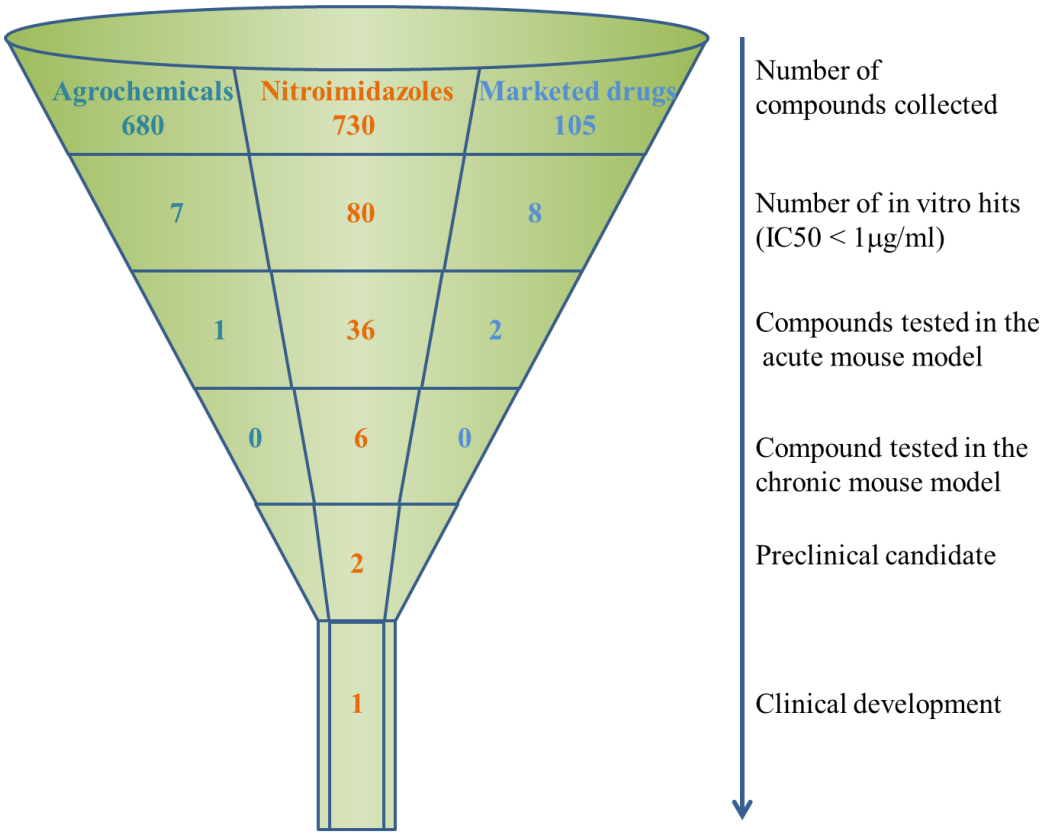


Fig Hit rate in HAT screening

1-Aryl-4-nitro-1*H*-imidazoles

A series of 43 1-aryl-4-nitro-1*H*-imidazoles was assayed for antitrypanosomal activity (Chapter 4). 15 compounds showed good selectivity against *T. b.rhodesiense* with IC50's < 1 μM. Especially 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1*H*-imidazole (Cpd16) and 1-(3,4-dichlorophenyl)-4-nitro-1*H*-imidazole (Cpd 31) showed excellent in vitro activity with IC50-values of 0.16 μM and 0.1 μM, respectively. Both compounds cured a first stage disease mouse model at an oral dose of 25 mg/kg/day and 50 mg/kg/day, respectively, given on four consecutive days. In the stage 2 HAT infection model involving brain infection 100% cure was achieved at an oral dose of 50 mg/kg (Cpd 16) or 100mg/kg (Cpd 31) administered twice daily for five days. Only few

compounds are known to cure this stringent mouse model, for instance melarsoprol or some experimental diamidines [69].

Ames test (bacterial mutagenicity) was performed with standard Salmonella tester strains which have the classical bacterial nitroreductases. The contribution of the bacterial nitro-reductase to the observed mutagenicity was estimated by testing against nitro-reductase deficient strains. The tested compounds induced mutations in the standard tester strains, but mutagenicity was not detected in the corresponding nitro-reductase deficient strains. The exception was Cpd 31 which showed only reduced mutagenicity. The mutagenicity observed for Cpd 16 was solely due to the action of bacterial nitro-reductases. The micronucleus test is another method to assess the genotoxicity of a compound. This test detects chromosomal damages and aneugenicity in all mammalian cells. All tested compounds were negative in this test. The results clearly show that the family of 1-aryl-4-nitro-1*H*-imidazoles merits further exploration for anti-protozoal drug discovery. 4-nitro-1-{4-(trifluoromethoxy)phenyl}-1H-imidazole (Cpd 16) cured both first stage and second stage HAT disease mouse models and did not show any genotoxicity. This compound can be considered as promising lead for further development into a new oral drug for human African trypanosomoasis.

Fexinidazole

Over 700 nitroheterocyclic compounds, mostly nitroimidazoles, were systematically reviewed and profiled including antiprotozoal activity, ADME-Tox, and mutagenicity (Chapter 1). The compounds had been obtained from diverse sources, from pharma industry as well as from academic groups. The goal of the screening campaign was the identification of drug candidates against HAT (Fig. 1) and the identification of new leads against Chagas disease and leishmaniasis. All collected compounds were tested in vitro against *T. b. rhodesiense*, *T. cruzi*, *L. donovani*, and for cytotoxicity against L6 rat skeletal myoblast cells. Non cytotoxic compounds which showed activity against one of the parasites were further pursued in toxicity tests and in the corresponding rodent models. At the end of the screening campaign fexinidazole was identified as the most promising drug candidate for the treatment of HAT. Fexinidazole (HOE239) is a 2-substituted 5-nitroimidazole and had been in clinical development in the 1970's and 1980's as a broad-spectrum antimicrobial drug by Hoechst AG [70,71]. The in vivo antitrypanosomal activity was described at that time. Fexinidazole was successfully tested in a *T. cruzi* mouse model [71] and Jennings et al

(1983) [72] described the in vivo activity of fexinidazole against African trypanosomes. However, the development of fexinidazole was not pursued at that time.

Fexinidazole is rapidly metabolized in to fexinidazole sulfoxide and fexinidazole sulfone. The parent compound and the two principle metabolites showed trypanocidal activity in vitro in the range of 0.2 – 0.9 μg/ml against a panel of *T. b. rhodesiense* and *T. b. gambiense* strains (Chapter 2), including sensitive and drug resistant laboratory strains as well as recent clinical isolates. Fexinidazole and its metabolites require up to 48 hours exposure in order to induce maximal effect in vitro. In spite the modest in vitro activity fexinidazole and metabolites cured the acute mouse model at an oral dose of 100 mg/kg administered on 4 consecutive days. The chronic disease mouse model for stage 2 of the disease was also cured by all three compounds at a 5-day orally administered dose of 200mg/kg. Fexinidazole is well absorbed by the oral route and rapidly metabolised into the sulfoxide and sulfone derivatives, both showing high plasma levels (Chapter 1). The pharmacokinetic data suggest that the excellent in vivo activity of fexinidazole is likely due to the cumulative exposure to the three compounds. The three compounds are very well distributed in the body with different but overlapping kinetics, thus ensuring effective exposure in both the hemolymphatic system and the brain. The half-life of fexinidazole in mice, rats and dogs is 1 to 3 h after oral treatment, whilst the half-life of the sulfoxide is 2 to 7 h and that of the sulfone up to 24 h. No accumulation of the compounds was observed in rats, and the drug is excreted through faeces (59%) and urine (30%) within 48 h. The distribution to the brain was confirmed in mice and rats. There is no reason to assume that the brain penetration of the three lipophilic compounds could be critical.

A full regulatory toxicology package has been prepared. Overall, fexinidazole was well tolerated and there were no specific concerns identified. In the classical in vitro Ames test, fexinidazole was positive. But this effect is dependent on the presence of bacterial nitroreductases. Fexinidazole was carefully tested in a set of in vitro and in vivo assays to detect possible signals of mammalian genotoxicity; all tests were negative.

The mechanism of action of fexinidazole is not yet understood, but recent studies have shown that the trypanocidal activity of nitroheterocyclic compounds on parasites depends on type I nitroreductase [50,51]. Fexinidazole and its metabolites were shown to have low single electron potentials. The nitroreductive enzymes in mammalian cells

can only reduce compounds with relatively high redox potentials. In contrast, the bacterial nitroreductases can act at much lower redox potentials. This could explain the positive results in the standard Ames test and the reduced or abolished activity in nitroreductive-deficient strains.

The rediscovery of fexinidazole as a drug candidate is the success of a pragmatic approach. With an extensive compound mining within a family of known active compounds and in true sense to Sir J. Black, a new drug candidate was identified in short time. A set of 700 compounds was collected and assayed for anti-parasitic activity and genotoxicity. Within 2 years a candidate for preclinical development was selected and 3 years later a clinical phase I trial was initiated. In the completed phase I trial [73] in healthy volunteers of African origin fexinidazole showed safety and was well tolerated. Phase II/III clinical trial [74] was initiated in late 2012. Stage 2 patients are treated either with Fexinidazole for 10 days (4 days with 1800mg/day and 6 days with 1200mg/day) or with NECT. This so far successful drug discovery and development program shows that it is worthwhile to dig into post research efforts.

Fexinidazole and the combat against HAT

T. b. gambiense is causing more than 95% of the reported HAT cases and *T. b. rhodesiense* less than 5%. Between 1999 and 2010 the reported cases decreased significantly for *T. b. gambiense* from 27862 to 6985 and for *T. b. rhodesiense* from 615 to 155. The disease is endemic in 36 countries; in 50% of the countries there are no HAT cases and in 40% of countries there are fewer than 20 HAT cases per year. The most affected countries are Uganda, Chad, South Sudan, Central African Republic and the Democratic Republic of Congo, where over 90% of *T. b. gambiense* cases were found.

The London Declaration on NTDs (2012) [75] and the WHO Roadmap on NTDs [76] target the elimination of gambiense HAT as public-health problem by 2020. The threshold for elimination was defined as less than 1 new case per 10’000 inhabitants in at least 90% of endemic foci. The ultimate goal is to interrupt the transmission of the disease to avoid past experiences where the disease re-emerged after a massive reduction. The absence of transmission will result in zero reported cases and it is envisaged to reach this goal by 2030 [77]. The diagnosis of sleeping sickness involves serological test, parasitological confirmation of infection, and determining the stage of

disease. The disease stage of each diagnosed patients must be determined with a lumbar puncture to avoid exposing a stage 1 patient to the risks and burden of the stage 2 treatments. The current treatment options are either 10 days of daily intravenous melarsoprol with its toxicity, the very complicated eflornithine monotherapy with 56 infusions over 14 days, or the recent improvement of NECT (10 days oral nifurtimox and 7 days of 12 hourly eflornithine infusions). Since a few years, NECT is the first-line treatment for stage 2 *T. b. gambiense* cases. The drawback of this treatment is the intravenous administration of eflornithine which is logistically complex. Patients are found in remote rural areas and need to be hospitalized in poorly equipped hospitals. A non-toxic, effective and easy to use drug for both stages of HAT, ideally in combination with an easy field diagnostic, would make the goal of HAT elimination realistic.

Fexinidazole is the first new clinical drug candidate in thirty years with the potential for treating advanced-stage sleeping sickness. If the clinical development of fexinidazole can be successfully completed, then this will be a major breakthrough in the control and even the elimination of HAT in Africa. Fexinidazole would be the first oral drug for both stages of HAT, well tolerated and effective in a 10 day treatment. The fact that it can be used for both stages of sleeping sickness, would have an impact on the complicated diagnosis. The determination of the stage of disease would not be mandatory, and the lumbar puncture could be waived. Fexinidazole would decisively simplify the treatment for the patients as well as the logistics. Based on the simple chemistry and short synthesis of fexinidazole, the treatment costs are estimated to be not more than US$ 50. It seems that fexinidazole is very stable, which is helpful for the development of a formulation for use in the tropics. Of course, there are still many hurdles to overcome before fexinidazole is registered and can be launched, but it is certainly the most promising candidate since many years.

Conclusion

The present work confirms that the approach of whole-cell assays, also called phenotypic screening, is a valuable alternative to the approach of target-based screening. In a relatively short time new chemical scaffolds and drug candidates have been identified. For the identification of new lead structures, it is worthwhile to test compounds that were developed for other purposes, as the examples of agrochemicals and marketed drugs show. The rediscovery of fexinidazole underlines that an accurate

and extensive revisiting of past research and data mining can help in the discovery of new drug candidates. Drug discovery is a multidisciplinary task and a team effort is required to bring a drug into clinical development in such a short time. Fexinidazole is the first drug candidate in clinical phase II/III trial since decades. It would be the first oral drug for the treatment of stage 1 and stage 2 of HAT. If fexinidazole overcomes all obstacles then it will be a major breakthrough in the combat against human African sleeping sickness.

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