Screening, identification, structure-activity, and mode of action studies with new antitrypanosomal leads of plant and fungal origin

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Dekan

to…

Mama

Bruederhärz

Didi

Oma und Opi

…in love and gratitude

"Hey! This is an excellent microbe. It is big for a microbe, easily visible, easy to breed in mice. It kills them proper and always. Where could I find a better microbe than this trypanosome, to help me find the magic bullet which is needed for the therapy? Alas! I wish I could find a dye that would heal one mouse, one tiny little mouse."

Paul Ehrlich, early 1900

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Abbreviations

ADME	Absorption distribution metabolism excretion
BBB	Blood brain barrier
b.i.d.	Twice a day
CD	Circular dichroism
CNS	Central nervous system
CYN	Cynaropicrin
d	Day
DCM	Dichloromethane
DFMO	Eflornithine
DMSO	Dimethylsulfoxide
DNDi	Drugs for Neglected Disease initiative
ESI-MS	Electronspray ionization-mass spectroscopy
EtOAc	Ethyl acetate
GSH	Glutathione
HAT	Human African trypanosomiasis
HPLC	High pressure liquid chromatography
HR-MS	High resolution mass spectroscopy
HTS	High throughput screening
IC_{50}	50% growth inhibitory concentration
i.p.	Intraperitoneal
MeOH	Methanol
MMV	Medicines for Malaria Venture
MS	Mass spectroscopy
MS/MS	Tandem mass spectroscopy
NCE	New chemical entity
NECT	Nifurtimox-eflornithine combination treatment
NMR	Nuclear magnetic resonance
NP	Natural product
ODC	Ornithine decarboxylase
PK	Pharmacokinetic
p.o.	Per oral
SAR	Structure-activity relationship

SI	Selectivity index
STL	Sesquiterpene lactone
$T(SH)_2$	Trypanothione
UV	Ultraviolet
WHO	World Health Organization

Summary

Human African trypanosomiasis (HAT) is a neglected disease caused by the protozoan *Trypanosoma brucei*, which is transmitted during blood-feeding tsetse fly bites. The disease is endemic covering 36 sub-Saharan African countries and mainly impacts poor people living in remote areas, for which satisfactory treatment does not exist. As such, this protozoal disease would never be viewed as viable target market for the pharmaceutical industry. Therefore, it is referred to as a neglected disease.

Man rarely become infected with the more virulent *T. b. rhodesiense* form, found in Eastern and Southern Africa, and more often with the chronic *T. b. gambiense* form, which occurs in West and Central Africa. Once the trypanosomes cross the blood brain barrier (BBB) the patients fall into a comatose state accompanied by neurological breakdowns and apathy resulting in death when left untreated. Chemotherapy remains the principal treatment for HAT and is based on four drugs: suramin, pentamidine, melarsoprol, eflornithine, and a recent approved eflornithine-nifurtimox combination. Reported severe side effects (e.g. melarsoprol), treatment failures of up to 25%, administration difficulties, and expensive medication urgently demand for safe, orally administered drugs, that are effective against both stages of HAT.

Natural sources like plants and fungi provide a rich biological diversity with unique pharmacophores created by evolution. According to the WHO, 65% of the world's population still relies on traditional medicines as a primary source of healthcare.

This thesis describes the search of new natural products (NPs) from nature. Over the last seven years we collected 724 plants and 64 fungi. The material was subsequently extracted and tested *in vitro* against *T. b. rhodesiense*, *Plasmodium falciparum* (the causative agent of malaria), *Leishmania donovani* (leishmaniasis), and *T. cruzi* (Chagas disease) to find potential hits. From the total 2151 extracts, 17.9% showed activity of more than 50% at 4.81 µg/mL test concentration against at least one parasite, and 3.4% showed potency of more than 50% at 0.81 µg/mL test concentration, respectively. Overall the plant extracts had six times higher "hit-rates" (15.3%) than the fungi extracts (2.6%), both resulting in high potencies against *T. b. rhodesiense* and *P. falciparum*. Yet, with up to 5 millions fungi, which outnumber higher plants by 16:1, the kingdom remains a relatively poorly studied source of NPs. Three fungal extracts had determined IC_{50}s below 10 ng/mL, making them up to three orders of magnitude lower than the most potent plant extracts, which indicate the antiprotozoal potential of fungi. These findings were underlined by the truffle *Elaphomyces granulates in vivo* activity when tested intraperitoneally (i.p.) at 50 mg/kg/d. *T. b. rhodesiense* infected mice remained parasite free for 14 days compared to the controls, which were euthanized after 7 days postinfection.

The liquid extract library contains 177 plant extracts produced from traditionally used antimalarial Iranian plants and from plants, which were reported in herbal books as antimalarial remedies in European Renaissance herbals. When activities of antimalarial traditionally used remedies were compared to randomly selected plants, a five times higher "hit-rate" was found for ethno-medically used plants (19.7%) than for randomly selected plants (4.5%).

One of the antitrypanosomal hits was a dichloromethane (DCM) extract of the cornflower *Centaurea salmantica* with a growth inhibition of 61% tested at 4.81 µg/mL against *T. b. rhodesiense*. HPLC-based activity profiling led to the identification of the sesquiterpene lactone (STL) cynaropicrin (CYN), which was the first plant NP to show *in vivo* efficacy in *T. b. rhodesiense* infected mice, treated i.p. at 10 mg/kg/b.i.d. for four consecutive days. Despite of more than 10'000 known STLs is a better understanding of the structural features, which contribute to activity, expedient. The established structure-activity relationship (SAR) study included 18 natural STLs and demonstrated that antitrypanosomal and cytotoxic effect depended on their α,β-unsaturated enone moieties. Many bioactivities of STLs have been attributed to a nucleophilic Michael-addition of these functional motifs to biological thiols. Considering that trypanosomes depend on their unique trypanothione-based redox system to deal with oxidative stress and to maintain a reducing intracellular milieu and that CYN contains reactive exocyclic α,β-unsaturated methylenes, we anticipated that the mechanism of action depended on a direct interference with glutathione (GSH) and trypanothione ($T(SH)_2$) in the cells. After 5 min. of CYN's exposure to trypanosomes, the intracellular thiol pool was completely depleted and a GS-CYN-monoadduct as well as a $T(S\text{-}CYN)_2$-bisadduct were formed. This led to apoptosis of the trypanosomes over 40 min. linked to phenotype transformations from the typical slender to a stumpy-like form. Additionally, ornithine quantification studies by tandem mass spectroscopy (MS/MS) showed that ornithine decarboxylase (ODC) is a potential secondary target for CYN.

To improve CYN's pharmacokinetic (PK) profile the α,β-unsaturated exocyclic double bond at the lactone was masked to create an amine prodrug with increased aqueous solubility and reduced unspecific binding to biological thiols. Through subsequent bioactivation the prodrug would be converted back to CYN and it would display a higher concentration on the target side. The lead optimization did not reward any better antitrypanosomal *in vivo* efficacy after oral application, but the prodrug had an improved *in vivo* cytotoxic profile. Further PK studies with other orally applied STL amino derivatives are needed to demonstrate if the use of amino STLs as prodrugs is a reasonable approach to improve STLs suitability as antitrypanosomal drug.

Zusammenfassung

Die Afrikanische Schlafkrankheit ist eine vernachlässigte Krankheit, die durch die Protozoen *Trypanosoma brucei* verursacht und während eines Blutmahls der Tsetsefliege übertragen wird. Die Krankheit ist in 36 subsaharischen Ländern Afrikas endemisch und betrifft hauptsächlich arme Einwohner in abgelegenen Orten, für welche zufriedenstellende Behandlung nicht zur Verfügung steht. Weil sie die teuren Therapien sich nicht leisten können, wird diese Protozoenerkrankung als nicht-profitabler Markt für die pharmazeutische Industrie angesehen.

Die Menschen werden selten mit der ansteckenderen *T. b. rhodesiense* Form, die in Ost- und Südafrika aufgefunden wird, infiziert, als mit der chronischeren *T. b. gambiense* Form, welche in West- und Zentralafrika auftritt. Wenn die Trypanosomen einst die Bluthirnschranke überquert haben, fallen die Patienten in einen komatösen Zustand, der von neurologischen Zusammenbrüchen und Teilnahmslosigkeit begleitet wird. Wenn die Patienten keine Behandlung bekommen, führt dies unweigerlich zum Tode. Die Chemotherapie verbleibt die einzige Kontrolle der Afrikanischen Schlafkrankheit und basiert auf vier Medikamente: Suramin, Pentamidin, Melarsoprol, Eflornithin, und eine kürzlich freigegebene Eflornithin-Nifurtimox Kombination. Gemeldete schwerwiegende Nebenwirkungen (z.B. von Melarsorpol), erfolglose Behandlungen in bis zu 25% der Fälle, schwierige Verabreichung, und die teure Medikation verlangen dringend sicherere, oral verfügbare Medikamente, die effektiv gegen beide Stadien der Afrikanischen Schlafkrankheit sind.

Natürliche Quellen wie Pflanzen und Pilze liefern eine reiche biologische Diversität mit einzigartigen Pharmakophoren, die von der Evolution kreiert wurden. Gemäss WHO haben 65% der Weltpopulation Zugang zu traditionell-verwendeter Medizin.

Diese Arbeit beschreibt die Suche nach neuen Naturstoffen. Während den letzten sieben Jahren haben wir 724 Pflanzen und 64 Pilze gesammelt, das Material extrahiert und *in vitro* gegen *T. b. rhodesiense*, *Plasmodium falciparum* (Erreger der Malaria), *Leishmania donovani* (Leishmaniose), und *T. cruzi* (Chagas Krankheit) getestet, um potentielle Hits zu finden. Von insgesamt 2151 Extrakten zeigten 17.9% eine Aktivität von mehr als 50% bei der Testkonzentration von 4.81 µg/mL gegen mindestens einen Parasiten, respektive 3.4% zeigten mehr als 50% Hemmung bei 0.81 µg/mL Testkonzentration auf. Insgesamt hatten die Pflanzenextrakte einen sechsmal höheren Anteil aktiver Hits (15.3%) als die Pilzextrakte (2.6%). Mit bis zu fünf Millionen übertreffen Pilze die Anzahl an Pflanzen 16:1, jedoch verbleiben sie eine verhältnismässig schlecht erforschte Naturstoffquelle. Drei Pilzextrakte hatten IC_{50}s unter 10 ng/mL, welche im Vergleich zu den getesteten Pflanzenextrakten bis zu

drei Ordnungsgrössen kleiner waren. Dieses Resultat wird vom Trüffel *Elaphomyces granulates in vivo* Aktivität, welcher intraperitoneal mit einer Dosis von 50 mg/kg/Tag getestet wurde, unterlegt. Die mit *T. b. rhodesiense* infizierten Mäuse waren für 14 Tage parasitenfrei im Gegensatz zur Kontrolle, welche nach 7 Tagen Postinfektion getötet wurden.

Die getestete Flüssigextrakt-Bibliothek beinhaltete 177 Pflanzenextrakte, die von traditionell genutzten iranischen Pflanzen und von Pflanzen, welche in Kräuterbüchern aus der europäischen Renaissanceepoche gegen Malaria dokumentiert sind, hergestellt wurden. Wenn die Aktivitäten der traditionell verwendeten Pflanzen mit den zufällig ausgewählten Pflanzen verglichen wurden, war die Hitrate für die traditionell genutzten Pflanzen (19.7%) fünf Mal höher als die der zufällig ausgewählten Pflanzen (4.5%) (definiert als > 50 % Hemmung bei 4.8 µg/mL).

Einer der aktiven Hits gegen *T. b. rhodesiense* war der Dichlormethanextrakt der Kornblume *Centaurea salmantica* mit einer Wachstumsinhibition von 61% bei 4.81 µg/mL Testkonzentration. HPLC-basiertes Aktivitätsprofiling führte zur Identifizierung des Sesquiterpenlacton Cynaropikrin, welche der erste Naturstoff ist, der bei *T. b. rhodesiense* infizierten Mäuse *in vivo* Wirksamkeit aufzeigte, welche mit 10 mg/kg/b.i.d. intraperitoneal für 4 Tage behandelt wurden. Trotz 10‘000 bekannten Sesquiterpenlactonen ist ein besseres Verständnis für Strukturmerkmale, die zur einer Steigerung der Aktivität beitragen, sinnvoll. Die darauffolgende Struktur-Aktivitäts-Beziehungs-Studie beinhaltete 18 Sesquiterpenlaktone und zeigte auf, dass die trypanosomale Wirksamkeit und die Zytotoxizität auf dem Vorhandensein von α,β-ungesättigten Enon-Gruppen zurückzuführen ist. Viele biologische Wirksamkeiten der Sesquiterpenlactone wurden der nukleophilen Michael-Addition von α-Methylen-γ-lacton Gruppen mit Thiolen zugeschrieben. Unter der Betrachtung, dass die Trypanosomen von ihrem einzigartigen auf trypanothion-basierenden Redoxsystem abhängig sind, um den oxidativen Stress einzudämmen und das intrazelluläre reduzierende Milieu aufrechtzuerhalten, und dass Cynaropikrin reaktive α,β-ungesättigte Methylengruppen besitzt, haben wir antizipiert, dass der zelluläre Wirkmechanismus des Cynaropikrins von dessen direkter Interferenz mit Glutathion und Trypanothion zusammenhängt. Nach 5-minütiger Aussetzung zu den Trypanosomen war der intrazelluläre Thiolpool komplett aufgebraucht und ein GS-CYN-Monoaddukt sowie ein $T(S\text{-}CYN)_2$-Bisaddukt wurden geformt, welche zu einer Apoptosis der Trypanosomen während 40 min. führte. Während diesem Zeitfenster veränderte sich der Phänotyp von ihrer typischen „slender“ zu einer „stumpy“-ähnlichen Form. Zusätzlich, zeigten Ornithinquantifizierungsstudien mit Tandem-Massenspektrometrie, dass die Ornithindecarboxylase ein zusätzliches Target für Cynaropikrin ist.

Um das pharmakokinetische Profil von Cynaropikrin zu verbessern, wurde die α,β-ungesättigte exozyklische Doppelbindung am Lacton maskiert, um ein Amin-Prodrug mit gesteigerter Wasserlöslichkeit und reduzierter unspezifischen Bindungen an Thiolen herzustellen. Durch subsequente Bioaktivierung des Prodrugs würde das Molekül zurück zu Cynaropikrin konventiert werden was zu einer erhöhten Konzentration am Zielort führen würde. Die Optimierung hatte nach einer oralen Applikation des Prodrugs nicht zu einer gesteigerten antitrypanosomalen *in vivo* Aktivität geführt, jedoch zeigte der Prodrug ein verbessertes *in vivo* Zytotoxizitäts-Profil auf. Weitere pharmakokinetische Studien mit zusätzlichen oralen verabreichten Sesquiterpenlacton-Aminoderivaten sind nötig, um zu demonstrieren, ob die Nützlichkeit der Amino-Prodrugs als trypanosomale Arzneistoffe angemessen ist.

CHAPTER 1

General introduction

On the WHO list of the most frequent worldwide causes of death by illness one found seventeen infectious diseases [1]. Among them are protozoan infections like malaria, sleeping sickness and schistosomiasis, which affect hundreds of millions worldwide resulting in significant mortality and social and economic consequences. The diseases mainly impact poor people living in remote areas, urban slums, and conflict zones with limited access to adequate health care services. For these diseases satisfactory treatment does not exist in terms of limitations in efficacy, severe side effects, high production costs, and complex administration patterns. As such, protozoal diseases would never be viewed as viable target markets for the pharmaceutical industry. Therefore they are referred to as neglected diseases [2]. In fact it nowadays costs the pharmaceutical industry 1 billion dollars and more than 10 years to develop a new drug [3]. Not surprisingly, the pharmaceutical industry would rather focus on Western life style diseases like diabetes (2010, nearly 26 million people have diabetes in the United States, 132 billion costs each year [4]), heart disease (63% of all deaths in the world [5]), and cancer (globally accounted for 7.6 million deaths 2008 [6]) to make a financial profit to cover the tremendous development costs than on the less lucrative tropical protozoal diseases. Therefore, development of new antiprotozoal drugs is a challenge. The good news is that in the last few years several non-profit drug research and development organizations like the Drugs for Neglected Disease Initiative (DNDi) [7], Medicines for Malaria Venture (MMV) [8], the World Health Organization (WHO), as well as academic centers significantly changed the way antiprotozoal drug development is done. DNDi and MMV function as project managers and bring together the components necessary to restock the drug pipeline. Additionally, enhanced funding possibilities coming largely from the Bill & Melinda Gates Foundation who spent 1.9 Bio. dollars for global health in 2011[9], Wellcome Trust [10] and the Sandlers Family Supporting Foundation [11], indicates a silver lining on the horizon. Funding of research is a crucial issue in antiprotozoal drug development, because the drugs will have to be cheap to produce.

1.1. Human African trypanosomiasis (sleeping sickness)

1.1.1. Vector and parasite

One of the most neglected diseases is Human African trypanosomiasis (HAT) with 30'000 estimated cases per year [12]. The disease is restricted to 36 sub-Saharan countries based on the distribution of its vector, the tsetse fly *Glossina sp*. *Glossina palpalis* occurs in West and Central Africa in habitats with tropical forests and transmits *Trypanosoma brucei gambiense* whereas *Glossina morsitans* (Figure 1)*, Glossina pallidipes,* and *Glossina swynnertoni*, breed in grassland, savannah, and woodland of Eastern and Southern Africa and transmit the parasite *Trypanosoma brucei rhodesiense* [13] (Figure 2). People become infected only sporadically with the rare, but more virulent *T. b. rhodesiense* form, which reflects 5% of the reported cases [14]. Patients infected with the chronic form of HAT caused by *T. b. gambiense* are still able to work over long periods despite the infection. Animals can host the pathogen, especially *T. b. rhodesiense*. Thus domestic and wild animals such as cattle, sheep, and goats are important parasite reservoirs [15].

Figure 1. *Glossina morsitans* (Wilson [16])

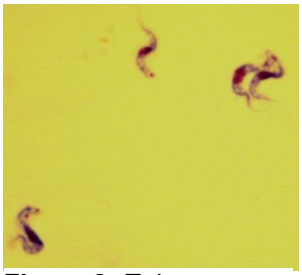


Figure 2. *T. b. rhodesiesene* STIB 900 strain; Giemsa stain (2012, Zimmermann)

1.1.2. Clinical manifestation features

Unicellular trypanosomes are transmitted by the bite from a blood-feeding tsetse fly. At the puncture site a chancre appears. This is a sign of the localized proliferation of pathogens within the tissue accompanied by an inflammatory response and odema. The trypanosomes then spread into the lymphatic system and later enter the blood flow, which causes irregularly relapsing fevers with swollen lymph glands. This first phase of the infection is called the hemolymphatic stage. This phase endures dependent on the species for days (*T. b. rhodesiense*) or weeks (*T. b. gambiense*). The following second stage is caused by the invasion of the parasites through the blood brain barrier (BBB) into the cerebrospinal fluid, which is characterized by severe headache, apathy, and a progressive breakdown of neurological functions. In the comatose state the patients drift into what gave sleeping sickness its name [14].

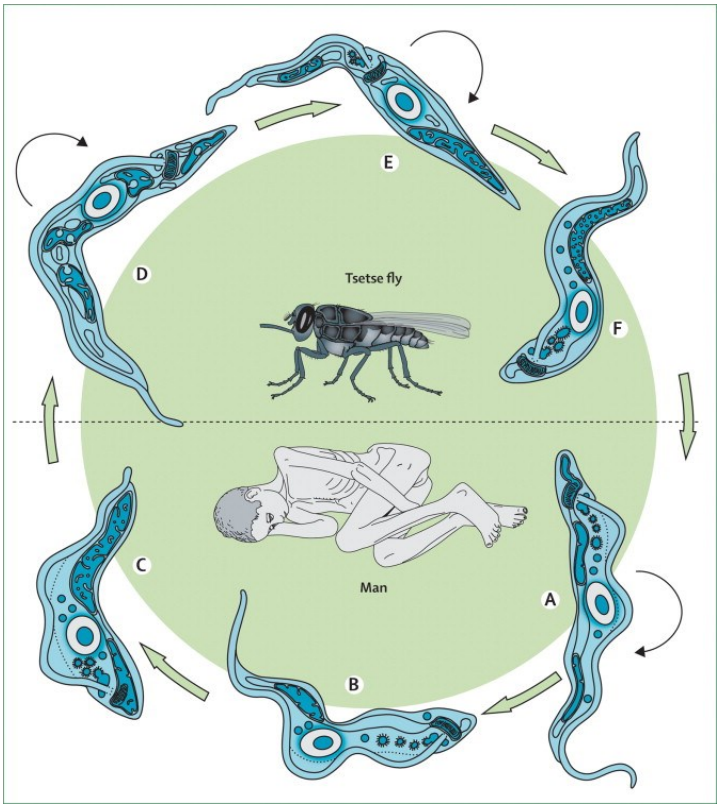


Figure 3. In man the bloodstream forms show polymorphism with (A) dividing slender forms, (B) intermediate forms, (C) stumpy forms. In the tsetse fly vector, bloodstream forms transform into (D) dividing midgut forms, then to (E) the migrating epimastigote forms, which develop in the salivary glands to (F) the infective metacyclic forms, which are injected during the next blood meal into the mammalian host [2].

1.1.3. Treatment

Since the clinical features of the first stage of the disease are not sufficiently specific and resemble a general malaise syndrome, exhaustive laboratory examinations of the population are required. In Africa resources are often scarce, particularly in remote areas where the disease is found. As a result, many individuals may die before they can ever be diagnosed and treated. The earlier the disease is identified, the better is the prospect of cure [14]. If patients are left untreated the disease is 100% fatal. Vaccination is not an option because of antigenic variation where the parasites repeatedly change their surface coat und thus evade the immune system [17]. Therefore chemotherapy remains the principal control of HAT, despite setbacks due to resistance [18,19]. The current treatment of sleeping sickness is based on whether the trypanosomes have infiltrated the central nervous system (CNS) (stage 2) or not (stage 1). This makes the chemotherapy of sleeping sickness difficult because the most effective drugs do not cross BBB and are thus not able to kill the parasites in the CNS. Additionally, treatment is limited and complex due to the only few available drugs, which have poor safety and unfavourable pharmacokinetic (PK) profiles. At present the licensed anti-HAT drugs are manufactured by the pharmaceutical companies Sanofi-Aventis (pentamidine, melarsoprol, and eflornithine) and Bayer Health Care (suramin). The drugs are donated for free to the WHO, which distributes them to the patients in Africa [12]. The following two drugs for the first stage infection are recommended by WHO (Figure 4).

Pentamidine (Pentacarinat®):

After it was shown 1937 that trypanosomes consume an enormous amount of sugar in order to reproduce [20], Yorke and Lourie tested synthalin, a known oral antidiabetic drug, *in vivo* [21]. Although synthalins mode of action had nothing to do with the glucose level, the

drug showed a trypanocidal effect. Further modification was done leading to a series of aromatic diamidines. Among them is pentamidine, which is chemically related to the antidiabetic drug phenformin [22]. At present pentamidine is still the drug of choice for treating the first stage *T. b. gambiense* infection.

Suramin (Germanil®):

Back in the early 1900s, Paul Ehrlich and his assistant Shiga tested more than 100 synthetic dyes for their *in vivo* utility to treat horses with Mal de Caderas, a disease caused by *Trypanosoma evansi*. The mice they used for *in vivo* experiments, however, all turned either blue or yellow from the dyes and were not healed from the infection. One of Paul Ehrlich's ideas was to change the structure of one of his dyes to gain better solubility in the mice's blood. Ehrlich called the compound trypan red, a member of the Congo red series of cotton dyes, which initially healed the mice infected with *T. evansi*, but not other trypanosomes species [23,24]. Later, the benzopurpurine trypan blue (still used in mammalian cell viability assays), provided by the pharmaceutical company Bayer, was found to be effective in eliminating all parasites *in vivo* with a single injection, but still stained the mice's skin bluish as an unacceptable side effect. For this reason, Bayer investigated its colorless, but antitrypanosomally active naphthalene derivatives. This resulted in the breakthrough discovery of Bayer 205, later renamed as suramin, which is still in use in the early stage of the *T. b. rhodesiense* infection [25]. Because of its sulphuric acid function, 99% of suramin binds to the plasma protein albumin. Therefore suramin does not penetrate the cerebral fluid, ruling out its use in second stage of disease [26].

Since the parasites infiltrate the CNS in stage 2, the drug has to cross the BBB to reach the parasites. The following two drugs for the second stage infection are recommended by WHO (Figure 4).

Melarsoprol (Arsobal®):

In 1905, the Canadian doctor Wolferstan found that the arsenical acid Atoxyl was active against trypanosomes in mice [27]. Despite its failure in a trial in East Africa [28] the relative success of Atoxyl paved the way for research on arsenicals as chemotherapeutic agents for HAT. Thirty years later, Friedheim developed the trivalent arsenic drug melarsorpol. Although highly toxic and accompanied by severe side effects – 5-10% of the patients develop an encephalopathy of which 50% die [29,30] - the drug is the only option against the second stage of *T. b. rhodesiense* infection [31].

Eflornithine (Ornidyl®):

Eflornithine, also known as DFMO (α-difluoromethylornithine), was originally developed for tumor chemotherapy based on its irreversible ornithine decarboxylase (ODC) inhibition, an enzyme involved in polyamine biosynthesis [32,33]. The rapid turnover of the mammalian ODC ruled out DFMO as an anticancer drug. In the 80's, Bacchi cured a *T. b. brucei* infected mouse model with DFMO without any severe side effects. This amazing breakthrough lead to several clinical trials followed by a cure in second-stage *T. b. gambiense* infected humans, and its registration in 1990 [34]. A quite interesting recent advance in the clinical treatment of HAT has been the combination treatment called NECT with orally administered nifurtimox (Lampit®), a nitrofuran derivative developed for the treatment of Chagas disease, and intravenously given DFMO for second stage *T. b. gambiense* HAT treatment. The WHO accepted this combination therapy and included it in the WHOs list of Essential Medicines in 2009 [35]. Despite DFMO being the only advance in the past 25 years in HAT chemotherapy, a clear improvement with reduced toxicity and treatment duration has been seen, but the requirement for intravenous administration is still a limitation. It is hoped that the broad implementation of the NECT regimen may avert the further development of DFMO resistance [36].

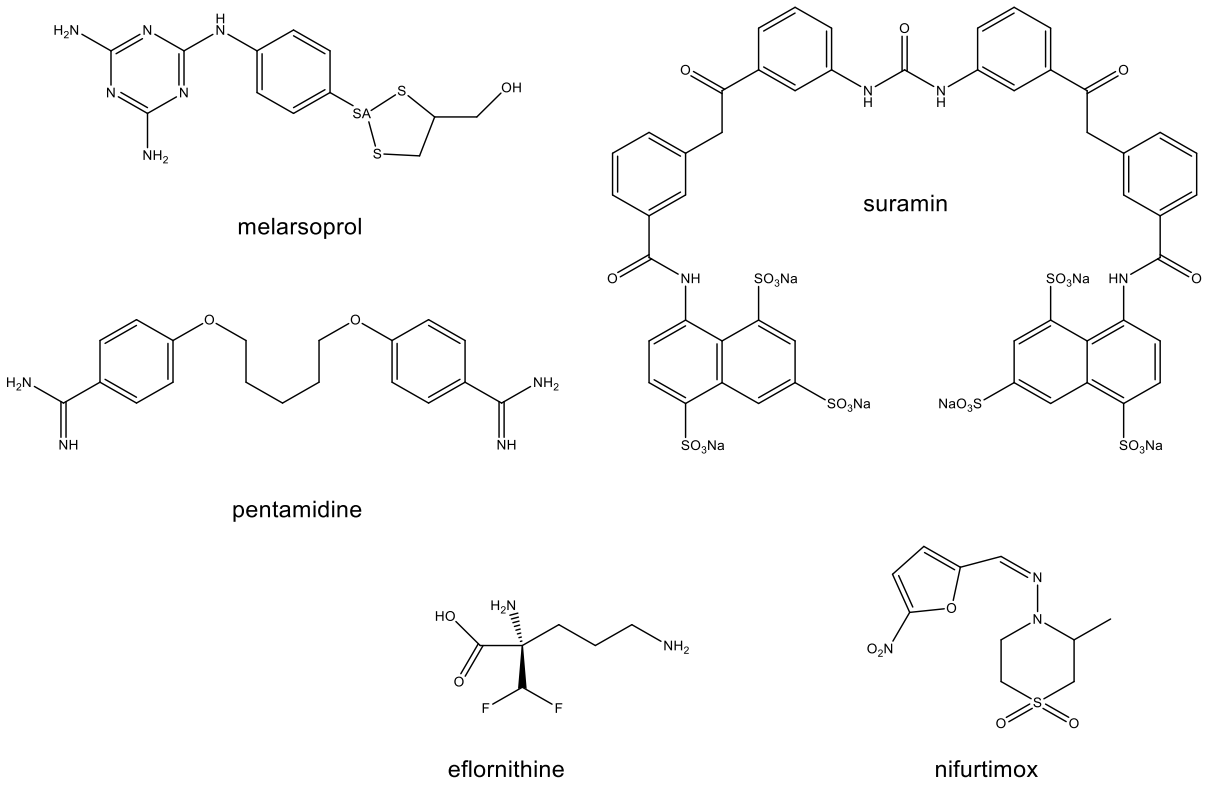


Figure 4. Current treatment options for first and second stage of sleeping sickness disease.

1.1.4. Perspectives for the future

Only DFMO has been registered in the last 50 years for use against HAT reflecting the gap in the drug pipeline. Currently, treatment is still limited in terms of severe side effects (e.g. melarsoprol), treatment failures up to 25% [37,38] administration difficulties, expensive medication, and lack of drug choice for second stage HAT *T. b. rhodesiense*. What is urgently needed is a safe, orally administered single dose drug, effective against both stages of HAT, which then eliminates the need for stage medication and raises the potential for the eradication of sleeping sickness disease. In this next section upcoming drug candidates are discussed, which are in clinical trials for registration of new chemical entities (NCE) against HAT (Figure 5).

Nitroimidazoles

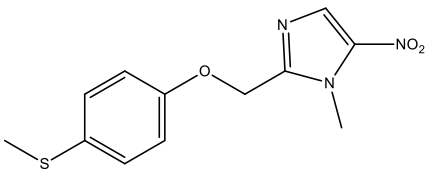
Originally developed by Hoechst as an antimicrobial, fexinidazole, a 2-substituted 5-nitroimidazole, was shown to be active against trypanosomes in the 1980s, where it prevented parasitemic relapses due to CNS infections of *T. brucei* in mice [39]. However, fexinidazole's development was not pursued at this time. The long forgotten drug was rediscovered as a promising candidate due to a screening of more than 700 nitroheterocyclics against *T. brucei*. Despite its weaker potency (IC_{50} of 1.7 µM against *T. b. rhodesiense)* than melarsorpol (IC_{50} of 0.009 µM against *T. b. rhodesiense)*, but non-specific cytotoxicity, the drug cured first stage HAT *T. b. rhodesiense* and *T. b. gambiense* infected mice with a oral dose of 100 mg/kg/d given for four consecutive days and second stage animal model with a oral dose of 100 mg/kg/b.i.d. for five consecutive days [40,41]. Based on a full set of preclinical studies conducted in accordance with the regulatory requirements for pharmaceuticals for human use, a phase I clinical trial was performed in 2009. In 2012 a phase II/III clinical trial was started where patients were treated orally for 10 days with a daily single dose [42] in order to register the drug for second stage HAT of both sub-species.

Benzoxaboroles

Scynexis identified a class of boron-containing compounds as novel leads against *T. brucei* [43]. The initial screening revealed SCYX-6759 as the most potent compound with BBB permeability. It cured second stage HAT infected mice when they were treated i.p. with 50 mg/kg/d for 14 consecutive days. At an oral dose of 50 mg/kg/b.i.d. for 7 consecutive days it showed a much lower efficacy [44]. Further optimization to improve oral bioavailability was done, which brought forward SCYX-7158, a clinical drug candidate with extensiv brain exposure using a reduced dose profile with 5 mg/kg for four consecutive days. Toxicity and ADME studies were unproblematic [45], which got SCYX-7158 the clearance for a phase I clinical trial started in March 2012 [46].

Diamidines

That aromatic diamidines have trypanocidal potential has been known since the development of pentamidine, which is still in use to treat first stage *T. b. gambiense*. The starting point was DB 75 (furamidine) with its prodrug DB289 (pafuramidine) [47,48]. The lead showed excellent *in vivo* efficacy, but lacked oral bioavailability in comparison to its prodrug, which was the first orally available drug candidate for first stage sleeping sickness to enter into clinical trials. Unfortunately DB289 had to be abandoned due to liver- and nephrotoxicity in phase III clinical trials. The diamidine project revealed several even more potent diamidines than the ones mentioned before: DB868, an aza analogue of DB289 cured the second stage HAT model and was well tolerated in monkeys [49]. Due to lack of financial support and the fact that already two other promising clinical candidates in the pipeline, the project is currently stopped (personal communication, Tanja Wenzler, Swiss TPH).



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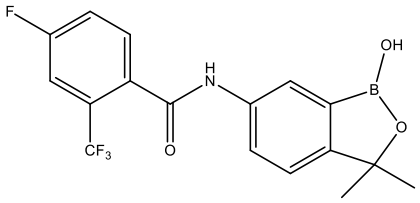
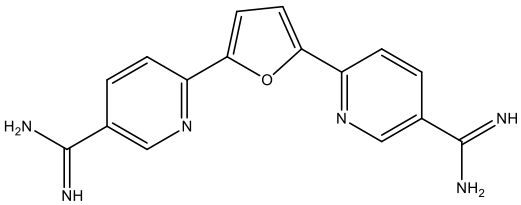


SCYX-7158



DB829

Figure 5. Drug candidates, which are in the clinical trials for registration of new chemical entities against HAT.

1.2. Drug discovery from nature

Considering how difficult it is for a drug to gain market approval and how little it takes to kill a drug candidate, it is pivotal to keep full drug development pipelines and sustain early drug discovery programs. The contemporary approach to identifying such compounds is automated high-throughput screening (HTS) of large and chemically diverse synthetic compound libraries generated by combinatorial chemistry allowing rapid screening and identifying new leads. Sometimes the screening is narrowed down by using more targeted libraries that are thought to be enriched with compounds with a desired type of activity (e.g. kinase) [50].

Another possibility to discover new drugs is to use nature as a potential source. Natural products (NP) derived small molecules are still proving to be an invaluable repository of medicines for mankind. Newman, Cragg, and Sandler recently analyzed all NCEs that enterd the market as registered drugs over the last 30 years [51] and showed that more than 50% of NCEs were NPs, semi-synthetically produced NP derivatives, or else inspired by

NPs. This reflects the significant influence of nature as source for new drugs. Given that NPs have historically provided many novel drugs leads (e.g. khellin, taxol, artemisinin), it might be expected that the industry does everything to identify new metabolites from living organisms, but instead they have decreased their NP research facilities in the past decades, because research on NPs poses several challenges that have to be faced: Hits are likely to have complex structures with an abundance of centers of stereochemistry. Secondary metabolites are limited in quantity in their organisms of origin due to seasonal or environmental variations, which makes the supply of subsequent re-collection difficult. Access to biological material is sometimes limited due to the specific geographic growth area. Local botanists, which are familiar with the flora of the region to properly identify the origin, are needed. Furthermore, species could be endangered and are therefore not allowed to be collected. Often, the structures of active compounds will be already known, which makes it impossible to file patents [52]. So clearly, drug discovery from nature is problematic. So why should we still proceed with drug discovery from nature?

Despite of the huge excitement accompanying the introduction of combinatorial chemistry, the output of active “hits” of <0.001% among these synthetic compound libraries has often been disappointing [53]. In fact, according to Newman’s analysis in 2006 of newly approved drugs in the last 25 years only one drug originated from a HTS screen of a combinatorial chemistry library [54]. With over a 100 NP derived compounds currently undergoing clinical trials and further 100 in preclinical projects it seems that the interest to use natural chemical diversity for drug discovery is now growing once again [55]. NPs provide a unique chemical diversity created by evolution and cover a broad section of chemical space, which is an advantage compared to synthetically produced drugs that can be an inspiration for the creation of compounds with improved pharmacological properties. There has been a trend to use NP’s privileged scaffolds as the cores for compound libraries made by combinatorial chemistry. With this application it becomes possible to create novel NP derived structures that can be patented. Furthermore, there have been many recent improvements of bioassay guided-fractionation technologies to isolate and purify NPs and advances in nuclear magnetic resonance (NMR) and circular dichroism (CD), which make drug discovery from nature more efficient [56, 57] and more compatible with HTS drug discovery campaigns.

Natural sources such as plants have been used as medicines for thousands of years and were the first and for a long time the only available source to treat mankind’s diseases. With rapid global industrialization a part of the past knowledge will no doubt disappear. According to the WHO, 65% of the world’s population still relies on traditional medicines as a

primary source of healthcare [58], because they neither have access nor can they afford nowadays pharmaceutical medicines.

1.3. Antiprotozoal drug discovery approach using nature as potential source

1.3.1. Establishment of extract libraries and antiprotozoal extracts testing

The typical process of discovering NP hits starts with the screening of large libraries, which themselves may include crude extracts, semi-pure mixtures or purified NPs. Extracts may contain many hundreds of compounds belonging to many different biosynthetic classes. The choice of the extraction solvent determines the chemical composition of an extract. Commonly used solvents are dichloromethane (DCM), hexane, ethyl acetate (EtOAc), and methanol (MeOH). Cell based (*in vitro*) tests are widely used to screen such collections in antiprotozoal drug discovery and only a few micrograms of a crude extract are needed to perform the bioassays. But also target-based assay or even systems with infected animals (*in vivo*) are commonly used tools.

The NP lead discovery projects described in this thesis started with establishing liquid extract libraries derived from plants and fungi, of which many were based on traditionally used medicines. The plant and fungal material was successively extracted with solvents of increasing polarity (*n*-hexane, EtOAc, MeOH), which yielded a set of three extracts for every sample. After drying, the extracts were re-dissolved in dimethylsulfoxide (DMSO; final concentration 10 mg/mL) and stored in 2D-barcoded 96-well plates at -80°C. *In vitro* screening of the extract library against the living parasites *T. b. rhodesiense*, *Plasmodium falciparum* (causative agent of malaria), *Leishmania donovani* (causative agent of leishmaniasis), and *T. cruzi* (causative agent of Chagas disease) was performed at test concentrations of 0.81 µg/mL (low concentration) and 4.81 µg/mL (high concentration) Extracts, which showed more than 50% inhibition against one or more parasite at 4.81 µg/mL were defined as “hit” and further processed to identify the active ingredients [59,60].

1.3.2. Isolation and elucidation of natural products from antiprotozoal active plant and fungi extracts

The most commonly used strategy to identify active NPs from extracts is the bioassay-guided isolation. The extracts are fractioned using chromatographic methods such as open column chromatography whereas fractions are successively tested in bioassays. This approach is time consuming, labor intensive, and expensive [61]. In our laboratory, we therefore established a more efficient approach called HPLC-based activity profiling yielding a much faster drug discovery platform operating in 96-well plate with high standardization and automation. We successfully applied this strategy to find new compounds against the

causative agents of protozoal tropical diseases [62]: If an extract had been shown active in the initial screening, the extract was separated over an analytical scale HPLC and microfractions are collected each minute over 35 min. into a 96-well plate. In parallel spectroscopic data (UV, HR-MS, ESI-MS) were gathered. The microfractions in the plate were subsequently tested in *in vitro* bioassays. The overlay of the HPLC chromatogram with *in vitro* activity results from the fractions enabled the identification of the active fractions and their constituents. The active compounds were then isolated by semi-preparative and/or preparative HPLC after a large scale extraction. Structures were elucidated by 1 and 2-dimensional NMR spectra. For the assignment of the absolute configuration of the NPs circular dichroism (CD) in combination with quantum chemical CD calculations was used. Chirality is often a major issue in NP structure elucidation due to their possession of many centers of stereochemistry [63].

1.3.3. Natural products antiprotozoal *in vitro* and *in vivo* evaluation

The activity of the isolated compounds was assessed using cell-based proliferation assays to determine half maximum inhibition concentrations (IC_{50s}). In parallel, cytotoxicity (rat myoblast cells, L6-cell line) assays were done to determine the compounds selectivities. These were expressed as the selectivity index (SI; ratio IC_{50} L6-cells/ parasitic IC_{50}). We considered a compound to be a "hit" if it had an IC_{50} of < 0.2 µg/mL against *T. b. rhodesiense* and *P. falciparum* with a SI of more than 10. But, even when *in vitro* SI values were high (>100), one can not reasonable extrapolate the toxicity situation to the *in vivo* model. Therefore, *in vivo* cytotoxicity evaluation is necessary to select a maximal non-toxic treatment dose. A cumulative dose of 150 mg/kg i.p. was used to screen pre-toxicity in non-infected mice. In general, the first experiment to determine *in vivo* antiprotozoal activity was to treat infected mice at a dose of 50 mg/kg/d i.p. for four consecutive days. On day 7 postinfection a blood smear was done and the parasites were counted. A cure was defined when the animal showed no parasites after 60 days postinfection. Due to the efficacy results the treatment scheme can be adapted in terms of application route and dose. For modelling late stage sleeping sickness the GVR35 mouse CNS model has been established to determine a drug's BBB permeability and CNS efficacy (Figure 6) [64].

1.4. Potential of secondary metabolites from nature against antiprotozoal diseases

Great efforts have been undertaken over the last decades by numerous research groups and many NPs with antiprotozoal activities have been reported in several reviews [65-68]. Just recently Schmidt reported about 800 *in vitro* active antiprotozoal NPs, of which 32 were tested in animal disease models. In the case of *T. brucei* 126 NPs were reported to have been tested *in vitro* and 2 *in vivo*, whereby one compound had shown *in vivo* parasitemia reduction [69, 70].

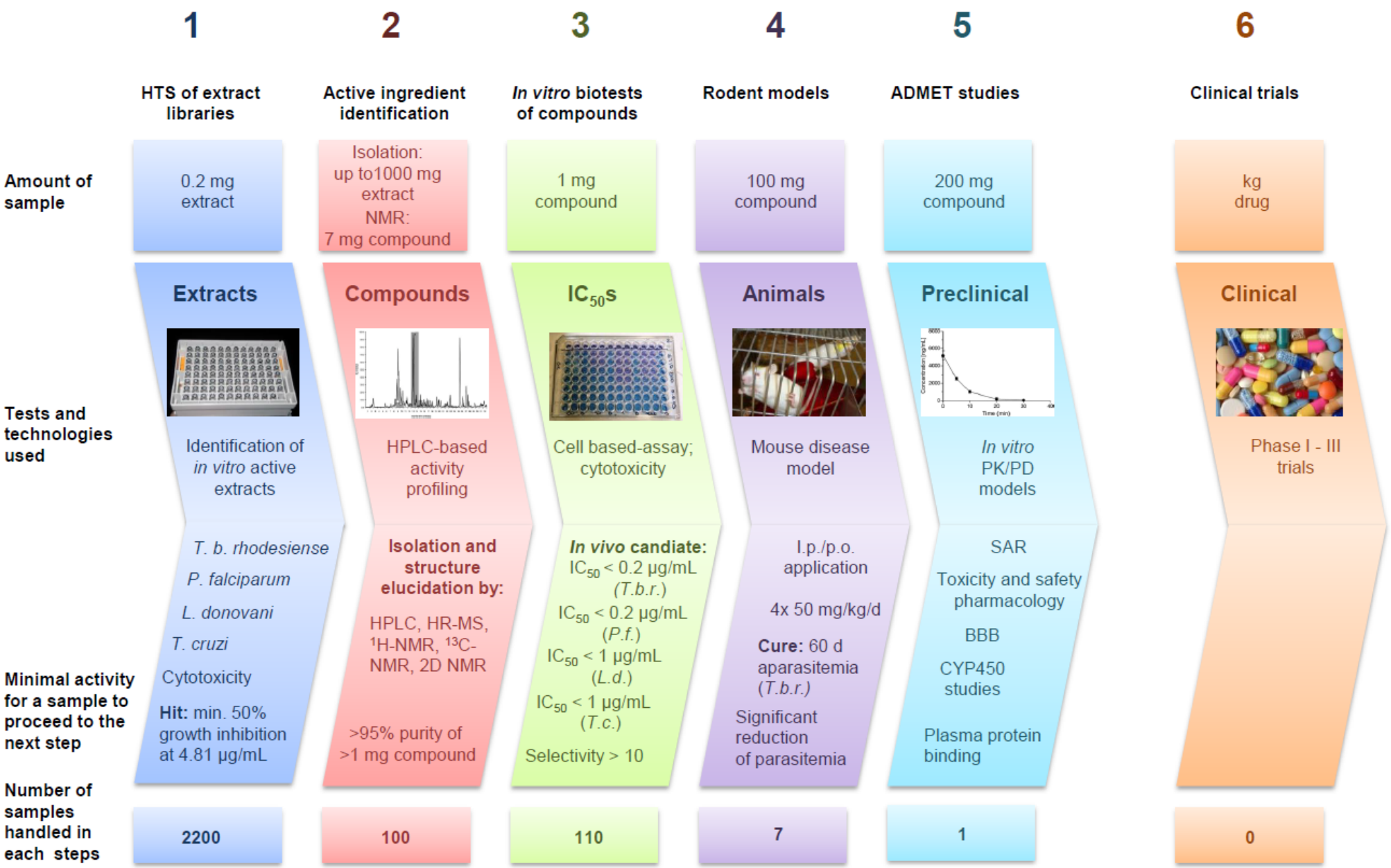


Figure 6: Scheme of antiprotozoal lead discovery steps 1-5 described in this thesis [62, 64, 71-73, 95, 96] leading up to clinical stage 6 [40] (2013, Zimmermann)

Our focused in-house liquid extract library, established over the last seven years to find new antiprotozoal leads, contains a total of 2151 extracts, which were produced from 724 plants and 64 fungi. The *in vitro* HTS campaign against *T. b. rhodesiense*, *P.falciparum*, *L. donovani*, and *T. cruzi* showed that 17.9% of the extracts had an activity of more than 50% at 4.81 µg/mL test concentration against at least one parasite and that 3.4% showed potency of more than 50% at 0.81 µg/mL test concentration [71-73]. The most active ones were chosen to identify the active ingredients by our established HPLC-based activity profiling approach [62]. The rapidly follow-up led to 110 isolated compounds of which 13 inhibited *T. b. rhodesiense* and 3 inhibited *P. falciparum* below 0.5 µM. From these active compounds seven were selected as *in vivo* candidates whereas one NP successfully reduced parasitemia in *T. b. rhodesiense* infected mice (Figure 6) [74-89].

Additionally, the most active NPs against *T. b. rhodesiense* bloodstream forms reported in the last years should be mentioned here: Terpinen-4-ol, a terpene with a characteristic spicy odor, had an IC_{50} of 0.02 µg/mL and a SI of > 1000) [69]. This compound was however not tested *in vivo*. Thomas Schmidt and his co-workers published many active sesquiterpene lactone (STL) derivatives including helenalin, isolated from *Arnica* and *Helenium* species, which was one of the most active compounds with an IC_{50} of 0.051 µM (SI 19.5). Unfortunately, the STL developed *in vivo* toxicity and thus led to its failure [90]. In 2006, a series of 69 flavonoids and flavanoid analogues were tested *in vitro* and *in vivo*. The most promising hit was 7,8-dihydroxyflavone (IC_{50} 0.068 µM; SI 116), which was chosen for *in vivo* tests. Surprisingly, the compound was assessed *in vivo* against *T. b. brucei* instead towards *T. b. rhodesiense*: Infected mice were treated with an i.p. dose of 50 mg/kg/d for four consecutive days. Mice had to be euthanized after 13 days postinfection due to increasing parasitemia [91]. Another interesting example is the marine alkaloid pyridoacridone, which had an IC_{50} of 7.1 nM (SI >100), which is comparable to the IC_{50} of the positive control melarsoprol (IC_{50} 5 nM). Here too, no *in vivo* results can be found in databases [92]. But the most astonishing IC_{50} found in the literature was sinefungin, a natural produced nucleoside by *Streptomyces* with IC_{50} of 0.4 nM and SI more than 10^6, which was 10 fold more active than melarsoprol. Mice infected with *T. b. brucei* were cured when it was administered i.p., but nephrotoxicity in goats blocked any further studies. *In vivo* studies with *T. b. rhodesiense* infected mice were not done (Figure 7) [93, 94].

In summary, many NPs with potent *in vitro* antitrypanosomal activity have been reported, for which *in vivo* testing would be justified, but in many cases no reports on such *in vivo* studies exist. Reasons could be that the isolated amount was not sufficient to go on with animal tests or *in vivo* tests were performed, but due to a negative outcome the results were not published. *In vitro* active compounds with lacking *in vivo* activity should not be simply abandoned, but instead structural modification should be done to increase their bioavailability and efficacy. Considering the high numbers of screened NPs against tropical diseases it is astonishing that only two made it to the market: quinine and artemisinin. Both have also been the leads for further semi-synthetically produced compounds against malaria.

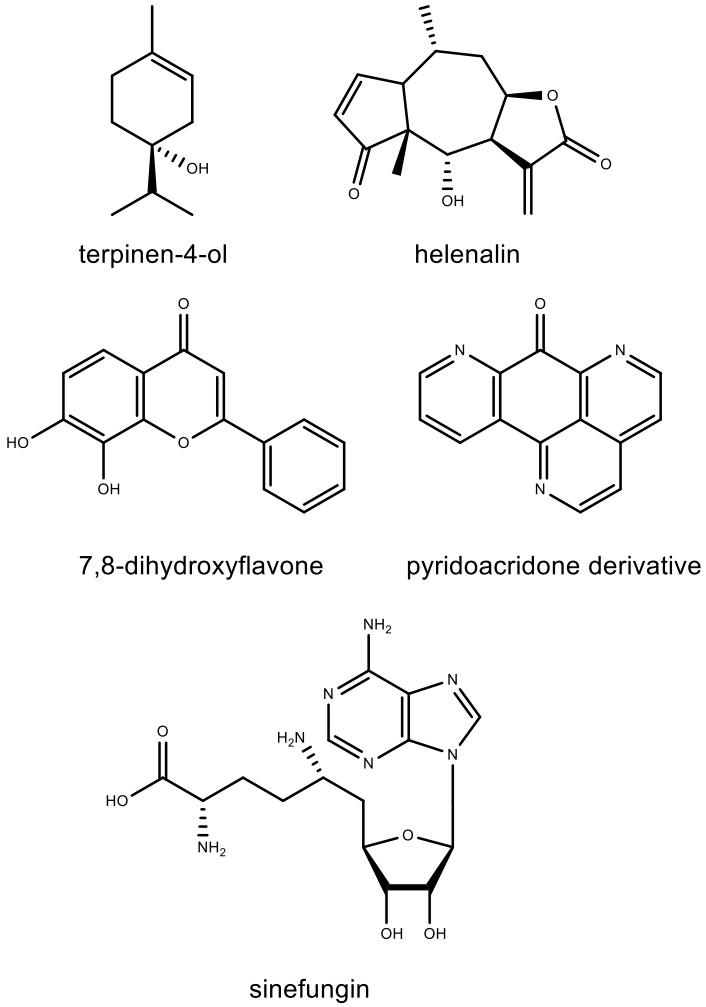


Figure 7. The most active NPs *against T. b. rhodesiense* bloodstream forms.

From all these reports it becomes evident that further studies to find new lead or drug candidates from nature will be highly rewarding.

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CHAPTER 2

Antiprotozoal screening of European macromycetes and European plants

First publication:

The paper highlights the potential of macrofungi for drug discovery by presenting a screen of 200 extracts against *P. falciparum* and *T. b. rhodesiense*. Three fungal extracts had determined IC_{50}s below 10 ng/mL making them up to three orders of magnitude lower than the most potent plant extracts. These findings were underlined by the truffle *Elaphomyces granulates in vivo* activity when tested at 50 mg/kg/d i.p. for four consecutive days in the *T. b. rhodesiense* acute mouse model where mice remained parasite free for 14 days [1].

In vitro testing of the liquid extract library against P. falciparum and T. b. rhodesiense, determination of extracts IC_{50}s against P. falciparum, T. b. rhodesiense, and L6-cells (cytotoxicity), writing of the manuscript, and preparation of both tables were my contributions to this publication.

Second publication:

Based on a survey of remedies used in Renaissance Europe to treat malaria, a library of 254 extracts from 61 plants for *in vitro* antiplasmodial activity was studied. HPLC-based activity profiling of *Arctium nemorosum* led to the identification of onopordopicrin, a germacranolide STL, as a potent inhibitor against *P. falciparum* (IC_{50} of 6.9 µM). With an IC_{50} of 0.37 µM (SI 8.2) against *T. b. rhodesiense* was onopordopicrin one of the most potent NPs reported so far [2].

In vitro testing of the liquid extract library against P. falciparum, HPLC-based activity profiling (biological part) of Hyssopus officinalis and Arctium nemorosum, IC_{50} determination of all isolated compounds against P. falciparum, T. b. rhodesiense, and L6-cells (cytotoxicity), writing of the manuscript, and preparation of figures (except of Fig. 1 and 4) and tables were my contribution to this publication.

Stefanie Zimmermann

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Mushrooms: the unexploited source of drugs. An example of an antitrypanosomal screen.

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Abstract

Macrofungi are a rich source of natural products, but have been far less used in drug discovery, than higher plants. In this study we explore their antiprotozoal potential in a screen of 192 extracts from 64 European macromycete species against the causal agents of malaria, human African trypansosomiasis, leishmaniasis, and Chagas disease. Several extracts showed very potent and specific antiprotozoal effects. Most noteworthy were the extract of *Cordyceps ophioglossoides*, which inhibited *Leishmania donovani* with an IC_{50} of 30 ng/mL and *Plasmodium falciparum* with an IC_{50} of 40 ng/mL. *Elaphomyces punctatus* was active against *Trypanosoma brucei rhodesiense* (ethyl acetate extract IC_{50}: 20 ng/mL; methanol extract IC_{50}: 20 ng/mL) and *P. falciparum* (ethyl acetate extract IC_{50}: 30 ng/mL; methanol extract IC_{50}: 60 ng/mL). This medium throughput antiprotozoal screen of macromycetes demonstrates that the hit rate is similar to the screening results of higher plants. The observed potency and selectivity of several fungi extracts was thus three orders of magnitude stronger than what is typically observed in plant screens. Macrofungi are an underutilized, but very promising source for antiprotozoal drug discovery.

Keywords:

Macrofungi, screening, *Trypanosoma, Plasmodium, Leishmania, antiprotozoal*

Introduction

Whilst more than half of our native higher plant flora has been studied phytochemically and more than a quarter for biological activities [1], fungi remain a relatively poorly studied source of natural products in modern drug discovery**.** With estimated 1.5 to 5 million species [2, 3] fungi outnumber higher plants by at least 16:1, yet only about 100,000 species have been taxonomically classified [4]. Macrofungi such as mushrooms, toadstools, puffballs, truffles, etc., are fungi that form conspicuous spore-bearing fruiting bodies visible to the naked eye. They comprise about 10% of all fungi [5] and their species numbers in temperate regions are at least comparable to those of higher plants, but they are less well inventoried. A Swiss mushroom field guide [6], for instance, lists 2'486 native species compared to the roughly 3'000 native taxa of higher plants [7]. Most research on fungal metabolites has been focused on a few narrow taxonomic groups within the Ascomycetes, like the genera *Penicillium* (penicillins*)* or *Tolypocladium* (cyclosporins), which can be easily cultured. Most slow growing or uncultivable groups of fungi, like most macrofungi were never studied [3, 8]. Starting in the 1940s natural products from terrestrial fungi -alongside other microorganisms- became a main focus of the pharmaceutical industry as antibiotics like the penicillins, cephalosporins, and polyketides, later followed by many more indications such as the immunosuppressive cyclosporins, anti-helmintic ivermectin, as well as the best selling drug of all times the statin Lipitor™, developed from the fungal metabolite mevastatin [9].

In rare cases when mushrooms were chemically studied they provided remarkable lead compounds like retapamulin, a semisynthetic derivative of pleuromutilin from the mushroom *Clitopilus passeckerianus* marketed by Glaxo Smith Kline for the topical treatment of skin infections [10], irofulvene a derivative of the sesquiterpene illudin from *Omphalotus illudens* [11]*,* currently investigated in clinical trials for prostate, ovarian, and other cancers, or strobilurin from the mushroom *Strobilurus tenacellus*, which led to the development of the strobilurine fungicides - now a multi-billion dollar market [12]. Despite being a potential treasure trove, macrofungi remain a chemically poorly studied group of organisms. The reason for this may lie in their overwhelming taxonomic complexity and ephemerity and rareness of sporophore production. Many species are not easily cultivable, largely due to highly specialized lifestyles (e.g. mycorrhizal, endophytic, parasitic and other symbiotic relationships), and therefore large amounts of fungal matter for chemical investigation are hard to obtain. The last decades saw great technological advances in the field of analytics and biotesting technologies making it possible to venture deep into areas of biological and chemical diversity previously not accessible. Our group has previously establishing methodologies, which allow to chemically study larger numbers of samples in smaller amounts than ever before, by using technologies like HPLC-based activity profiling to identify actives in samples containing just mg amounts [13, 14]. This study is all about harnessing

these technological advances to systematically probe mushroom chemodiversity as a source of potential antiprotozoal leads. In this study we have ventured into the world of European macrofungi to determine whether they are a promising source of potential antiprotozoal natural products. We collected wild fungi, established a library of 192 extracts, and screened them against the pathogens, which cause malaria, human African trypanosomiasis, leishmaniasis, and Chagas disease.

Each year, every 14th person in the world is infected with malaria and 1-2 million die of it – most of them infants [15]. Thirty million people annually contract and 120'000 die of one of the protozoan "neglected tropical diseases" [16], which include Chagas disease, human African trypanosomiasis, and leishmaniasis [17]. To treat trypanosomatid infections (*Trypanosoma* and *Leishmania*) there are only few drugs on the market and their pharmacological profiles are insufficient by modern standards. These sicknesses have in common, that they are all insect borne diseases and strongly linked to poverty. Malaria drugs are now reasonably affordable, available and safe, yet few in number and increasingly compromised by resistances [18]. New drug leads with new modes of action to combat these perils of mankind are urgently needed.

Materials and Methods

Establishment of a fungal library

A collection of 64 mushrooms was compiled in September 2011 and identified at a species level. Fungal specimens were collected in two Austrian federal states (Styria, Lower Austria) in various forest types, mostly in beech-fir-spruce forest and in productions forests dominated by spruce. The selection of species was guided both by available material (to ensure that sufficient material for extraction be available) and by taxonomy, to represent the phylogenetic and ecological diversity of macrofungi as good as possible with a restricted sampling. The collection bias towards basidiomycetes is representative of the respective communities of fleshy macromycetes. Prior knowledge of medical mushrooms was not used for species selection. All fungi were dried in a fungal drying apparatus (Dörrex, SIGG, CH) and voucher specimens were deposited at the Department of Pharmaceutical Sciences, University of Basel. The voucher numbers are shown in **Table S1**. For extraction 1 g of sample was used, in some cases when not enough material was available as little as 0.28 g was taken. First the fungal material was finely ground using a ZM1 ultra centrifugal mill (Retsch; Haan, Germany). Powdered material was then successively extracted first with petrol ether, then ethyl acetate and finally methanol (all solvents from Scharlau, Barcelona, Spain) using an accelerated solvent extraction system ASE (ASE 200, Dionex, Switzerland; 3 cycles at 120 bar, and 70°C) to give a set of three extracts of increasing polarity for every sample, totalling

192 extracts. The extracts were formatted into a library of solutions at 10 mg/ml DMSO in three 2D-barcoded 96 well plates, of which three copies were made (Zinsser Analytics, France). The plates were stored at –80° C, until use.

Antiprotozoal screening

Antiprotozoal screening was repeated twice at 0.8 and 4.8 µg/mL. The most active extracts were tested in serial dilution in two independent assays to determine their 50% inhibitory concentration (IC_{50}).

Protozoan parasites and cell line

Trypanosoma brucei rhodesiense (STIB 900) were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with 0.2 mM 2-mercaptoethanol as described by Baltz et al. [19] with the following modifications: 1 mM sodium pyruvate, 0.5 mM hypoxanthine, and 15% heat-inactivated horse serum. Cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C. *Trypanosoma cruzi* trypomastigote forms (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) were cultured in RPMI 1640 medium supplement with 10% fetal bovine serum and 2 mM L-glutamine [20] and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Antiplasmodial activity was determined against the chloroquine- and pyrimethamine-resistant *Plasmodium falciparum* K1 strain. The parasites were maintained by the method of [21] in a humidified atmosphere consisting of 4% CO_2, 3% O_2, and 93 % N_2 at 37 °C. Rat skeletal myoblast cells (L6-cells) were seeded in RPMI 1640 medium supplemented with 2 µM L-glutamine, 5.95 g/L HEPES, 2 g/L $NaHCO_3$ and 10% fetal bovine serum as previously reported [13]. The cultures were routinely maintained by weekly passages at 37 °C under a humidified 5% CO_2 atmosphere.

Bioassays

***Trypanosoma brucei rhodesiense* (STIB 900 strain) bioassay**

Evaluation of *in vitro* antiprotozoal activity against *T. b. rhodesiense* was done using the Alamar Blue assay to determine IC_{50}s as previously described [22]. Serial threefold dilution were prepared in 96-well micro titer plates and 2000 *T. b. rhodesiense* STIB 900 bloodstream forms in 50 µL were added to each well except for the negative controls. Melarsoprol (Arsobal®, purity> 95%, Sanofi-Aventis, Meyrin, Switzerland) was used as reference drug. After 70 h of incubation 10 µL of Alamar blue marker (12.5 mg resazurin (Sigma-Aldrich, Buchs, Switzerland) dissolved in 100 mL of distilled water) was added, and color change was developed for 2 to 6 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices Cooperation, Sunnyvale, CA) with an excitation wavelength of 536 nm and an emission wavelength of 588 nm was used to read the plates. The IC_{50} values

were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

***Trypanosoma cruzi* bioassay**

In vitro testing against *T. cruzi*: Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium. After 24 h the medium was removed and replaced by 100 µL containing 5000 trypomastigote forms of *T. cruzi*. After 48 h the medium was removed and replaced by 100 µL of fresh medium with the serial threefold drug dilutions. The plates were incubated under a humidified 5% CO_2 atmosphere at 37°C for an additional 96 h. Then chlorophenyl red β-D-galactopyranoside agent (CPRG)/Nonident (50 µL) (Sigma-Aldrich) was added to all wells and a color change was developed within 2 to 6 h. The plates were read photometrically at 540 nm. Data were evaluated and IC_{50} values calculated with Softmax Pro software (Molecular Devices) [20]. Benznidazole (purity > 95%, Sigma-Aldrich) was used as a standard drug.

***Plasmodium falciparum* bioassay**

In vitro testing against *P. falciparum*: Antiplasmodial activity was determined with the [^{3}H]-hypoxanthine incorporation assay [23]. Chloroquine (purity > 95%, Sigma-Aldrich) and artesunate (purity > 95%, Mepha, Switzerland) were used as standard drugs. Briefly, infected human red blood cells (final parasitemia and hematocrit were 0.3% and 1.25%, respectively) in RPMI 1640 medium were exposed to twofold serial drug dilutions in 96-well micro titer plates. After 48 h of incubation, 50 µL of [^{3}H]-hypoxanthine (0.5 µCi) were added to each well. The plates were incubated for further 24 h before being harvested using a Betaplate cell harvester (Wallac, Zürich, Switzerland) onto glass-fiber filters and then washed with distilled water. The dried filters were inserted into plastic foils with 10 mL scintillation fluid. The radioactivity was counted with a Betaplate liquid scintillation counter (Wallac) as counts per minute per well at each drug concentration and compared to the untreated controls. IC_{50} values were calculated from sigmoidal inhibition curve.

Rat myoblast cell L6-cytotoxicity assay

The cytotoxicity assay was performed using the Alamar Blue assay [22] described above with rat skeletal myoblasts (L6-cells) seeded in 100 µL RPMI 1640 in 96-well micro titer plates. After 24 h the medium was removed and replaced by 100 µL of fresh RPMI 1640 with or without a serial threefold drug dilution. Podophyllotoxin (purity > 95%, Sigma-Aldrich) was used as the reference drug. After 70 h of incubation under a humidified 5% CO_2 atmosphere, 10 µL of the Alamar blue marker (see above) was added to all wells. The plates were incubated for an additional 2 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices) was used to read the plates using an excitation wavelength of 536 nm

and an emission wavelength of 588 nm. The IC_{50}s were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

Acute mouse sleeping sickness model

This model mimics the first stage of the human African trypanosmiasis. Adult female NMRI mice were purchased from Janvier (St. Berthevin, France). They weighed between 20 and 25 g at the beginning of the study and were kept under standard conditions in macrolon type III cages with food pellets and water *ad libitum* at 22 °C and 60-70% humidity. All protocols and procedures used in this study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland (authorization N° 739; 11.12.2009). The samples were first dissolved in 100% DMSO followed by addition of distilled H_2O to a final DMSO concentration of 10%. For the establishment of the *in vivo* antitrypanosomal activity, the mice were infected intraperitoneally with 1 x 10^4 STIB900 bloodstream forms. Experimental groups of four mice were treated orally once a day on four consecutive days from day 3 to day 6 post infection. A control group of four mice was infected, but remained untreated. The determination of the parasitemia was done on day 7 post infection. Six µL of tail blood were diluted in 24 µL sodium citrate (3.2%), whereby the first µL was discarded to obtain circulating blood. Five µL of this mixture were transferred to a glass slide and covered with an 18 x 18 mm cover slide. The sample was examined under a light microscope (200-fold magnification) and parasites were counted in 3 of the 16 squares of the grid.

Results and Discussion

A total of 192 extracts, which represent three extracts of different polarity (petrol ether, ethyl acetate, and methanol) from different 64 fungi were screened against the protozoan parasites that cause malaria, human African trypanosomiasis, Chagas disease, and leishmaniasis. The results of the fungal extract screening (**Table S1**) showed two extracts from one fungus (*Elaphomyces granulates*) with a 100% inhibition at the lower test concentration against *T. brucei rhodesiense. Pycnoporus cinnabarinus* showed 77% inhibition of *P. falciparum* at the higher concentration and *Elaphomyces granulates* indicated a 75% inhibition of *P. falciparum* at the higher concentration. The most promising extracts *Cordyceps ophioglossoides* (EtOAc extract), *Elaphomyces granulates* (EtOAc and MeOH extract), and *Pycnoporus cinnabarinus* (EtOAc and MeOH extract) were tested and IC_{50} were determined against *T. b. rhodesisense, P.falciparum*, *L. donovani*, und *T. cruzi.* Additionally, cytotoxicities against rodent cell line were established to show their selectivity toward the protozoan parasites. None of these active extracts were generally toxic towards the rodent cell line and the cytotoxic effects were moderate with selectivities of more than 100 in the case of *Elaphomyces granulatus*. With the next step we study the extracts by HPLC-based activity profiling to isolate and identify their active constituents.

Our research collaboration has in recent years been involved in the screening of many thousand plant extracts against protozoan parasites and the subsequent identification of active natural products from them [24-33].

Hereby we have learned that in these screening systems the test concentration of 4.81 µg/mL were a stringent selection criteria to identify just a small percentage of samples (< 5%) as actives. At the six-fold dilution (0.81 µg/mL) only very few of these would be promising. Applying these filters of defining actives, this screen of European macrofungi shows a similar "hit rate" as many screens of plant extract libraries [34] with 2 out of 64 samples (3.1%) showing total inhibition of at least one parasite at the lower screen concentration. What really puts the fungi in a class of their own though, is that the few actives showed astonishing activities and selectivities. Several active extracts showed IC_{50}s below 60 ng/mL (**Table 1**) and that they were up to three orders of magnitude stronger than anything we had experienced with plant extracts.

These results were supported by *in vivo* antitrypanosomal activity of the *Elaphomyces* EtOAc extract when mice were treated *ip* with 50 mg/kg/body weight/day for four consecutive days. In comparison to the control group, which were euthanized after a week, were treated mice parasite-free for 14 days postinfection.

It is noteworthy that this screen did not detect generally toxic fungi as in fact *Elaphomyces* is regarded as non toxic or even edible when cooked [35]. This study suggests that extracts of macrofungi can show antiparasitic effects more potent than the far better studied plant extracts and the relative frequency of this could be similar or higher than for plant extracts. This study is the starting point for a chemical study of the most promising fungi to identify their active constituents.

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Table 1 Antiprotozoal effects (IC_{50} in µg/mL ± standard of the mean SD) of 5 selected extracts against the 4 protozoal parasites *T.b.rhodesisense, T. cruzi, L. donovani*, and *P. falciparum*, as well as the cytotoxic effects against rat myoblast (L6) cells.

extract	Extract solvent	*T.b. rhodesiense*	*T. cruzi*	*L. donovani*	*P. falciparum*	L6
Cordyceps ophioglossoides	EtOAc	1.1 ± 0.1	5.7 ± 8.6	0.03 ± 0.01	0.4 ± 0.02	3.8 ± 0.4
Elaphomyces granulatus	EtOAc	0.02 ± 0.001	4.8 ± 1.0	0.3 ± 0.1	0.03 ± 0.03	> 20
Elaphomyces granulatus	MeOH	0.02 ± 0.001	8.6 ± 0.01	1.2 ± 0.1	0.06 ± 0.04	> 20
Pycnoporus cinnabarinus	EtOAc	5.9 ± 2.3	9.6 ± 0.1	2.8 ± 0.8	1.7 ± 0.06	2.6 ± 1.3
Pycnoporus cinnabarinus	MeOH	4.0 ± 0.3	7.7 ± 3.6	2.1 ± 1.0	1.6 ± 0.2	1.1 ± 0.1

SUPPORTING INFORMATION

Mushrooms: the unexploited source of drugs. An example of an antitrypanosomal screen.

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Table S1 Antiplasmodial and antitrypanosomal activity (growth inhibition in % ± standard of the mean SD) of 196 fungal extracts against STIB 900 strain and *Plasmodium falciparum* (K1) strain. Bioassays were carried out in two independent experiments, at test concentrations of 4.81 (higher concentration= HC) µg/mL and 0.81 µg/mL (lower concentration= LC), respectively. The positive control was artesunate (100% inhibition in all bioassays) and melarsoprol.

			T.b. rhodesiense STIB 900		*P. falciparum* K1	
Species	**Extract solvent**	**Organism number**	**HC ± SD**	**LC ± SD**	**HC ± SD**	**LC ± SD**
Boletus radicans Pers.	PE	MM001	6.5 ± 9.2	10.6 ± 3.9	21.1 ± 9.1	24.3 ± 6.9
	EtOAC	MM001	10.2 ± 4.5	5.4 ± 7.6	21.1 ± 0.0	33.2 ± 17.5
	MeOH	MM001	9.8 ± 6.3	9.9 ± 2.2	24.3 ± 16.1	14.2 ± 10.6
Flammulina velutipes (Curtis) Singer	PE	MM002	4.2 ± 2.1	7.1 ± 4.7	17.0 ± 10.5	13.5 ± 2.8
	EtOAC	MM002	4.6 ± 6.4	3.0 ± 1.8	6.6 ± 1.1	13.0 ± 10.5
	MeOH	MM002	6.4 ± 5.3	10.6 ± 2.0	15.2 ± 9.0	0.4 ± 0.6
Piptoporus betulinus (Bull.) P. Karst	PE	MM003	6.9 ± 4.0	1.6 ± 2.2	13.0 ± 7.0	15.0 ± 2.1
	EtOAC	MM003	1.5 ± 2.1	1.8 ± 2.5	34.4 ± 10.8	7.8 ± 7.1
	MeOH	MM003	8.7 ± 12.3	5.6 ± 7.9	21.2 ± 19.5	23.1 ± 9.3
Lactarius vellereus (Fr.) Fr	PE	MM004	9.5 ± 7.1	10.2 ± 4.0	26.4 ± 11.2	18.0 ± 2.7
	EtOAC	MM004	11.5 ± 11.6	7.8 ± 10.4	28.0 ± 11.4	17.2 ± 7.3
	MeOH	MM004	6.5 ± 9.1	0.0 ± 0.0	23.6 ± 10.1	2.4 ± 1.6
Russula badia Quél.	PE	MM005	6.2 ± 8.8	2.9 ± 1.6	16.0 ± 20.0	23.9 ± 2.1
	EtOAC	MM005	4.9 ± 6.9	1.3 ± 1.8	23.1 ± 7.6	13.5 ± 1.5
	MeOH	MM005	6.9 ± 4.7	0.0 ± 0.0	13.8 ± 13.6	8.5 ± 12.0
Amanita citrina (Schaeff.) Pers.	PE	MM006	4.8 ± 6.8	0.0 ± 0.0	17.7 ± 6.1	1.8 ± 2.5
	EtOAC	MM006	2.3 ± 3.2	2.6 ± 3.7	2.0 ± 2.8	4.4 ± 0.5
	MeOH	MM006	0.0 ± 0.0	0.0 ± 0.0	19.8 ± 13.9	5.6 ± 1.9
Amanita muscaria (L.) Lam.	PE	MM007	0.0 ± 0.0	2.7 ± 3.8	9.4 ± 1.8	0.7 ± 1.0
	EtOAC	MM007	7.0 ± 9.9	4.1 ± 5.7	19.0 ± 11.1	14.1 ± 4.1
	MeOH	MM007	3.4 ± 4.8	3.1 ± 4.3	16.1 ± 14.3	16.9 ± 6.7
Hygrocybe virginea (Wulfen) P.D. Orton & Watling	PE	MM008	7.9 ± 5.9	3.2 ± 4.5	28.4 ± 3.3	12.8 ± 13.5
	EtOAC	MM008	4.4 ± 6.2	0.0 ± 0.0	19.1 ± 5.2	9.5 ± 5.0
	MeOH	MM008	0.0 ± 0.0	1.1 ± 1.6	12.8 ± 1.9	37.0 ± 6.1
Hygrocybe psittacina (Schaeff.) P. Kumm.	PE	MM009	3.7 ± 5.2	3.9 ± 1.7	8.0 ± 3.9	7.3 ± 10.3
	EtOAC	MM009	2.0 ± 1.5	0.8 ± 1.1	1.9 ± 2.7	1.6 ± 2.2
	MeOH	MM009	0.0 ± 0.0	1.1 ± 1.6	15.0 ± 4.1	4.6 ± 6.5
Lactarius semisanguifluus R. Heim & Leclair	PE	MM010	0.0 ± 0.0	0.0 ± 0.0	17.6 ± 5.5	2.2 ± 3.1
	EtOAC	MM010	0.0 ± 0.0	0.0 ± 0.0	20.8 ± 4.0	0.3 ± 0.4
	MeOH	MM010	0.0 ± 0.0	3.6 ± 5.1	14.6 ± 7.5	0.0 ± 0.0

Hygrobe conica (Schaeff.) P. Kumm.	PE	MM011	2.8 ± 4.0	4.1 ± 4.5	18.1 ± 15.6	10.6 ± 0.5
	EtOAC	MM011	0.3 ± 0.4	4.5 ± 6.3	14.9 ± 5.4	5.3 ± 7.1
	MeOH	MM011	7.5 ± 2.8	3.5 ± 2.8	17.2 ± 2.7	8.9 ± 2.9
Chroogomphus rutilus (Schaeff.: Fr.) O.K. Mill.	PE	MM012	21.9 ± 5.6	8.7 ± 4.4	17.2 ± 14.6	8.9 ± 5.0
	EtOAC	MM012	8.0 ± 11.2	2.2 ± 3.1	19.3 ± 8.9	1.3 ± 1.8
	MeOH	MM012	9.0 ± 2.8	6.9 ± 4.5	15.9 ± 17.0	3.5 ± 5.0
Scleroderma citrinum Pers.	PE	MM013	0.9 ± 1.2	1.4 ± 1.9	15.9 ± 9.4	5.0 ± 7.1
	EtOAC	MM013	0.8 ± 1.1	0.0 ± 0.0	34.9 ± 13.7	5.4 ± 2.3
	MeOH	MM013	0.0 ± 0.0	0.6 ± 0.8	11.2 ± 10.1	2.0 ± 0.9
Xerocomus badius (Pers.) E.-J. Gilbert	PE	MM014	2.6 ± 2.5	2.4 ± 3.4	13.9 ± 7.4	4.0 ± 0.4
	EtOAC	MM014	1.3 ± 1.8	1.0 ± 1.3	9.4 ± 6.3	2.9 ± 4.1
	MeOH	MM014	6.1 ± 8.6	2.9 ± 1.3	19.8 ± 8.6	12.3 ± 0.4
Xerocomus chrysenteron (Bull.) Quel.	PE	MM015	0.3 ± 0.4	6.4 ± 0.1	18.5 ± 17.3	7.0 ± 0.9
	EtOAC	MM015	6.8 ± 6.6	3.7 ± 5.2	25.6 ± 13.2	8.7 ± 4.9
	MeOH	MM015	5.1 ± 4.7	1.0 ± 1.3	16.7 ± 1.7	3.9 ± 5.0
Chalciporus piperatus (Bull.) Bataille	PE	MM016	5.2 ± 4.3	5.7 ± 0.5	18.5 ± 9.8	9.0 ± 12.7
	EtOAC	MM016	17.8 ± 10.0	0.0 ± 0.0	29.4 ± 13.7	5.9 ± 1.5
	MeOH	MM016	6.2 ± 0.1	3.1 ± 4.3	10.7 ± 15.1	0.3 ± 0.4
Leccinum scabrum var. *melaneum* (Smotl.) Dermek	PE	MM017	0.0 ± 0.0	0.0 ± 0.0	7.0 ± 2.2	3.8 ± 4.3
	EtOAC	MM017	0.0 ± 0.0	4.2 ± 5.9	14.5 ± 11.5	2.3 ± 3.2
	MeOH	MM017	2.8 ± 0.4	2.4 ± 3.4	15.7 ± 7.0	0.0 ± 0.0
Leccinum versipelle (Fr. & Hök) Snell	PE	MM018	1.8 ± 2.5	0.0 ± 0.0	9.6 ± 4.9	0.7 ± 1.0
	EtOAC	MM018	5.8 ± 8.1	1.8 ± 0.8	25.4 ± 7.4	8.8 ± 5.3
	MeOH	MM018	0.0 ± 0.0	2.5 ± 3.5	17.2 ± 12.7	0.0 ± 0.0
Leccinum brunneogriseolum Lannoy & Estadès	PE	MM019	9.4 ± 1.8	2.7 ± 1.4	21.1 ± 4.7	15.5 ± 8.8
	EtOAC	MM019	3.7 ± 5.2	4.8 ± 6.7	21.2 ± 4.7	8.2 ± 10.8
	MeOH	MM019	2.6 ± 2.6	0.9 ± 0.9	16.5 ± 13.3	1.6 ± 2.2
Paxillus involutus (Batsch) Fr.	PE	MM020	3.5 ± 4.9	6.0 ± 2.5	13.7 ± 10.3	3.5 ± 5.0
	EtOAC	MM020	0.0 ± 0.0	4.4 ± 6.2	30.2 ± 27.5	1.8 ± 2.6
	MeOH	MM020	0.0 ± 0.0	0.0 ± 0.0	21.0 ± 4.6	10.8 ± 2.6
Hydrophobus agathosmus (Fr.) Fr.	PE	MM021	0.0 ± 0.0	1.5 ± 2.1	20.3 ± 7.4	1.9 ± 2.6
	EtOAC	MM021	1.3 ± 1.8	2.8 ± 4.0	15.0 ± 11.0	7.2 ± 1.4
	MeOH	MM021	0.1 ± 0.1	1.4 ± 2.0	6.1 ± 1.1	0.0 ± 0.0
Lactarius turpis (Weinm.) Fr.	PE	MM022	2.2 ± 3.0	5.1 ± 7.2	28.4 ± 3.6	19.4 ± 4.2
	EtOAC	MM022	0.0 ± 0.0	3.1 ± 4.3	17.7 ± 3.6	2.3 ± 2.6
	MeOH	MM022	4.4 ± 6.2	2.1 ± 1.9	16.4 ± 1.8	14.4 ± 10.3
Lactarius glyciosmus (Fr.) Fr.	PE	MM023	9.3 ± 3.1	6.9 ± 2.2	21.1 ± 2.1	9.9 ± 3.7
	EtOAC	MM023	6.2 ± 4.1	6.7 ± 5.2	19.9 ± 10.7	3.5 ± 1.1
	MeOH	MM023	7.9 ± 1.0	3.0 ± 1.8	21.8 ± 10.0	6.6 ± 2.3
Macrocysridia cucumis (Pers.) Joss.	PE	MM024	0.8 ± 1.1	5.0 ± 7.0	17.1 ± 10.5	2.6 ± 3.6

	EtOAC	MM024	0.0 ± 0.0	0.1 ± 0.1	16.2 ± 6.5	3.9 ± 0.6
	MeOH	MM024	0.0 ± 0.0	2.0 ± 2.8	26.2 ± 1.2	6.7 ± 9.4
Tricholoma cf. *fulvum* (Fr.) Bigeard & H. Guill.	PE	MM025	0.0 ± 0.0	2.2 ± 3.1	21.1 ± 7.9	15.2 ± 0.1
	EtOAC	MM025	4.1± 5.7	4.7 ± 6.6	12.8 ± 9.2	1.0 ± 0.9
	MeOH	MM025	5.8 ± 4.0	1.0 ± 1.3	23.7 ± 10.0	15.4 ± 15.3
Clitopilus prunulus (Scop.) P. Kumm	PE	MM026	3.7 ± 5.0	2.7 ± 3.8	18.3 ± 2.8	13.7 ± 10.5
Clitopilus prunulus (Scop.) P. Kumm	EtOAC	MM026	2.8 ± 3.4	0.3 ± 0.4	17.6 ± 9.3	12.1 ± 16.5
	MeOH	MM026	12.5 ± 5.2	5.3 ± 0.1	26.9 ± 12.8	16.8 ± 2.3
Climacocystis borealis (Fr.) Kotl. & Pouzar	PE	MM027	2.7 ± 3.7	6.1 ± 0.9	29.3 ± 5.9	12.1 ± 12.1
	EtOAC	MM027	11.6 ± 3.0	0.0 ± 0.0	20.5 ± 8.2	11.9 ± 4.4
	MeOH	MM027	14.4 ± 5.1	6.6 ± 7.1	30.2 ± 4.2	13.1 ± 1.6
Cortinarius bolaris (Pers.) Fr.	PE	MM028	8.8 ± 2.1	4.3 ± 0.9	16.6 ± 0.8	8.4 ± 2.8
	EtOAC	MM028	7.1 ± 0.9	4.7 ± 6.6	22.4 ± 0.1	0.8 ± 1.1
	MeOH	MM028	14.6 ± 9.0	5.1 ± 2.8	14.1 ± 2.0	6.8 ± 6.2
Mycetinis alliaceus (Jacq.) Earle ex A.W. Wilson & Desjardin	PE	MM029	3.8 ± 5.3	7.5 ± 4.0	17.5 ± 5.5	5.0 ± 1.3
	EtOAC	MM029	11.9 ± 3.4	4.9 ± 3.0	24.2 ± 9.3	14.1 ± 6.0
	MeOH	MM029	3.4 ± 1.8	3.4 ± 1.1	28.8 ± 1.7	9.1 ± 2.9
Clitocybula lacerata (Scop.) Singer ex Métrod	PE	MM030	15.2 ± 9.2	19.4 ± 16.3	27.2 ± 11.5	29.0 ± 7.2
	EtOAC	MM030	19.1 ± 14.2	13.0 ± 11.0	34.9 ± 13.5	16.2 ± 17.3
	MeOH	MM030	17.2 ± 17.6	16.3 ± 17.7	33.0 ± 18.0	19.1 ± 7.1
Hydnum repandum L.	PE	MM031	15.0 ± 10.1	13.0 ± 7.8	32.1 ± 20.2	25.7 ± 16.7
	EtOAC	MM031	11.2 ± 8.2	3.3 ± 4.6	7.7 ± 10.8	7.1 ± 10.0
	MeOH	MM031	13.3 ± 18.8	7.4 ± 10.4	25.9 ± 12.0	12.3 ± 15.2
Fomitopsis pinicola (Sw.) P. Karst.	PE	MM032	7.8 ± 11.0	0.8 ± 1.1	28.4 ± 16.7	4.4 ± 6.2
	EtOAC	MM032	3.6 ± 5.0	3.9 ± 5.5	34.6 ± 0.5	6.9 ± 3.5
	MeOH	MM032	8.2 ± 10.3	8.4 ± 9.8	30.4 ± 6.8	23.3 ± 12.7
Pycnoporus cinnabarinus (Jacq.) P. Karst	PE	MM033	9.6 ± 12.2	7.7 ± 10.8	27.2 ± 13.6	5.6 ± 7.9
	EtOAC	MM033	10.3 ± 14.6	4.3 ± 6.0	68.2 ± 5.2	14.5 ± 11.5
	MeOH	MM033	30.5 ± 26.4	7.2 ± 10.1	77.2 ± 16.7	22.7 ± 13.2
Russula densifolia Secr. Ex Gillet	PE	MM034	5.6 ± 7.9	0.0 ± 0.0	20.8 ± 18.5	16.8 ± 14.1
	EtOAC	MM034	1.0 ± 1.3	2.2 ± 3.0	34.9 ± 17.6	22.8 ± 17.4
	MeOH	MM034	1.1 ± 1.6	2.3 ± 3.3	30.0 ± 13.9	21.3 ± 13.3
Pleurocybella porrigens (Pers.) Singer	PE	MM035	6.8 ± 9.6	7.0 ± 4.7	19.3 ± 3.0	9.0 ± 3.9
	EtOAC	MM035	13.4 ± 12.2	5.6 ± 7.8	25.8 ± 24.7	2.1 ± 3.0
	MeOH	MM035	8.9 ± 12.5	7.5 ± 10.5	16.1 ± 5.8	12.8 ± 3.2
Porphyrellus porphyrosporus (Fr. & Hök) E.-J. Gilbert	PE	MM036	5.8 ± 8.2	0.6 ± 0.8	21.4 ± 11.8	9.3 ± 3.3
	EtOAC	MM036	10.3 ± 8.9	8.1 ± 1.0	22.2 ± 7.3	9.2 ± 13.0
	MeOH	MM036	9.9 ± 6.4	8.2 ± 4.9	14.9 ± 4.8	10.8 ± 15.2
Russula cavipes Britzelm.	PE	MM037	10.4 ± 5.3	5.5 ± 1.2	30.5 ± 0.4	15.4 ± 13.2
	EtOAC	MM037	4.9 ± 6.9	4.5 ± 2.5	25.9 ± 9.2	18.9 ± 11.4

	MeOH	MM037	0.0 ± 0.0	1.3 ± 1.8	25.0 ± 13.9	16.3 ± 7.9
Lactarius salmonicolor R. Heim & Leclair	PE	MM038	4.0 ± 5.6	2.0 ± 2.8	26.3 ± 10.5	10.9 ± 15.3
	EtOAC	MM038	0.2 ± 0.2	1.9 ± 2.6	20.4 ± 15.9	13.1 ± 11.2
	MeOH	MM038	5.8 ± 8.2	3.4 ± 4.7	14.6 ± 2.6	10.8 ± 5.6
Tricholomopsis decora (Fr.) Singer	PE	MM039	5.4 ± 4.7	2.2 ± 3.0	15.3 ± 3.3	0.0 ± 0.0
	EtOAC	MM039	2.8 ± 4.0	1.1 ± 1.6	15.1 ± 7.1	13.6 ± 12.5
	MeOH	MM039	0.0 ± 0.0	6.2 ± 8.8	10.9 ± 2.3	0.0 ± 0.0
Tricholoma sciodes (Pers.) C. Martín	PE	MM040	8.9 ± 6.1	3.7 ± 3.3	25.4 ± 2.8	10.8 ± 7.4
	EtOAC	MM040	4.3 ± 6.0	1.7 ± 2.4	24.9 ± 11.7	5.9 ± 8.3
Tricholoma sciodes (Pers.) C. Martín	MeOH	MM040	7.7 ± 5.6	4.1 ± 4.5	10.2 ± 0.5	1.2 ± 1.6
Amanita submembranaceae (Bon) Gröger	PE	MM041	2.4 ± 3.4	7.5 ± 0.1	28.2 ± 15.2	18.4 ± 8.7
	EtOAC	MM041	3.0 ± 2.3	9.8 ± 0.5	24.9 ± 24.8	17.4 ± 11.3
	MeOH	MM041	3.6 ± 1.9	2.1 ± 2.9	20.1 ± 14.9	22.0 ± 16.4
Inocybe cervicolor (Pers.) Quél.	PE	MM042	3.8 ± 5.4	8.7 ± 6.4	22.8 ± 24.9	11.6 ± 16.3
	EtOAC	MM042	2.5 ± 3.5	3.3 ± 4.7	11.6 ± 6.9	6.7 ± 0.7
	MeOH	MM042	6.1 ± 8.6	4.3 ± 6.1	15.2 ± 1.6	3.1 ± 4.4
Inocybe corydalina Quél.	PE	MM043	11.5 ± 1.0	2.9 ± 2.6	5.3 ± 6.9	4.9 ± 6.9
	EtOAC	MM043	0.7 ± 0.9	0.0 ± 0.0	16.3 ± 13.4	1.0 ± 1.4
	MeOH	MM043	3.6 ± 5.1	3.0 ± 4.2	29.2 ± 2.2	15.8 ± 0.1
Hypomyces viridis (Alb. & Schwein.) P. Karst. on *Russula* spec.	PE	MM044	5.6 ± 7.9	6.8 ± 1.8	22.9 ± 2.1	6.7 ± 9.4
	EtOAC	MM044	5.1 ± 6.8	4.6 ± 0.8	17.6 ± 0.8	10.2 ± 5.9
	MeOH	MM044	0.0 ± 0.0	0.0 ± 0.0	25.2 ± 12.0	9.8 ± 13.8
Thelephora palmata (Scop.) Fr.	PE	MM045	0.5 ± 0.6	2.8 ± 2.6	19.7 ± 16.2	13.9 ± 19.6
	EtOAC	MM045	4.0 ± 5.7	0.0 ± 0.0	17.4 ± 9.2	9.1 ± 10.5
	MeOH	MM045	2.9 ± 4.0	3.1 ± 4.4	21.7 ± 23.3	11.0 ± 15.5
Hydnellum peckii Banker	PE	MM046	7.4 ± 10.4	2.6 ± 3.7	11.0 ±15.6	1.5 ± 2.1
	EtOAC	MM046	8.6 ± 12.1	5.9 ± 8.3	17.7 ± 0.1	0.0 ± 0.0
	MeOH	MM046	11.8 ± 16.6	6.3 ± 0.8	11.8 ± 0.4	2.3 ± 3.2
Cantharellus lutescens (Pers.) Fr.	PE	MM047	0.2 ± 0.3	0.0 ± 0.0	24.6 ± 8.6	17.4 ± 8.2
	EtOAC	MM047	2.2 ± 3.1	3.8 ± 5.3	32.2 ± 0.3	10.9 ± 5.9
	MeOH	MM047	1.6 ± 2.2	1.9 ± 2.5	15.2 ± 1.1	15.8 ± 15.9
Craterellus tubaeformis (Fr.) Quél.	PE	MM048	6.5 ± 9.2	3.6 ± 0.9	17.2 ± 9.1	12.6 ± 6.3
	EtOAC	MM048	0.5 ± 0.7	0.0 ± 0.0	26.8 ± 5.6	14.8 ± 7.9
	MeOH	MM048	0.0 ± 0.0	0.0 ± 0.0	18.9 ± 16.2	7.3 ± 10.3
Hydnellum concrescens (Pers.) Banker	PE	MM049	0.0 ± 0.0	1.8 ± 2.5	22.1 ± 11.8	19.0 ± 12.8
	EtOAC	MM049	3.4 ± 3.0	2.2 ± 3.1	22.2 ± 18.7	13.4 ± 18.8
	MeOH	MM049	11.7 ± 16.5	5.5 ± 7.7	14.8 ± 7.4	0.0 ± 0.0
Sarcodon imbricatum (L.) P. Karst.	PE	MM050	3.8 ± 5.3	1.7 ± 2.4	8.7 ± 0.5	1.9 ± 2.6
	EtOAC	MM050	3.6 ± 5.0	1.2 ± 1.6	18.9 ± 4.9	2.5 ± 3.5
	MeOH	MM050	0.9 ± 1.2	0.0 ± 0.0	6.9 ± 8.0	0.0 ± 0.0

Boletopsis leucomelaena (Pers) Fayod	PE	MM051	5.8 ± 4.2	2.5 ± 1.2	25.2 ± 9.2	12.9 ± 2.1
	EtOAC	MM051	5.3 ± 7.4	5.5 ± 0.3	17.8 ± 0.6	2.5 ± 3.0
	MeOH	MM051	8.3 ± 10.0	5.3 ± 3.3	20.6 ± 0.7	6.8 ± 2.7
Gloeophyllum sepiarium (Wulfen) P. Karst.	PE	MM052	0.0 ± 0.0	0.1 ± 0.1	27.9 ± 2.8	21.7 ± 2.2
	EtOAC	MM052	0.0 ± 0.0	0.5 ± 0.6	25.2 ± 14.0	14.9 ± 7.5
	MeOH	MM052	1.5 ± 2.1	1.8 ± 2.5	25.1 ± 16.6	23.5 ± 4.7
Clavulina cristata (Holmsk.) J. Schröt.	PE	MM053	4.1 ± 2.0	7.2 ± 0.2	25.8 ± 21.5	14.5 ± 18.7
	EtOAC	MM053	4.8 ± 6.4	2.2 ± 3.1	21.5 ± 1.6	12.8 ± 8.3
	MeOH	MM053	3.7 ± 5.2	2.7 ± 3.7	13.7 ± 12.1	3.3 ± 4.6
Cordyceps ophioglossoides (Ehrh.) Link	PE	MM054	53.8 ± 8.8	16.5 ± 16.6	28.0 ± 1.8	4.7 ± 3.9
	EtOAC	MM054	39.9 ± 13.1	12.3 ± 10.6	99.9 ± 0.1	75.0 ± 6.9
	MeOH	MM054	22.7 ± 6.4	7.5 ± 5.2	6.9 ± 9.7	1.2 ± 1.7
Elaphomyces granulatus Fr.	PE	MM055	100.0 ± 0.0	31.7 ± 19.2	65.2 ± 1.0	7.7 ± 6.3
	EtOAC	MM055	100.0 ± 0.0	100.0 ± 0.0	100 ± 0.0	56.4 ± 5.1
	MeOH	MM055	99.7 ± 0.5	100.0 ± 0.0	98.6 ± 2.1	38.7 ± 5.5
Tricholoma sulphureum (Bull.) P. Kumm.	PE	MM056	11.8 ± 11.5	3.3 ± 2.7	6.8 ± 5.4	3.4 ± 4.8
	EtOAC	MM056	7.6 ± 10.7	0.0 ± 0.0	13.3 ± 18.8	0.0 ± 0.0
	MeOH	MM056	11.0 ± 15.6	7.3 ± 3.0	9.5 ± 0.3	3.7 ± 5.2
Cortinarius venetus var *monanus* M.M. Moser	PE	MM057	12.9 ± 17.7	5.0 ± 2.1	22.0 ± 9.7	5.0 ± 7.0
	EtOAC	MM057	8.8 ± 12.4	2.7 ± 2.8	27.8 ± 7.0	11.6 ± 5.0
	MeOH	MM057	9.9 ± 8.5	5.9 ± 4.2	18.3 ± 1.6	5.6 ± 3.2
Cortinarius elegantior (Fr.) Fr.	PE	MM058	5.9 ± 2.4	7.3 ± 6.7	9.7 ± 13.7	10.9 ± 15.4
	EtOAC	MM058	12.5 ± 3.7	10.9 ± 10.7	11.0 ± 13.0	14.0 ± 19.0
	MeOH	MM058	19.3 ± 10.0	11.9 ± 9.4	13.7 ± 19.3	6.8 ± 9.6
Lactarius badiosanguineus Kühner et Romagn.	PE	MM059	14.1 ± 8.5	12.2 ± 5.2	15.1 ± 16.7	9.3 ± 13.2
	EtOAC	MM059	11.2 ± 5.2	7.8 ± 0.1	26.8 ± 4.4	10.9 ± 15.4
	MeOH	MM059	12.1 ± 0.3	17.3 ± 12.0	11.1 ± 14.4	17.5 ± 15.8
Lactarius scrobiculatus (Scop.) Fr.	PE	MM060	24.1 ± 21.9	20.2 ± 18.3	18.4 ± 12.3	6.8 ± 9.5
	EtOAC	MM060	17.8 ± 1.3	16.5 ± 8.9	23.0 ± 13.9	7.9 ± 11.2
	MeOH	MM060	2.9 ± 4.0	2.3 ± 3.2	7.0 ± 9.9	5.4 ± 7.6
Lactarius picinus Fr.	PE	MM061	4.8 ± 3.3	0.0 ± 0.0	8.2 ± 2.2	10.4 ± 14.7
	EtOAC	MM061	4.8 ± 2.4	10.1 ± 12.0	11.0 ± 15.6	9.1 ± 12.9
	MeOH	MM061	4.8 ± 3.8	1.8 ± 2.5	9.5 ± 13.4	7.6 ± 10.8
Hygrocybe persistens (Britzelm.) Sing. var. langei (Kühner) Bon	PE	MM062	1.3 ± 1.8	0.0 ± 0.0	12.1 ± 17.0	9.3 ± 13.1
	EtOAC	MM062	6.3 ± 4.7	0.1 ± 0.1	8.0 ± 11.2	5.7 ± 8.1
	MeOH	MM062	0.0 ± 0.0	0.0 ± 0.0	14.1 ± 19.9	6.4 ± 6.0
Tricholoma pseudonictitans Bon	PE	MM063	2.0 ± 2.8	1.8 ± 2.5	20.3 ± 19.8	8.9 ± 12.6
	EtOAC	MM063	9.5 ± 4.9	0.8 ± 1.1	19.3 ± 27.2	7.7 ± 10.8
	MeOH	MM063	15.1 ± 14.4	1.9 ± 2.5	8.9 ± 3.7	4.0 ± 5.7
Meripilus giganteus (Pers.) P. Karst.	PE	MM04	9.9 ± 8.8	7.2 ± 3.8	24.2 ± 5.9	10.9 ± 15.3

Meripilus giganteus (Pers.) P. Karst.	EtOAC	MM064	3.0 ± 4.2	0.0 ± 0.0	8.5 ± 11.3	3.5 ± 5.0
	MeOH	MM064	2.2 ± 3.1	0.0 ± 0.0	26.4 ± 18.7	22.6 ± 31.9

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Screening and HPLC-Based Activity Profiling for New Antiprotozoal Leads from European Plants

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Abstract

Based on a survey of remedies used in Renaissance Europe to treat malaria, we prepared and screened a library of 254 extracts from 61 plants for antiplasmodial activity *in vitro*. HPLC-based activity profiling was performed for targeted identification of active constituents in extracts. One of the most remarkable results was the identification of onopordopicrin, a germacranolide sesquiterpene lactone isolated from *Arctium nemorosum* as a potent inhibitor of *P. falciparum* with an IC_{50} of 6.9 µM. It was tested similarly against *Trypanosoma brucei rhodesiense*, the parasite which causes African sleeping sickness. With an IC_{50} of 0.37 µM, onopordopicrin was one of the most potent natural products reported so far. Cytotoxicity was determined against rat myoblast L6 cells (IC_{50}: 3.06).

Keywords

European plants • *Plasmodium falciparum* • *Trypanosoma brucei rhodesiense* • *Arctium nemorosum* • Onopordopicrin

Introduction

Malaria is still the most deadly parasitic disease in the world, leading to one million deaths every year, mostly in Sub-Saharan Africa. In 1976 Trager and Jensen [1] developed a method for propagation of *Plasmodium falciparum* in human erythrocytes. This parasite

species is the cause of malaria tropica, the most severe form of malaria in humans [2]. This assay opened up the possibility of screening large numbers of samples for antiplasmodial activity. One particular source of samples which deserves special attention is plants that local populations use to treat malaria in regions where it is endemic. This is a reasonable approach to lead discovery considering that nowadays *P. falciparum* malaria is treated with artemisinin-based combination therapies (ACT) such as artemether/ lumefantrine, artesunate/amodiaquine, artesunate/mefloquine and artesunate/sulfadoxine-pyrimethamine [3]. Artemisinin is a natural compound isolated from the medicinal herb *Artemisia annua* L. (Asteraceae) which was used in China to treat fever including malaria. Combination of artemisinin derivatives with other antimalarial drugs is necessary, firstly to increase efficacy and secondly to prevent resistances [3].

More than 1200 plant species from 160 families have been described as traditional antimalarial remedies, and hundreds of extracts and purified compounds from such plants have shown antiplasmodial activities tested in the last decades [4]. Bero et al [5] recently reviewed more than 300 antiplasmodial (IC_{50}s < 11 µM) plant compounds isolated from traditionally used plants, all of which had been published in the three years from 2005 through 2008. Among them were 31 compounds with IC_{50} below 2 µM, which were termed "promising" compounds [5]. They argued that "ethnopharmacological approaches appear to be a promising way to find plant metabolites that could be used as templates for designing new derivatives with improved properties". Willcox recently reviewed clinical studies including 18 case studies and 39 cohort studies on traditional herbal malaria treatments [6].

Malaria is nowadays mostly seen as a tropical disease, and it is common in tropical countries where most indigenous knowledge and plants are collected. Yet, all through history and until the 20th century malaria caused by *P. vivax* and *P. malariae* was widespread in Europe [7]. We recently did a search in eight herbals in German language from the 16th and 17th centuries and identified 314 plants that had been used to treat what was then known as tertian (*P. vivax*) and quartan (*P. malariae*) fever. However, one finding from this study was that only five percent of these plants had ever been studied for antiplasmodial effects and only one had been assayed *in vivo* [8].

Considering the apparent lack of exploitation of this knowledge, we set out for a screening involving plants identified in our survey. We purchased or collected 61 European medicinal plants from various sources (see Table S1 in the Supporting Information), 34 of which had been described as antimalarials in the herbals [8]. The other 27 were taken as a control group, as they too were commercial medicinal plants or plants from genera which had medicinal uses. The plants were dried, separated in different parts (roots, aerial parts, flowers, etc) and extracted successively with *n*-hexane, ethyl acetate, and methanol to give three extracts of increasing polarity for each sample. This focused extract library was then tested for *in vitro* inhibition of *P. falciparum*. Follow-up of active extracts was by HPLC-based activity profiling to identify the active constituents [9]. We recently adapted and validated this approach for the miniaturized and efficient identification of antiprotozoal compounds in complex plant and fungal extracts [10] and have successfully applied it to discovery of new antiprotozoal natural products in other library-based discovery projects [11–17].

Results and Discussion

Two hundred and fifty-four extracts were prepared from 61 plants and tested for antiplasmodial activity at two test concentrations (0.8 and 4.8 µg/ml) according to an established procedure. The identity of the plants, their botanical authority, their origin, the plant part used, the extraction solvent and the % inhibition with the standard deviation as the mean of the three repetitions are all shown in Table S1.

Nine of the 10 most active extracts in this screen (at 4.8 µg/ml) were from plants with a documented antimalarial use in Renaissance herbals [8] (Table S1). These included the petroleum ether extract of the aerial parts of *Peucedanum ostruthium* (L.) Koch (71% inhibition), the ethyl acetate extract of the roots of *Asparagus officinalis* L. (75%), the ethyl acetate extract of the aerial parts of *Artemisia abrotanum* L. (69%), the petroleum ether extract of the leaves of *Artemisia absinthium* L. (68%), the ethyl acetate extract of the roots of *Inula conyzae* (Griess.) Meikle (66%), the ethyl acetate extract of the flowers of *Humulus lupulus* L. (96%), the ethyl acetate extract of the roots of *Anacyclus pyrethrum* (L.) Link (65%), the ethyl acetate extract of the aerial parts of *Hypericum perforatum* L. (98%), and the petroleum ether extract of the aerial parts of *Hyssopus officinalis* L. (66%). Finally, the ethyl acetate extract of the leaves of *Arctium nemorosum* Lej., which had no record of antimalarial use, was particularly effective (99%).

Some of the active plants, including *Peucedanum ostruthium*, *Asparagus officinalis*, *Humulus lupulus*, *Artemisia absinthium* and *Hypericum perforatum* had previously been studied for antiplasmodial effects by other groups (see [8]) and were therefore not followed up in this study. We decided to proceed with HPLC-based activity profiling with the active extracts from *Arctium nemorosum* and *Hyssopus officinalis* to determine their active constituents. The lack of previous phytochemical studies of *A. nemorosum* made this plant species in particular interesting to us.

Hyssopus officinalis L.

The HPLC-based activity profile of the *H. officinalis* extract showed that the activity was concentrated in one major time window at minute 26, which also contained the most dominant peak in the chromatogram (Fig. 1).

HR-HPLC-MS (method shown in the Supporting Information) analysis of the peak with the retention time 25.3 minutes showed m/z 293.4263 $[M-H]^-$, which was indicative of a molecular formula of $C_{18}H_{30}O_3$ (calc. for 294.4290).

The compound corresponding to this peak was then isolated by the following procedure: Two kilograms of dried and finely ground aerial parts of *Hyssopus officinalis* (Dixa AG Herbs and Spices, St. Gallen, Switzerland) were extracted three times with eight L of *n*-heptane, which yielded 19 g of a dark viscous extract. MPLC was done to separate 5 g extract into 129 fractions (Method 1). Fractions were analysed by HPLC-ESI-MS and TLC (see Supporting Information) after every separation step to identify fractions containing targeted compound **1**. Fractions F_{80-85} were pooled and preparative HPLC (see Supporting information) was applied to isolate 0.5 mg of **1**. By comprehensive use of HR-MS, 1D and 2D NMR, and comparison with literature [17], **1** was identified as 13-oxo-9*Z*,11*E*-octadeca-dienoic acid (**1**, Fig. 2). This compound was previously reported from *H. officinalis* [18].

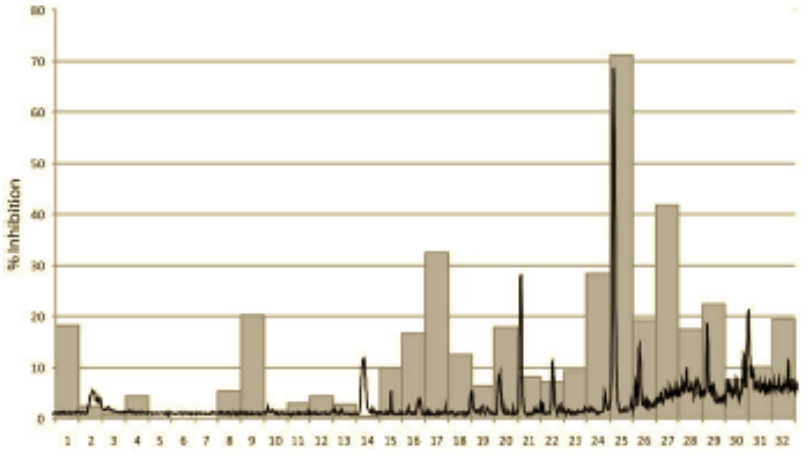
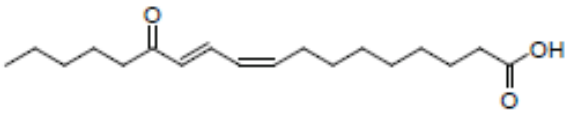


Fig. 1. Activitiy profiling of *Hyssopus officinalis*. The antiplasmodial activity of the 32 one-minute microfractions is plotted against the mass trace of the petrol ether extract (ESI-ESI positive scan *m/z* 150–1500).

This prompted us to test a series of saturated and unsaturated fatty acids for their antiplasmodial effects alongside **1**. These compounds were 10-cis-heptadecenoic acid, 11-cis-eicosenoic acid, 13-cis-docosenoic acid, methyl linoleate, linoleic acid, caproic acid, docosanoic acid, arachidic acid, 9,12,15-all-cis-octadecaterienic acid, and caprylic acid. Neither **1** nor any of these fatty acids had more than 50% inhibition at a test concentration of 10 µg/mL.



1

Fig. 2. 13-Oxo-9Z,11E-octadecadienoic acid

Arctium nemorosum Leij.

The HPLC-based activity profile of the *A. nemorosum* extract showed that the major peak of activity was concentrated in one major time window at minute 12. This time window also contained the most dominant peak in the chromatogram (Fig. 4). HR-HPLC-MS analysis (method in the Supporting Information) of the peak with retention time 12.6 min showed *m/z* 719.3088 $[2M+Na]^+$, which was indicative of a molecular formula of $C_{29}H_{24}O_6$ (calc. for 348.1580).

Fig. 3. *Arctium nemorosum* Leij., May 2011 (by S. Zimmermann), The river Rhine in the background.

The aerial parts of *A. nemorosum* (28.6 g) were milled and extracted exhaustively with EtOAc to afford 1.16 g of dried extract. The active peak at RT 12.5 seen in the HPLC-based activity profiling (Fig. 4) was isolated by semi-preparative HPLC (Method 1) yielding 5.71 mg. The structure was established by comprehensive analysis of HR-MS and 2D NMR data as the germacranolide sesquiterpene lactone onopordopicrin (**2**, Fig. 5). NMR and MS data was in accordance with literature [19]. Onopordopicrin has been previously reported as constituent in leaves of *Onopordum acanthium* L. and *Arctium lappa* L. [19, 20].

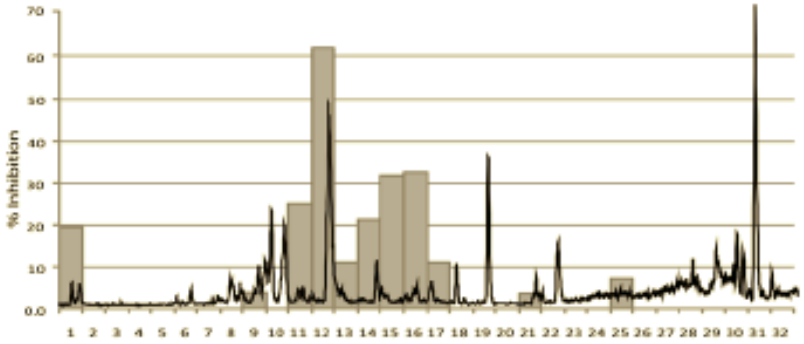
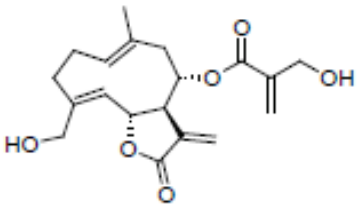


Fig. 4. Activity profiling of *Arctium nemorosum*. The antiplasmodial activity of the 32 one-minute microfractions is plotted against the mass trace of the ethyl acetate extract (ESI-ESI positive scan *m/z* 150–1500).

Onopodopicrin (**2**) was an effective inhibitor of *P. falciparum* parasite growth, with an IC_{50} of 6.89 ± 0.56 µM. The compound shares some structural features, such as the lactone ring with an exocyclic methylene group, and a 2-(hydroxymethyl) acrylate side chain, with cynaropicrin, which we recently identified as an *in vivo* active compound in the acute *T. brucei rhodesiense* sleeping sickness mouse model [16]. Therefore, we tested **2** against *T. brucei rhodesiense in vitro* as well. Onopordopicrin (**2**) showed potent activity with an

IC_{50} value of 0.37 ± 0.01 µM. Against rat myoblast cells (L6 cells) it was cytotoxic with an IC_{50} of 3.06 ± 1.1 µM, and thus a selectivity index (SI) of 8.3.



2

Fig. 5. Onopodopicrin

In summary, 254 extracts from 61 medicinal plants (Table S1) were prepared and screened for antiplasmodial effects. Thirty-four of the plants had been described as antimalarials in German Renaissance herbals [8] and 27 were regarded as a control group. Amongst the most potent 10 extracts, nine were from those plants which had had an antimalarial use. *Arctium nemorosum,* a plant which was very active, was not reported in the herbals [8]. However, the closely related species *Arctium lappa* had been used as a traditional malaria remedy (Table S1). The extracts of *A. lappa* were included in this screening, too, and showed slightly lower activity against *P. falciparum* than *A. nemorosum* (Table S1). HPLC-based activity profiling was done with two of the active extracts, namely the petroleum ether extract of *Hyssopus officinalis* and the ethyl acetate extract of *Arctium nemorosum*. In both cases the active compounds were rapidly identified and isolated. In the case of *H. officinalis* the isolated compound was 13-oxo-9*Z*,11*E*-octadecadienoic acid, an unusual keto fatty acid. Tested as a pure compound its IC_{50} was higher than the highest test concentration of 10 µg/ml. A possible explanation for this may be that due to its high concentration in the extract, the compound nevertheless showed up in the activity profile (Fig. 1). Thus, the presence of "false positives", that is, major and with weak activity must be considered when applying these approaches. Also, it may be that another compound we could not detect was present in this time window and was responsible for the observed activity.

In the case of *Arctium nemorosum* onopordopicrin (**2**) was identified as being chiefly responsible for the observed activity. Its *in vitro* antiplasmodial (*P. falciparum* IC_{50}= 6.89 ± 0.56) and antitrypanosomal (*T. b. rhodesiense* IC_{50}= 0.37 ± 0.01) effects were similar to those of cynaropicrin (*P. falciparum* IC_{50}= 3.00 ± 0.28; *T. b. rhodesiense* IC_{50}= 0.28 ± 001, L6-cells IC_{50}= 2.19 ± 0.27), which has previously been shown to possess *in vivo* antitrypanosomal activity [17]. HPLC-based activity profiling is thus a fast and efficient method for identifying active compounds in complex extracts [9, 10]. The selection of traditionally used plants for a focused screening as a rational approach to lead discovery is supported here.

Experimental

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Scharlau (Barcelona, Spain). HPLC grade water was obtained by an EASY-pure II (Barnstead; Dubuque IA, USA) water purification system.

Plant material

Plant material was purchased from commercial sources or collected by the author (M. Adams), and voucher specimens were deposited at the Department of Pharmaceutical Sciences, University of Basel. Origins and voucher numbers are shown in the Supporting Information.

Extraction of plant material for screening

Plants were dried and separated into different parts (roots, aerial parts, flowers) and finely ground using a ZM1 ultra centrifugal mill (Retsch; Haan, Germany). 1 g of powdered material was then successively extracted first with petroleum ether, then ethyl acetate and finally methanol using an accelerated solvent extraction system ASE (ASE 200, Dionex, Switzerland; 3 cycles at 120 bar, and 70°C) to give a set of three extracts of increasing polarity for every sample. The extracts were formatted into a library of solutions at 10 mg/ml DMSO in 2D-barcoded 96 well plates (Zinsser Analytics, France) and stored at −80° C, until used for screening and for HPLC-based activity profiling.

Screening of extracts for antiplasmodial activity

The antiplasmodial screening was performed as previously described [8]. Tests were repeated three times in duplicate, at test concentrations of 0.8 and 4.8 µg/ml.

HPLC-based activity profiling

Selected extracts were separated by analytical HPLC for activity profiling as previously reported [10], with the following modifications: extracts were separated by HPLC into 96-deep well plates (ScreenMates 96 well, Matrix Technology, Hudson, USA) and dried within 2 h in a parallel evaporator (EVX-96 Apricot Evaporex, Monrovia, USA), with an N_2-stream (60 °C upper part/ 40°C lower part). The dried microfractions were dissolved in 50 µl methanol and transferred into 96 well conical plates (V-well plates, Thermo Scientific, USA). After rinsing with 50 µl MeOH, the plate was again dried using the same condition as described above. The dried microfractions were dissolved in 5 µL of DMSO and then diluted with 95 µL of PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). This gave the stock solutions which could be used for the assays [10]. On-line collection of mass spectral data was done by LC ion trap ESI-MS and HR-TOF-MS [10]. Details are given in the Supporting Information.

Bioassays

Screening of extracts, HPLC-based activity profiling and pure compounds were tested as previously described [10, 17]. Tests were done in three independent assays in duplicate. For details please refer to the Supporting Information.

Acknowledgement

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Supporting Information

Supporting information containing Table S1 with the antiplasmodial activity of 254 plant extracts as well as details on the analytical methods are available in the online version (Format: PDF, Size: < 0.2 MB): http://dx.doi.org/10.3797/scipharm.1111-13.

Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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Supporting Information to

Screening and HPLC-Based Activity Profiling for New Antiprotozoal Leads from European Plants

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Tab. S1. Antiplasmodial activity (growth inhibition in % ± standard deviation [SD]) of 254 plant extracts against Plasmodium falciparum. Bioassays were carried out in duplicate of three independent experiments, at test concentrations of 4.81 µg/mL and 0.81 µg/mL, respectively. The positive control was artesunate (100% inhibition in all bioassays).

Plant Family	Plant species	Historical source	Src.	Voucher specimen	Plant part	extract solvent	growth inhib. at 4.81 µg/mL ± SD[a]	growth inhib. at 0.81 µg/mL ± SD[a]
Adoxaceae	*Sambucus ebulus* L.	Br., Lo., Ta.2	A	P01663	leaves	PE	12.3 ± 6.9	1.9 ± 1.4
				P01664	leaves	EtOAc	9.1 ± 12.2	1.5 ± 2.1
				P01665	leaves	MeOH	3.3 ± 4.7	1.3 ± 1.8
				P01669	fruits	PE	20.5 ± 15.0	0.2 ± 0.2
				P01670	fruits	EtOAc	15.8 ± 15.2	8.8 ± 11.8
				P01671	fruits	MeOH	15.9 ± 20.3	2.7 ± 3.8
			A	P01547	roots	PE	58.0 ± 10.3	6.8 ± 5.2
				P01548	roots	EtOAc	9.4 ± 5.1	5.4 ± 5.2
				P01549	roots	MeOH	29.0 ± 9.0	9.7 ± 7.5
	Sambucus nigra L.	Lo., Ta.2	A	P01681	flowers	PE	7.8 ± 6.6	2.2 ± 1.9
				P01682	flowers	EtOAc	7.5 ± 5.8	0.0 ± 0.0
				P01683	flowers	MeOH	9.9 ± 7.1	6.5 ± 4.8
Amaryllidaceae	*Allium ursinum* L.	–	A	P01592	aer. pts.	PE	7.9 ± 7.6	0.0 ± 0.0
				P01593	aer. pts.	EtOAc	5.5 ± 4.0	0.6 ± 0.9
				P01594	aer. pts.	MeOH	13.4 ± 5.9	1.1 ± 2.0
Apiaceae	*Angelica archangelica* L.	Ta.2	A	P01610	fruits	PE	16.0 ± 9.6	10.9 ± 5.7
				P01611	fruits	EtOAc	15.6 ± 11.7	14.5 ± 20.5
				P01612	gruits	MeOH	17.2 ± 8.2	8.6 ± 7.4
				P01613	fer. pts.	PE	32.9 ± 20.2	28.4 ± 24.2
				P01614	aer. pts.	EtOAc	35.0 ± 8.7	8.4 ± 6.8
				P01615	aer. pts.	MeOH	9.9 ± 7.1	7.7 ± 5.5
				P01616	roots	PE	36.6 ± 16.0	29.1 ± 41.1
				P01617	roots	EtOAc	41.6 ± 3.5	19.4 ± 6.1
				P01618	roots	MeOH	17.6 ± 1.8	12.7 ± 9.0
	Angelica sylvestris L.	Ta.2	B	SZ0001	fruits	PE	34.0 ± 5.0	5.2 ± 3.8
				SZ0002	fruits	EtOAc	51.0 ± 6.6	1.7 ± 2.5
				SZ0003	fruits	MeOH	4.9 ± 5.4	1.5 ± 1.1
				SZ0004	leaves	PE	6.7 ± 0.7	0.0 ± 0.0
				SZ0005	leaves	EtOAc	54.6 ± 3.0	4.4 ± 3.5
				SZ0006	leaves	MeOH	17.5 ± 23.1	5.0 ± 3.9
				SZ0007	roots	PE	35.6 ± 2.3	10.2 ± 2.6
				SZ0008	roots	EtOAc	52.3 ± 1.3	8.6 ± 6.1
				SZ0009	roots	MeOH	16.5 ± 2.5	2.3 ± 2.1
	Coriandrum sativum L.	Br., Ta.2	A	P01678	seed	PE	6.3 ± 9.0	2.9 ± 4.1
				P01679	seed	EtOAc	3.3 ± 1.0	7.8 ± 11.1
				P01680	seed	MeOH	6.9 ± 6.7	0.0 ± 0.0
	Eryngium campestre L.	Ma., Ta.1, Ta.2, Zw.	A	P01559	roots	PE	36.9 ± 11.7	4.8 ± 1.9
				P01560	roots	EtOAc	44.5 ± 6.5	0.0 ± 0.0
				P01561	roots	MeOH	13.6 ± 3.2	9.9 ± 5.5
				P01565	aer. pts.	PE	64.1 ± 2.3	1.6 ± 4.0
				P01566	aer. pts.	EtOAc	16.9 ± 5.5	0.1 ± 4.4
				P01567	aer. pts.	MeOH	34.4 ± 3.8	0.0 ± 0.0
Apiaceae	*Foeniculum vulgare* Mill. subsp. *vulgare* var. *dulce* (Mill.)	Ta.2	A	P01577	aer. pts.	PE	29.7 ± 2.2	2.5 ± 1.8
				P01578	aer. pts.	EtOAc	8.7 ± 3.5	0.0 ± 0.0
				P01579	aer. pts.	MeOH	5.1 ± 2.5	1.5 ± 2.1
	Peucedanum ostruthium (L.) Koch	Bo., Br., Ma., Zw.	A	P01587	rhizome	PE	11.7 ± 8.0	0.0 ± 0.0
				P01588	rhizome	EtOAc	16.4 ± 4.2	1.7 ± 2.4
				P01586	rhizome	MeOH	15.4 ± 9.8	0.0 ± 0.0
				P01590	aer. pts.	PE	71.1 ± 8.1	0.0 ± 0.0
				P01591	aer. pts.	EtOAc	7.8 ± 6.7	3.9 ± 5.5
				P01589	aer. pts.	MeOH	31.2 ± 3.2	1.2 ± 1.7
Asparagaceae	*Asparagus officinalis* L.	Ta.2, Zw.	A	P01463	roots	PE	26.4 ± 10.9	0.3 ± 0.3
				P01464	roots	EtOAc	75.5 ± 7.5	4.0 ± 4.8
				P01465	roots	MeOH	37.5 ± 0.8	1.6 ± 1.1
Aspleniaceae	*Asplenium scolopendrium* L.	Bo., Lo, Zw.	B	P01666	leaves	PE	4.7 ± 5.9	0.0 ± 0.0
				P01667	leaves	EtOAc	7.4 ± 5.3	0.0 ± 0.0
				P01668	leaves	MeOH	11.3 ± 14.6	0.0 ± 0.0
Asteraceae	*Achillea millefolium* L.	Ta.2	A	P01599	aer. pts.	PE	48.4 ± 4.1	0.0 ± 0.0
				P01600	aer. pts.	EtOAc	9.3 ± 1.1	0.0 ± 0.0
				P01598	aer. pts.	MeOH	16.6 ± 7.1	0.5 ± 0.3
	Achillea moschata Wulfen	–	A	P01499	aer. pts.	PE	42.6 ± 17.2	5.9 ± 5.0
				P01500	aer. pts.	EtOAc	36.5 ± 12.6	0.0 ± 0.0
				P01501	aer. pts.	MeOH	8.4 ± 7.2	0.0 ± 0.0
	Anacyclus pyrethrum (L.)	Bo., Ma., Ta.2, Zw.	B	P01637	roots	PE	15.0 ± 6.1	0.4 ± 0.6
				P01638	roots	EtOAc	65.3 ± 2.8	26.2 ± 32.0

Tab. S1. (Cont).

Plant Family	Plant species	Historical source	Src.	Voucher specimen	Plant part	extract solvent	growth inhib. at 4.81 µg/mL ± SD[a]	growth inhib. at 0.81 µg/mL ± SD[a]
Asteraceae	*Arctium lappa* L.	Zw.	A	P01472	leaves	PE	17.4 ± 1.5	2.0 ± 2.9
				P01473	leaves	EtOAc	33.3 ± 25.7	0.0 ± 0.0
				P01474	leaves	MeOH	8.9 ± 8.1	2.3 ± 3.2
				P01475	roots	PE	50.5 ± 3.0	54.7 ± 2.8
				P01476	roots	EtOAc	23.3 ± 4.0	11.2 ± 2.0
				P01477	roots	MeOH	4.1 ± 3.0	0.3 ± 0.5
	Arctium nemorosum Lej.	–	B	SZ0010	aer. pts	MeOH	53.6 ± 24.8	21.8 ± 29.1
				SZ0011	leaves	PE	16.4 ± 3.9	12.8 ± 2.9
				SZ0012	leaves	EtOAc	99.1 ± 0.4	14.1 ± 10.0
				SZ0013	leaves	MeOH	16.5 ± 3.2	4.3 ± 3.2
				SZ0014	fruits	PE	5.3 ± 3.8	2.3 ± 3.3
				SZ0015	fruits	EtOAc	55.6 ± 4.8	3.1 ± 4.2
				SZ0016	fruits	MeOH	5.8 ± 3.5	0.0 ± 0.0
				SZ0017	hollow stem	PE	2.7 ± 2.0	1.7 ± 2.4
				SZ0018	hollow stem	EtOAc	31.3 ± 5.6	1.3 ± 1.8
				SZ0019	hollow stem	MeOH	57.5 ± 3.4	17.5 ± 3.9
				SZ0020	roots	PE	5.0 ± 4.9	1.7 ± 2.1
				SZ0021	roots	EtOAc	31.0 ± 5.6	0.6 ± 0.8
				SZ0022	roots	MeOH	3.2 ± 4.5	0.0 ± 0.0
	Arnica montana L.	–	B	P01451	flowers	PE	18.2 ± 3.8	6.4 ± 4.7
				P01452	flowers	EtOAc	30.7 ± 6.8	4.0 ± 5.7
				P01453	flowers	MeOH	5.7 ± 4.4	0.0 ± 0.0
				P01454	roots	PE	23.1 ± 0.9	14.1 ± 1.3
				P01455	roots	EtOAc	36.1 ± 3.2	5.9 ± 8.3
				P01456	roots	MeOH	13.5 ± 8.0	5.6 ± 4.0
	Artemisia abrotanum L.	Bo., Ma., Ta.2	A	P01433	aer. pts.	PE	50.2 ± 2.4	23.5 ± 8.5
				P01434	aer. pts.	EtOAc	69.3 ± 3.4	18.3 ± 7.9
				P01435	aer. pts.	MeOH	17.8 ± 11.2	8.5 ± 6.0
	Artemisia absinthium L.	Ma., Ta.2, Zw.	B	P01672	leaves	PE	67.9 ± 11.2	5.8 ± 3.8
				P01673	leaves	EtOAc	55.7 ± 14.5	3.8 ± 4.9
				P01674	leaves	MeOH	6.3 ± 7.4	1.0 ± 1.5
	Artemisia dracunculus L.	–	A	P01545	aer. pts.	PE	16.5 ± 3.2	0.5 ± 0.7
				P01546	aer. pts.	EtOAc	4.3 ± 3.0	7.1 ± 5.0
				P01544	aer. pts.	MeOH	24.5 ± 3.2	1.7 ± 1.8
Asteraceae	*Artemisia vulgaris* L.	Ta.2	A	P01457	aer. pts.	PE	18.9 ± 7.0	6.3 ± 2.7
				P01458	aer. pts.	EtOAc	41.9 ± 11.6	0.0 ± 0.0
				P01459	aer. pts.	MeOH	0.0 ± 0.0	0.0 ± 0.0
				P01460	roots	PE	16.5 ± 5.9	5.2 ± 4.1
				P01461	roots	EtOAc	44.1 ± 9.7	5.0 ± 4.3
				P01462	roots	MeOH	17.3 ± 3.8	6.6 ± 5.7
			B	P01645	aer. pts.	PE	31.7 ± 3.4	15.2 ± 11.4
				P01646	aer. pts.	EtOAc	19.4 ± 10.7	0.4 ± 0.7
				P01647	aer. pts.	MeOH	6.0 ± 1.9	0.0 ± 0.0
	Carthamus tinctorius L.	–	A	P01502	flowers	PE	9.1 ± 9.6	1.2 ± 1.7
				P01503	flowers	EtOAc	10.3 ± 2.1	0.4 ± 0.6
				P01504	flowers	MeOH	9.0 ± 6.7	1.0 ± 1.5
	Centaurea cyanus L.	Bo., Lo.	A	P01527	flowers	PE	54.8 ± 9.1	22.3 ± 3.5
				P01528	flowers	EtOAc	19.2 ± 8.0	9.6 ± 6.3
				P01526	flowers	MeOH	28.0 ± 6.6	26.4 ± 7.5
	Centaurea montana L.	–	B	P01648	flowers	PE	40.7 ± 5.8	0.0 ± 0.0
				P01649	flowers	EtOAc	16.6 ± 5.3	18.2 ± 25.7
				P01650	flowers	MeOH	16.5 ± 2.6	0.0 ± 0.1
				P01651	leaves	PE	25.3 ± 8.7	6.5 ± 3.8
				P01652	leaves	EtOAc	10.0 ± 4.6	0.0 ± 0.0
				P01653	leaves	MeOH	8.4 ± 2.7	1.1 ± 1.1
	Cichorium intybus L.	Bo., Ta.2, Zw.	A	P01514	roots	PE	6.0 ± 4.4	0.0 ± 0.0
				P01515	roots	EtOAc	14.2 ± 1.9	2.0 ± 1.4
				P01516	roots	MeOH	1.8 ± 2.3	0.0 ± 0.0
				P01517	aer. pts.	PE	9.5 ± 1.9	0.0 ± 0.0
				P01518	aer. pts.	EtOAc	18.2 ± 11.4	0.1 ± 0.2
				P01519	aer. pts.	MeOH	6.5 ± 6.6	0.0 ± 0.0
			B	P01657	aer. pts.	PE	9.7 ± 8.6	0.0 ± 0.0
				P01658	aer. pts.	EtOAc	9.7 ± 13.7	0.0 ± 0.0
				P01659	aer. pts.	MeOH	3.5 ± 3.3	0.6 ± 0.8
				P01660	roots	PE	15.0 ± 12.3	4.5 ± 6.3
				P01661	roots	EtOAc	21.0 ± 3.1	1.1 ± 1.0
				P01662	roots	MeOH	12.1 ± 3.6	3.9 ± 3.7

Tab. S1. (Cont).

Plant Family	Plant species	Historical source	Src.	Voucher specimen	Plant part	extract solvent	growth inhib. at 4.81 µg/mL ± SD[a]	growth inhib. at 0.81 µg/mL ± SD[a]
Asteraceae	*Echinacea angustifolia* DC.	–	A	P01551	roots	PE	20.6 ± 6.9	4.2 ± 4.0
				P01552	roots	EtOAc	8.9 ± 6.1	1.3 ± 1.9
				P01550	roots	MeOH	12.2 ± 7.8	3.1 ± 3.1
	Echinacea purpurea (L.) Moench	–	A	P01554	roots	PE	29.2 ± 12.8	0.0 ± 0.0
				P01555	roots	EtOAc	19.0 ± 7.4	0.3 ± 0.2
				P01553	roots	MeOH	24.1 ± 9.0	0.0 ± 0.0
				P01557	aer. pts.	PE	23.3 ± 6.5	0.0 ± 0.0
				P01558	aer. pts.	EtOAc	21.9 ± 2.9	10.1 ± 5.2
				P01556	aer. pts.	MeOH	16.2 ± 0.6	1.4 ± 2.0
	Eupatorium cannabinum L.	Ma.	A	P01569	aer. pts.	PE	38.7 ± 9.6	5.2 ± 4.1
				P01570	aer. pts.	EtOAc	11.2 ± 9.1	0.9 ± 0.6
				P01568	aer. pts.	MeOH	17.4 ± 4.0	0.0 ± 0.0
				P01572	aer. pts.	PE	31.6 ± 3.2	5.6 ± 5.3
				P01573	aer. pts.	EtOAc	8.7 ± 1.9	0.0 ± 0.0
				P01571	aer. pts.	MeOH	16.4 ± 10.2	0.0 ± 0.0
	Inula conyzae (Griess.) Meikle	Bo.	B	SZ0023	roots	PE	25.9 ± 5.6	3.0 ± 2.2
				SZ0024	roots	EtOAc	66.4 ± 8.3	5.4 ± 6.0
				SZ0025	roots	MeOH	21.2 ± 15.0	0.0 ± 0.0
				SZ0026	leaves + flowers	PE	3.3 ± 3.7	13.2 ± 18.5
				SZ0027	leaves + flowers	EtOAc	7.5 ± 3.9	0.0 ± 0.0
				SZ0028	leaves + flowers	MeOH	2.6 ± 3.7	10.3 ± 14.5
	Silybum marianum (L.) Gaertn.	–	A	P01490	aer. pts.	PE	11.4 ± 9.4	0.0 ± 0.0
				P01491	aer. pts.	EtOAc	19.8 ± 7.3	0.0 ± 0.0
				P01492	aer. pts.	MeOH	6.8 ± 7.1	0.0 ± 0.0
				P01493	fruits	PE	8.0 ± 5.1	11.0 ± 2.5
				P01494	fruits	EtOAc	24.7 ± 5.5	0.0 ± 0.0
				P01495	fruits	MeOH	23.9 ± 8.5	0.5 ± 0.8
Asteraceae	*Tanacetum parthenium* L.	Br., Lo., Zw.	A	P01511	aer. pts.	PE	21.9 ± 10.3	0.0 ± 0.0
				P01512	aer. pts.	EtOAc	37.6 ± 11.4	0.0 ± 0.0
				P01513	aer. pts.	MeOH	3.6 ± 5.1	0.4 ± 0.5
Brassicaceae	*Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.	Bo., Lo, Ta.2, Zw.	A	P01445	roots	PE	9.4 ± 6.8	0.6 ± 0.9
				P01446	roots	EtOAc	29.8 ± 11.4	9.3 ± 3.8
				P01447	roots	MeOH	17.0 ± 4.5	2.1 ± 2.9
	Nasturtium officinale R. Br.	Zw.	A	P01602	aer. pts.	PE	26.1 ± 3.8	5.2 ± 5.0
				P01603	aer. pts.	EtOAc	7.3 ± 5.2	4.6 ± 3.8
				P01601	aer. pts.	MeOH	30.7 ± 12.7	8.5 ± 2.0
Cannabaceae	*Humulus lupulus* L.	Br., Lo., Ta.2	A	P01684	flowers	PE	31.3 ± 9.9	0.0 ± 0.0
				P01685	flowers	EtOAc	95.8 ± 2.6	4.0 ± 10.5
				P01686	flowers	MeOH	9.2 ± 5.6	3.1 ± 2.2
Caryophyllaceae	*Gypsophila muralis* L.	–	B	P01634	aer. pts.	PE	4.2 ± 3.5	0.0 ± 0.0
				P01635	aer. pts.	EtOAc	10.9 ± 8.1	8.5 ± 12.0
				P01636	aer. pts.	MeOH	4.7 ± 1.6	0.0 ± 0.0
Clusiaceae	*Hypericum perforatum* L.	Bo.,Fu., Lo., Ma., Ta.2	A	P01693	aer. pts.	PE	69.3 ± 17.2	32.3 ± 40.0
				P01694	aer. pts.	EtOAc	97.5 ± 1.2	20.0 ± 1.3
				P01695	aer. pts.	MeOH	16.8 ± 10.8	1.6 ± 2.3
Cucurbitaceae	*Bryonia alba* L.	–	A	P01487	roots	PE	18.0 ± 6.8	0.8 ± 0.6
				P01488	roots	EtOAc	13.7 ± 10.1	0.0 ± 0.0
				P01489	roots	MeOH	9.2 ± 5.0	0.0 ± 0.0
Ericaceae	*Arbutus unedo* L.	–	A	P01445	roots	PE	19.1 ± 7.8	0.0 ± 0.0
				P01446	roots	EtOAc	8.8 ± 10.4	0.7 ± 0.6
				P01447	roots	MeOH	4.4 ± 6.2	1.5 ± 2.1
Euphorbiaceae	*Euphorbia cyparissias* L.	–	A	P01572	aer. pts.	PE	11.0 ± 9.3	0.0 ± 0.0
				P01573	aer. pts.	EtOAc	18.4 ± 11.5	0.0 ± 0.0
				P01571	aer. pts.	MeOH	10.2 ± 7.3	0.0 ± 0.0
Fabaceae	*Anthyllis vulneraria* L.	–	A	P01439	flowers	PE	20.7 ± 5.0	13.5 ± 5.1
				P01440	flowers	EtOAc	20.8 ± 14.7	8.9 ± 5.8
				P01441	flowers	MeOH	15.8 ± 12.5	7.3 ± 3.7
	Robinia pseudoacacia L.	–	A	P01466	flowers	PE	26.7 ± 5.2	7.5 ± 1.8
				P01467	flowers	EtOAc	39.1 ± 5.6	8.0 ± 7.3
				P01468	flowers	MeOH	0.0 ± 0.0	0.0 ± 0.0
Gentianaceae	*Centaurium erythraea* Rafn.	Ma., Ta.1, Ta.2, Zw.	A	P01654	aer. pts.	PE	9.1 ± 6.5	5.4 ± 1.7
				P01655	aer. pts.	EtOAc	38.9 ± 4.2	0.0 ± 0.0
				P01656	aer. pts.	MeOH	3.6 ± 2.8	2.3 ± 3.2
	Gentiana lutea L.	Bo., Ma., Ta.2, Zw.	A	P01642	roots	PE	4.4 ± 2.1	7.9 ± 10.8
				P01643	roots	EtOAc	9.7 ± 5.4	0.0 ± 0.0
				P01644	roots	MeOH	0.3 ± 0.4	0.0 ± 0.0
Lamiaceae	*Galeopsis segetum* Neck.	–	A	P01578	aer. pts.	PE	18.1 ± 10.1	0.0 ± 0.0
				P01579	aer. pts.	EtOAc	7.6 ± 6.9	1.6 ± 2.2
				P01577	aer. pts.	MeOH	23.7 ± 7.6	2.4 ± 3.4

Tab. S1. (Cont).

Plant Family	Plant species	Historical source	Src.	Voucher specimen	Plant part	extract solvent	growth inhib. at 4.81 µg/mL ± SD[a]	growth inhib. at 0.81 µg/mL ± SD[a]
Lamiaceae	*Hyssopus officinalis* L.	Ma.	A	P01584	aer. pts.	PE	66.0 ± 8.3	0.0 ± 0.0
				P01585	aer. pts.	EtOAc	6.1 ± 4.3	1.8 ± 2.6
				P01582	aer. pts.	MeOH	64.1 ± 5.9	6.3 ± 5.3
	Nepeta cataria L.	Br.	A	P01605	aer. pts.	PE	35.2 ± 3.7	0.2 ± 0.3
				P01606	aer. pts.	EtOAc	5.8 ± 4.9	6.1 ± 8.7
				P01604	aer. pts.	MeOH	12.7 ± 2.4	0.0 ± 0.0
	Origanum dictamnus L.	–	A	P01542	aer. pts.	PE	16.4 ± 7.2	0.0 ± 0.0
				P01543	aer. pts.	EtOAc	33.1 ± 16.3	16.9 ± 4.8
				P01541	aer. pts.	MeOH	14.7 ± 7.6	3.9 ± 5.5
	Origanum vulgare L.	–	A	P01608	aer. pts.	PE	57.7 ± 15.0	5.3 ± 5.3
				P01607	aer. pts.	MeOH	53.3 ± 15.6	9.9 ± 9.4
	Stachys officinalis (L.) Trev.	Br.	A	P01478	aer. pts.	PE	30.5 ± 17.6	0.0 ± 0.0
				P01479	aer. pts.	EtOAc	30.5 ± 8.8	2.1 ± 1.5
				P01480	aer. pts.	MeOH	4.2 ± 5.8	0.0 ± 0.0
Piperaceae	*Piper cubeba* L.F.	–	A	P01520	fruits	PE	34.9 ± 2.4	18.3 ± 5.1
Polygonaceae	*Bistorta officinalis* Delarbre	–	A	P01481	aer. pts.	PE	7.1 ± 6.1	0.0 ± 0.0
				P01482	aer. pts.	EtOAc	23.3 ± 6.8	4.2 ± 3.0
				P01483	aer. pts.	MeOH	14.1 ± 5.7	0.0 ± 0.0
				P01484	roots	PE	11.1 ± 8.2	2.1 ± 2.3
				P01485	roots	EtOAc	12.6 ± 11.2	1.5 ± 2.1
				P01486	roots	MeOH	6.7 ± 7.5	0.0 ± 0.0
Ranunculaceae	*Aquilegia vulgaris* L.	–	A	P01442	aer. pts.	PE	18.1 ± 3.5	4.5 ± 4.0
				P01443	aer. pts.	EtOAc	42.7 ± 7.4	12.0 ± 0.8
				P01444	aer. pts.	MeOH	18.7 ± 2.3	5.7 ± 3.7
Rosaceae	*Alchemilla alpina* L.	–	A	P01533	aer. pts.	PE	67.3 ± 9.2	11.1 ± 3.2
				P01534	aer. pts.	EtOAc	12.6 ± 3.6	1.2 ± 1.7
				P01532	aer. pts.	MeOH	27.6 ± 5.4	10.6 ± 7.5
	Alchemilla vulgaris L. em. Fröhner	–	A	P01563	aer. pts.	PE	43.3 ± 2.6	5.8 ± 0.4
				P01564	aer. pts.	EtOAc	11.4 ± 0.6	3.1 ± 0.2
				P01562	aer. pts.	MeOH	11.1 ± 20.4	0.0 ± 0.0
	Agrimonia eupatoria L.	Br., Lo., Ma.,Ta.2, Zw.	A	P01699	aer. pts.	PE	47.9 ± 6.1	0.0 ± 0.0
				P01700	aer. pts.	EtOAc	20.4 ± 4.7	1.1 ± 1.1
				P01701	aer. pts.	MeOH	53.6 ± 17.6	21.8 ± 20.6
	Geum urbanum L.	–	A	P01505	roots	PE	8.6 ± 1.9	0.0 ± 0.0
				P01506	roots	EtOAc	14.7 ± 11.5	1.2 ± 1.7
				P01506	roots	MeOH	3.4 ± 3.1	0.0 ± 0.0
				P01507	aer. pts.	PE	11.0 ± 8.3	0.3 ± 0.4
				P01508	aer. pts.	EtOAc	25.0 ± 6.9	0.0 ± 0.0
				P01509	aer. pts.	MeOH	17.6 ± 8.1	3.6 ± 4.6
	Potentilla erecta (L.) Raeusch.	Br., Bo., Lo., Ta.2	A	P01696	roots	PE	7.6 ± 5.5	0.0 ± 0.0
				P01697	roots	EtOAc	23.8 ± 1.8	9.5 ± 3.8
				P01698	roots	MeOH	7.3 ± 1.9	1.8 ± 2.5
	Potentilla anserina L.	–	A	P01436	aer. pts.	PE	16.9 ± 12.3	7.7 ± 7.6
				P01437	aer. pts.	EtOAc	16.7 ± 15.9	18.9 ± 4.7
				P01438	aer. pts.	MeOH	24.4 ± 6.1	19.5 ± 8.9
	Potentilla aurea L.	Zw.	A	P01687	leaves	PE	11.1 ± 5.3	4.4 ± 4.9
				P01688	leaves	EtOAc	18.5 ± 1.6	6.1 ± 4.6
				P01689	leaves	MeOH	13.0 ± 6.3	5.3 ± 4.0
Rubiaceae	*Galium odoratum* (L.) Scop.	–	A	P01469	aer. pts.	PE	8.5 ± 9.7	0.0 ± 0.0
				P01470	aer. pts.	EtOAc	21.8 ± 4.5	0.0 ± 0.0
				P01471	aer. pts.	MeOH	20.4 ± 4.6	9.2 ± 13.1
Verbenaceae	*Verbena officinalis* L.	Bo., Ta.2, Zw.	B	P01690	aer. pts.	PE	8.8 ± 8.7	0.0 ± 0.0
				P01691	aer. pts.	EtOAc	40.9 ± 16.8	3.7 ± 3.5
				P01692	aer. pts.	MeOH	22.5 ± 19.0	1.5 ± 2.2

Source A: Plants were obtained from Dixa (St. Gallen, Switzerland).
Source B: Plants were collected in and around Basel by Dr. M. Adams in the summer of 2010.

Analytical methods

TLC

Thin layer chromatography plates (TLC silica gel 60 F254) were from Merck (Darmstadt, Germany). Mobile phase: ethyl acetate/*n*-heptane 30:70. Detection was done in a UV

chamber at 254 and 366 nm. Spots were also visualised with anis aldehyde-sulphuric acid reagent, which was prepared according to Wagner and Bladt [21].

HPLC ESI-MS

For micro fractionation and analysis of extracts an HPLC system consisting of a 1100 series low-pressure mixing pump with degasser module, column oven, and a 1100 series PDA detector (all Agilent, Waldbronn, Germany) was used. A Gilson 215 liquid handler with Gilson 819 injection module and 50 µl loop served as autosampler (Gilson; Mettmenstetten, Switzerland). The HPLC was coupled to an Esquire 3000 Plus ion trap mass spectrometer equipped with an electrospray (ESI) interface (Bruker Daltonics; Bremen, Germany). The MS parameters were as follows: Spectra were recorded under ion charge control conditions (ICCD 30 000) at a scan speed of 30 000 m/z/s with a Gauss filter with of 0.2 m/z. Nitrogen was used as a drying gas a flow rate of 10 L/min and as nebulising gas at a pressure of 30 psi. The nebulizer temperature was set 300 ° C. In the positive ion mode spectra were detected from 150–1500 m/z. Capillary voltage was set at -4500 V, endplate offset at -500 V. capillary exit at 109.8 V, skimmer voltage at 65.0 V, and trap drive at 39.8. The negative ion mode was also recorded from 150–1500 m/z. Capillary voltage was set at 4500 V, endplate offset at -500 V. capillary exit at -111.8 V, skimmer voltage at - 40 V, and trap drive at 43.7. A SunFire RP-18, 3.5 µm, 3 x 150 mm (Waters GmbH, Eschborn, Germany) was used for HPLC ESI-MS. A gradient consisting of A (H_2O + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid) was used, starting at 90% A–10 % B and leading to 0% A–100% B in in 30 min, followed 100% B for 5 minutes. The flow rate was 0.5mL/min. Data acquisition and processing for HPLC system was performed using HyStar 3.0. software (Bruker Daltonics).

MPLC

A Büchi Sepacore system consisting of a control unit C-620, a fraction collector C-660, an UV photometer C-635, and two pump modules C-605 was used, with the following method. The column consisted of a cartridge (Büchi, ø 40 x150 mm) containing pressed silica gel (Silica gel 60, 0.040-0.063 mm, Merck, Darmstadt, Germany). A gradient system was used consisting of A (heptane) and B (ethyl acetate), starting at 100 % A and 0% B, and leading to 70% A and 30% B in 33 minutes, then to 20 % A and 80 % B in 31.5 minutes. The flow rate was 30 mL/min. Fractions were collected every 30 seconds. The sample was dissolved in A:B 1:1 at a concentration of 50 mg/mL and the injection volume was 10 ml.

Semi-preparative HPLC

Semi-preparative HPLC was done on an Agilent 1100 series HPLC system consisting of an 1100 series quaternary low-pressure mixing pump with degasser module, column oven, and a 1100 series PDA detector with a 1000 µL loop.) using a SunFire prep RP-18 column (5 µm, 10 x 150 mm, Waters GmbH, Eschborn, Germany). A gradient starting at 85% A (H_20 + 0.1% formic acid) and 15% B (acetonitrile + 0.1% formic acid) and leading to 40% A and 60% B in 15 minutes, then to 100 % B in another 5 minutes. Finally the column was flushed with 100% B for 7 minutes. The flow rate was 5 mL/min. The sample was dissolved in MeOH at a concentration of 50 mg/mL and the injection volume was 300 µl.

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Preparative HPLC

Preparative HPLC was done on a SCL-10, HPLC system from Shimadzu (Kyoto, Japan).A SunFire™ prep C18 OBD™ (5 µm, 30x 150 mm, Waters, Ireland) was used. The gradient was isocratic for 30 min and consisted of acetonitrile:H_20 1: 1 at a flow rate of 30 ml/min. UV data were recorded from 220 to 500 nm. The samples were dissolved in acetonitrile at a concentration of 100 mg/ml and the injection volume was 300 µl.

High resolution Mass Spectrometry (micrOTOF)

High-resolution mass spectra were obtained on a micrOTOF ESI-MS system (Bruker Daltonics) connected to an Agilent 1100 series HPLC. Data acquisition and processing was performed using HyStar 3.0 software (Bruker Daltonics). Conditions for LC-TOF MS were as follows: spectra were recorded in the range of m/z 150–1500 in positive mode. Nitrogen was used as a nebulising gas at a pressure of 2.0 bar and as a drying gas at a flow rate of 9.0 L/min (dry gas temperature 240 °C). Capillary voltage was at 4500 V, endplate offset at -500 V, hexapole at 250.0 Vpp, skimmer 1 at −50 V and skimmer 2 at −22.5 V. Instrument calibration was performed using a reference solution of sodium formiate 0.1 % in isopropanol / water (1:1) containing 5 mM sodium hydroxide. Typical mass accuracy was ±2 ppm. The spectra were recorded in negative and positive mode in the range of m/z 150–1500.

NMR

NMR data were acquired at target temperature 18°C on a Bruker Avance III™ 500 MHz spectrometer (Bruker, Fällanden, Switzerland) operating at 500.13 MHz for ^{1}H, and 125.77 MHz for ^{13}C. A 1mm TXI microprobe with a z-gradient was used for ^{1}H-detected experiments; ^{13}C-NMR spectra were recorded with a 5 mm BBO probe head with z-gradient. NMR experiments were done as previously described [22]. For processing and evaluation Topspin 2.0 was used.

Bioassays

a. In vitro test against Trypanosoma brucei rhodesiense

Trypanosoma brucei rhodesiense (STIB 900) were grown in axenic medium as previously described [23].The compounds were tested using a modified Alamar Blue assay protocol [24] to determine the 50% inhibitory concentration (IC_{50}). Serial threefold drug dilutions were prepared in 96-well micro titer plates and 50 µl of *T. b. rhodesiense* STIB 900 bloodstream forms were added to each well except for the negative controls. Melarsoprol (Arsobal®, Sanofi-Aventis, Meyrin, Switzerland) was used as a reference drug. After 70 h of incubation Alamar blue marker (12.5 mg resazurin dissolved in 100 mL distilled water) was added. The plates were then incubated for an additional 2 to 5 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices Cooperation, Sunnyvale, CA) with an excitation wavelength of 536 nm and an emission wavelength of 588 nm was used to read the plates. The IC_{50} values were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

b. In vitro testing against Plasmodium falciparum

A modification of the [^{3}H]-hypoxanthine incorporation assay was used to determine the intra-erythrocytic antiplasmodial activity (Des Jardins 1979) of the extract library and

purified compounds in 96 well plates. Chloroquine (Sigma-Aldrich) and artesunate (Mepha, Switzerland) were used as standard drugs. Briefly, infected human red blood cells in RPMI 1640 medium (100 µL per well with 2.5% haematocrit and 0.3% parasitaemia) were exposed to twofold serial drug dilutions in 96-well micro titer plates. After 48 h incubation, 0.5 µCi [^{3}H]-hypoxanthine was added to each well. The plates were incubated for further 24 h before being harvested using a Betaplate cell harvester (Wallac, Zürich, Switzerland) The radioactivity was counted with a Betaplate liquid scintillation counter (Wallac) as counts per minute per well at each drug concentration and compared to the untreated controls. IC_{50} values were calculated from sigmoidal inhibition curves using Microsoft Excel. All assays were run in duplicate and repeated three times [25].

c. In vitro cytotoxicity testing

Cytotoxicity was assessed using a similar Alamar Blue assay protocol [23] whereby 4000 rat myoblast cells/well were seeded in RPMI 1640 medium. All following steps were according to the *T. b. rhodesiense* protocol. Podophyllotoxin (Sigma-Aldrich) was used as the reference drug.

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CHAPTER 3

The first natural product with *in vivo* activity against *T. b. rhodesiense*

Third publication:

From the initial HTS campaign of more than 2000 extracts against *T. b. rhodesiense* was one of the most promising extract a DCM of the cornflower *Centaurea salmantica* (Asteraceae) with a growth inhibition of 61% tested at 4.81 µg/mL. Subsequent HPLC-based activity profiling led to the identification of cynaropicrin, a STL of the guajanolide type. Due to its high *in vitro* activity with an IC_{50} of 0.3 µM (SI 7.8) against *T. b. rhodesiense* the compound was tested i.p. at 10 mg/kg/b.i.d. using the acute sleeping sickness mouse model. A 92% reduction of parasitemia compared to untreated controls on day seven postinfection was determined. This is the first report of a NP with potent *in vivo* activity against *T. b. rhodesiense* [1].

Upscaled extraction of plant material, isolation of cynaropicrin, interpretation of analytical data (HPLC-ESI-MS, HR-MS, NMR), removal of cynaropicrin's side chain, IC_{50} determinations against P. falciparum, T. b. rhodesiense, melarsoprol-, and pentamidine resistant T. b. rhodesiense strains, and cytotoxicity test, writing of the manuscript, and preparation of the figures and tables were my contribution to this publication.

Stefanie Zimmermann

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Cynaropicrin: The First Plant Natural Product with *In Vivo* Activity against *Trypanosoma brucei*

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Key words
- *Centaurea salmantica* L.
- Asteraceae
- antitrypanosomal
- human African trypanosomiasis (HAT)
- *Trypanosoma brucei rhodesiense*
- sesquiterpene lactone
- cynaropicrin

Abstract

A screen of 1800 plant and fungal extracts with subsequent HPLC-based activity profiling was done to identify new antiprotozoal leads from nature. This led to the identification of cynaropicrin (1) from the herb *Centaurea salmantica* L. (Asteraceae) as a potent *in vitro* inhibitor of *Trypanosoma brucei rhodesiense*. It preferentially inhibited *T.b. rhodesiense* (IC_{50} of 0.3 µM) and *T. brucei gambiense* (IC_{50} of 0.2 µM), compared to *Trypanosoma cruzi* (IC_{50} of 4.4 µM) and *Plasmodium falciparum* (IC_{50} of 3.0 µM). Testing against melarsoprol- and pentamidine-resistant strains (IC_{50}s of 0.3 µM and 0.1 µM, respectively) showed no cross-resistance. Intraperitoneal administration of 2 × 10 mg/kg body weight/day in the *T.b. rhodesiense* STIB 900 acute mouse model led to a 92% reduction of parasitemia compared to untreated controls on day seven post-infection. Removal of the 2-hydroxymethyl-2-propenoyl moiety of cynaropicrin led to a loss of toxicity towards *T.b. rhodesiense*. Cytotoxicities against rat myoblasts (L6 cells), human colon adenocarcinoma cells, and murine peritoneal macrophages were measured, and selectivity indices of 7.8, 62, and 9.5 were determined. This is the first report of a plant natural product with potent *in vivo* activity against *Trypanosoma brucei*.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is caused by the protozoan parasite *Trypanosoma brucei* and is cyclically transmitted by blood-feeding tsetse flies (*Glossina spp.* Wiedemann) [1]. About 95% of HAT cases are caused by *T.b. gambiense* which occurs in Central and Western Africa and causes a chronic form of sleeping sickness that can extend for months or years before clinical symptoms emerge. The other 5% of cases are caused by *T.b. rhodesiense* which is endemic to Eastern Africa and causes an acute, more virulent form of HAT [2,3]. The first stage of both HAT forms is a hemolymphatic systemic stage which appears as a nonspecific malaise syndrome, followed by the encephalitic stage, and the invasion of the parasites into the central nervous system (CNS), which provokes the progressive breakdown of neurological functions, including the disruption of the sleep cycle which gives the disease its name [3,4]. If either form of HAT is not treated adequately, the victims will inevitably die of it. Only a few drugs are on the market to treat sleeping sickness, and their pharmacological profiles are insufficient by modern standards. In the last fifty years, no new drugs have been launched to treat second stage *T.b. rhodesiense* HAT, as there is little economic incentive for big pharmaceutical companies to invest in their development. Therefore, safe new drugs for HAT are urgently needed [2].

Our contribution to the effort of antiprotozoal lead discovery is to screen plant and fungal extract libraries for *in vitro* activity against the protozoan parasites *Plasmodium falciparum*, *T.b. rhodesiense*, *Trypanosoma cruzi*, and *Leishmania donovani* and then identify the active constituents by HPLC-based activity profiling [5]. This microprofiling strategy, combined with online dereplication techniques, makes high-throughput lead discovery from complex extracts more feasible, because the time-, resource-, and labor-consuming steps of fraction processing and handling can be miniaturized and automated. Promising com-

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pounds identified by this approach are then carried forward into *in vivo* tests and preclinical evaluation [5–7].

Here, we report on the identification of cynaropicrin (**1**), a natural guajanolide sesquiterpene lactone from the herb *Centaurea salmantica* L. (Asteraceae), as a potent inhibitor of *T. b. rhodesiense in vitro* and *in vivo*. This compound has been previously reported from the species of the genera *Centaurea* L., *Cynara* L., and *Hemisteptia* (Bunge) F. E. L. Fischer et C. A. Meyer (Asteraceae) [8].

Materials and Methods

Plant material

The aerial parts of *C. salmantica* were collected in 2001 by Mr. M. Friedrich, Friedrich Nature Discovery (Euskirchen, Germany), in Tiflet, Morocco, and dried artichoke leaves (*Cynara scolymus* L.) were purchased from Kräuterhexe (Lengries, Germany). Voucher specimens (*C. salmantica*: P00265; *C. scolymus*: drug number: 797) are deposited at the Institute of Pharmaceutical Sciences, Division of Pharmaceutical Biology, University of Basel, Switzerland.

Extraction, isolation, and structure elucidation

The extraction, isolation, and structure elucidation of **1** as well as the preparation of **2** were performed as described in Supporting Information.

In vitro biotests

The cultivation of protozoan parasites and cell lines, the design of HPLC-based activity profiling, and all *in vitro* assays used in this study are shown in Supporting Information. Bioassays were done in duplicate in at least three independent repetitions, and IC_{50}s and respective standard errors were calculated using sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices) or MS Excel (Microsoft).

STIB900 acute mouse model

This model mimics the first stage of the human African trypanosomiasis. Adult female NMRI mice were purchased from RCC Janvier. They weighed between 20 and 25 g at the beginning of the study and were kept under standard conditions in macrolon type III cages with food pellets and water *ad libitum* at 22 °C and 60–70% humidity. All protocols and procedures used in this study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland (authorization N°739; 11.12.2009). The samples and the positive control pentamidine isothionate (Pentacarinat®, 1 × 5 mg/kg/d, purity > 95%; Sanofi-Aventis) were first dissolved in 100% DMSO followed by the addition of distilled H_2O to a final DMSO concentration of 10%. For the establishment of the *in vivo* antitrypanosomal activity, mice were infected intraperitoneally (ip) with 5×10^3 STIB900 bloodstream forms. Experimental groups of six and five mice were treated ip twice daily with a time interval of 7 to 8 h on four consecutive days from day 1 to day 4 post-infection. The positive control group was treated once ip on four consecutive days from day 1 to day 4. A control group of four mice was infected, but remained untreated.

The determination of the parasitemia was done on day 5 and 7 post-infection. Six µL of tail blood were diluted in 24 µL sodium citrate (3.2%), whereby the first µL was discarded to obtain circulating blood. Five µL of this mixture were transferred to a glass slide and covered with an 18 × 18-mm cover slide. The sample was examined under a light microscope (200-fold magnification), and parasites were counted in 3 of the 16 squares of the grid. The average parasitemia per milliliter mouse blood was calculated out of three enumerations.

Supporting information

Descriptions of the isolation and structure elucidation of cynaropicrin (**1**), the preparation of its deacyl-derivative (**2**), as well as all the *in vitro* bioassays used here are available as Supporting Information.

Results

We screened 1800 plant and fungal extracts for *in vitro* activities against *Plasmodium falciparum*, *Trypanosoma brucei rhodesiense*, and *Trypanosoma cruzi*. The dichloromethane extract of aerial parts of *Centaurea salmantica* inhibited *T.b. rhodesiense* by 61% at a test concentration of 4.7 µg/mL and was thus the most active extract in the screen against this parasite. HPLC-based activity profiling showed that the antitrypanosomal activity was localized around the time window of minute 13. ○ **Fig. 1** shows the overlay of the HPLC-PDA trace (245 nm) with the activity profile of the 32 one-minute microfractions. HPLC hyphenated methods (ion trap ESI-MS, ESI-TOF-MS and off-line microprobe NMR) and comparison with literature data [8] led to the identification of the main peak in the active microfraction as the guajanolide sesquiterpene lactone cynaropicrin (**1**) (○ **Fig. 2**). Larger amounts of cynaropicrin were subsequently purified from artichoke leaves (*Cynara scolymus* L., Asteraceae).

Cynaropicrin (**1**) had an IC_{50} of 0.28 ± 0.01 µM (mean ± standard error of the mean [SEM]) against *T.b. rhodesiense* bloodstream forms. Against the melarsoprol-resistant *T. b. rhodesiense* strain, the IC_{50} was 0.31 ± 0.01 µM; against the pentamidine-resistant strain 0.14 ± 0.02 µM, and against *T. brucei gambiense* the IC_{50} was 0.21 ± 0.01 µM. It was ten times less active against *P. falciparum* (IC_{50} of 2.99 ± 0.28 µM) and fifteen times less active against *T. cruzi* (4.43 ± 0.04 µM) than against *T.b. rhodesiense* wild type.

In the cytotoxic assays, **1** showed an IC_{50} of 2.19 ± 0.27 µM against rat skeletal myoblasts (L6 cells) and thus had a selectivity index of 7.8 (SI: IC_{50} L6 cells divided by IC_{50} for *T.b. rhodesiense*) (○ **Table 2**). Toxicity results were also determined against human colon adenocarcinoma cells (HT-29) (IC_{50} of 17.27 ± 0.19 µM, SI of 6.2), and to test toxicity against primary cells, we used murine macrophages (IC_{50} of 2.66 ± 0.6 µM, SI of 9.5) (○ **Table 2**). The activity of the deacyl derivative **2** was determined similarly against *T.b. rhodesiense* and L6 cells (○ **Tables 1** and **2**). Compound **2** was thus 31 times less active against *T.b. rhodesiense* and 3.4 times less toxic against L6 cells (○ **Table 2**).

The *in vivo* antitrypanosomal assays were done with five mice each with the doses of 2 × 5 mg/kg body weight/day (morning and evening application) and six mice each with the doses of 2 × 10 mg/kg body weight/day ip (morning and evening application) for four consecutive days after infection. The controls were done with four mice (◕ **Table 3**) using 1 × 5 mg/kg body weight/day. On day seven after infection the mice treated with cynaropicrin at 2 × 10 mg/kg/body weight/day ip had a slightly lower parasitemia (0.73×10^7 trypanosomes/mL) as compared to the treatment with 2 × 5 mg/kg/body weight (1.46×10^7 trypanosomes/mL). At twice the dose (2 × 10 mg/kg body weight/day), the parasitemia was 91.7% reduced, whereas the mice which were treated with the lower dose showed an 83.4% reduction of the para-

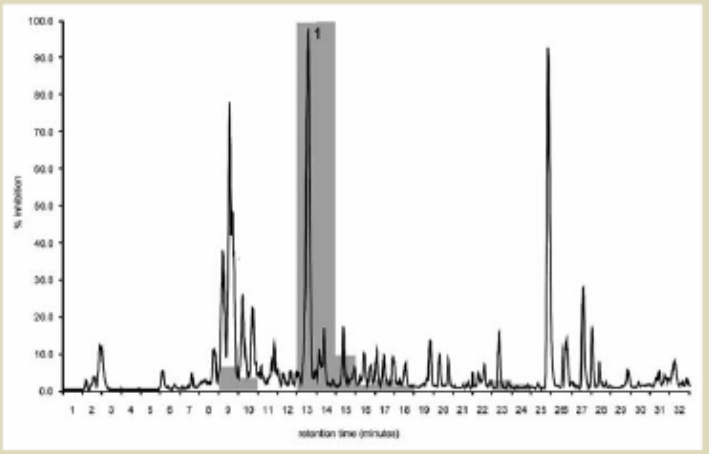

Fig. 1 HPLC-based activity profile of the dichloromethane extract of *Centaurea salmantica*. The grey bars represent the % inhibition of the 1-minute microfractions against *Trypanosoma brucei rhodesiense* compared to the control. The chromatogram in black shows the corresponding ESI-pos MS trace (m/z 150–1500) of the HPLC separation. The peak corresponding to cynaropicrin (**1**) eluted at t_R 13.3 min.

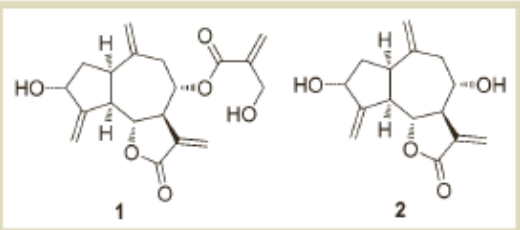

Fig. 2 Chemical structures of cynaropicrin (**1**) and deacyl derivative (**2**).

sitemia. No cytotoxic symptoms were observed at the lower dose. At 2 × 10 mg/kg body weight/day ip, one mouse showed piloerection and inflammatory effects on the ninth day after infection. Untreated control mice were euthanized due the high parasitemia at day 9 post-infection while treated mice were euthanized due the high parasitemia at day 11 and 12 after infection, which are 7 and 8 days after the termination of the treatment (○ **Table 3**). The positive control reduced the parasitemia completely for more than 30 days when the mice were treated once daily with 5 mg/kg body weight/day.

Discussion

With the use of HPLC-based activity profiling, we identified cynaropicrin (**1**) as a potent and preferential inhibitor of *Trypanosoma brucei rhodesiense in vitro*. Deacyl derivative (**2**) was 31 times less active against *T.b. rhodesiense* and 3.4 times less toxic against L6 cells than **1**, demonstrating the necessity of the 2-(hydroxymethyl) 2-propenoic acid side chain for the preferential activity towards *T.b. rhodesiense*. Given that resistance to known drugs is a major problem in antitrypanosomal therapy, we tested for cross-resistance with pentamidine- and melarsoprol-resistant laboratory strains [9]. Cynaropicrin (**1**) showed no *in vitro* cross-resistance with these strains; hence its uptake does not involve these transporters.

We studied the effects of **1** *in vivo* in the STIB 900 acute mouse model. A four-day treatment at 2 × 10 mg/kg body weight/day resulted in a 92% parasitemia reduction 3 days after termination of the treatment. Although these mice could not be cured, the parasitemia reduction is impressive and underlines the strong antitrypanosomal activity of cynaropicrin. To the best of our knowledge, cynaropicrin is the first secondary plant metabolite to show significant and preferential *in vitro* and *in vivo* inhibition of *T. brucei rhodesiense*. Numerous natural products from plants possess some degree of *in vitro* activity against trypanosomes [10–12], but only a few compounds with *in vivo* activity against *T. bru-*

Table 1 *In vitro* antitrypanosomal activities (IC_{50}[a]) of cynaropicrin (**1**) and deacyl derivative (**2**).

Tested compounds	*T.b. rhodesiense* STIB 900					*T.b. gambiense* (ITMAP 141267)
	Wild type	Melarsoprol resistant	R_1[b]	Pentamidine resistant	R_2[c]	
Melarsoprol	0.01 ± 0.01	0.62 ± 0.03	62.0	0.11 ± 0.01	11.0	0.03 ± 0.01
Pentamidine	0.02 ± 0.02	0.46 ± 0.12	23.0	0.14 ± 0.03	7.0	0.01 ± 0.02
1	0.28 ± 001	0.31 ± 0.01	1.1	0.14 ± 0.02	0.5	0.21 ± 0.01
2	4.92 ± 0.71	n.d.	–	n.d.	–	n.d.

[a] Values are expressed in µM. Each value corresponds to the mean ± standard error of the mean from at least three independent bioassays. [b] Resistance factor for melarsoprol-resistant *Trypanosoma brucei rhodesiense*: IC_{50} of resistant strain divided by IC_{50} of wild-type strain. [c] Resistance factor for pentamidine-resistant *Trypanosoma brucei rhodesiense*: IC_{50} of resistant strain divided by IC_{50} of wild-type strain. [d] n.d. = not determined

Table 2 *In vitro* cytotoxic activities of cynaropicrin (**1**) and deacyl derivative (**2**).

Compound	L6	SI[b]	HT-29	SI[c]	Macrophages	SI[d]
Podophyllotoxin	0.02 ± 0.01	-	0.03 ± 0.01	-	2.10 ± 0.01	-
1	2.19 ± 0.27	7.8	17.27 ± 0.19	61.7	2.66 ± 0.60	9.5
2	19.20 ± 3.20	3.9	n.d.[e]	-	n.d.	-

[a] Values are expressed in µM. Each value corresponds to the mean ± standard error of the mean from at least two independent bioassays. [b] Selectivity index (SI): calculated as (IC_{50} for L6-cells/IC_{50} for parasites). [c] Selectivity index (SI): calculated as (IC_{50} for HT-29/IC_{50} for parasites). [d] Selectivity index (SI): calculated as (IC_{50} for macrophages/IC_{50} for parasites). [e] n.d. = not tested

Table 3 *In vivo* antitrypanosomal activity of cynaropicrin (**1**) in *Trypanosoma brucei rhodesiense* STIB900 acute mouse model.

Dose	Number of mice	Parasitemia[a]		% Reduction[b]		Mean survival days[c]
		Day 5	Day 7	Day 5	Day 7	
Positive control Pentamidine 5 mg/kg/d[d]	4	0	0	100	100	> 30
Negative control	4	8.80×10^7	8.57×10^7			9
Cynaropicrin (**1**) 5 mg/kg/d[e]	5	1.40×10^7	1.46×10^7	84.2	83.4	11
Cynaropicrin (**1**) 10 mg/kg/d[e]	6	0.77×10^7	0.73×10^7	91.2	91.7	12

[a] Parasitemia [tryps/mL]. Trypanosomes were counted in a defined grid (18 mm × 18 mm). [b] Difference of the mean infection rate of the control group (= 100 %) to the test group is calculated and expressed as percent reduction. [c] Number of days post-infection until the animals were euthanized due to high levels of parasitemia. [d] Dose was administered once per day on four consecutive days, day 1 to day 4. [e] Dose was administered twice per day (morning and evening) on four consecutive days, day 1 to day 4

cei, and none with effects against *T.b. rhodesiense*, have been reported up to now.

A number of sesquiterpene lactones have been shown to possess *in vitro* activity against protozoans. Schmidt et al. [13] recently published a structure-(*in vitro*)-activity study of 40 sesquiterpene lactones against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum* as well as cytotoxicity data against L6 cells, where *T.b. rhodesiense* was shown to be the most sensitive towards the test compounds. The most active sesquiterpene lactones had IC_{50}s between 0.1 and 0.4 µM. Their activity correlated significantly with cytotoxicity against L6 cells, and the major determinant for activity were α,β-unsaturated carbonyl groups, which are known to act as Michael acceptors for biological nucleophiles [14].

In conclusion, cynaropicrin (**1**) can be considered as a lead for the development of novel antitrypanosomal drugs, and/or as a biological tool for the characterization of new potential drug targets. Its total synthesis has been recently described [15], facilitating a systematic modification of the parent structure. Studies on the pharmacokinetic properties and mode of action, as well as the structure-activity relationship are ongoing.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

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Supporting Information

Cynaropicrin: the first natural product with *in vivo* activity against *Trypanosoma brucei rhodesiense*

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Extraction, isolation, and structure elucidation of cynaropicrin

Sample preparation for the initial screening and HPLC-based activity profiling of micro-fractions were done as previously reported [1].

Isolation of cynaropicrin from *C. salmantica*: Dried aerial parts of *C. salmantica* (200 g) were defatted with 2 L of *n*- hexane (Scharlau), dried and then extracted exhaustively 3 times with 2 L of ethyl acetate (Scharlau) yielding 4.3 g of crude extract. The extractions were done in a glass column (480 x 50mm). 3.4 g of the ethyl acetate extract were separated by medium pressure liquid chromatography consisting of a control unit C-620, a fraction collector C-660, an UV photometer C-635, and two pump modules C-605. The MPLC unit was controlled with Sepacore Control software (version 1.0.3000.1) (all from Büchi). A glass column (480 x 50mm) filled with silica gel 60, 15-40 mesh (Merck), was used with a mobile phase consisting of a gradient of 60 % to 0 % *n*-hexane in ethyl acetate in 6 hours at a flow rate of 15 mL. Fractions were collected every minute and compared by thin layer chromatography (TLC, silica gel 60, F 254, Merck, ethyl acetate/*n*-hexane 7:3, anisaldehyde reagent, Rf **1**: 0.36); similar fractions were pooled to give enriched fraction C (428 mg) which was subjected to semipreparative HPLC on an Agilent 1100 series HPLC system, consisting of a 1100 series quaternary low-pressure mixing pump with degasser module, column oven, and a 1100 series PDA detector with a 1000 µL loop using a SunFire prep RP-18 column (5 µm, 10 x 150 mm.; Waters GmbH). The mobile phase was: A : water + 0.1% formic acid, and B: MeOH + 0.1% formic acid, with a gradient of 10-100% B in 30 minutes, followed by flushing for 5 min with 100% B. The flow rate was 5 mL/min. Absorption was monitored at 245 nm. The retention time of **1** in this HPLC system was 17.1 minutes. Five runs were done with 10 mg of extract each. Thus 28.1 mg of **1** were isolated. Its purity of over 96 % was calculated from the integrals of the ^{1}H-NMR spectrum.

Isolation of cynaropicrin from *Cynara scolymus:* 1 kg of dried leaves were defatted with *n*-hexane (Scharlau), dried and then extracted exhaustively with chloroform (Scharlau) yielding 64 g of extract. From this, pure cynaropicrin was isolated with semipreparative HPLC as described above. The total amount of pure **1** isolated for these studies was 800 mg with a purity of more than 95%.

NMR data were measured at 18 °C on a Bruker Avance III™ 500 MHz spectrometer (Bruker). ^{1}H and ^{13}C experiments were performed as previously described [2]. Topspin 2.0 was used as software for data processing and evaluation. Structures were elucidated with high resolution mass spectrometry and 1D and 2D NMR spectroscopy and were in good agreement with the literature [3].

Deacylated cynaropicrin (**2**) was prepared from cynaropicrin (**1**) by alkaline hydrolysis as described by Macías et al. [4]. Briefly, 400 mg cynaropicrin in 200 mL 10 % potassium carbonate were stirred for 24 h at 50 °C, then acidified with 1M HCl to pH 7, and extracted with ethyl acetate. The organic phase was washed three times with water, and **2** was isolated by semipreparative HPLC as described above to yield 1.0 mg (t_R 8.9 min.).

Protozoan parasites and cell lines

Trypanosoma brucei rhodesiense (STIB 900) was grown in minimum essential medium (MEM) with Earle's salts supplemented with 0.2 mM 2-mercaptoethanol as described by Baltz et al. [5] with the following modifications: 1 mM sodium pyruvate, 0.5 mM hypoxanthine, and 15% heat-inactivated horse serum. Cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C. *Trypanosoma brucei rhodesiense* (STIB 900) with induced resistance to melarsoprol- and pentamidine, as described by Bernhard et al [6], was cultured and tested similarly. *Trypanosoma brucei gambiense* (ITMAP 141267) was maintained in minimum essential medium (MEM) with Earle's salts supplemented with 0.2 mM 2-mercaptoethanol as described by Baltz et al. [5] with the following modifications: 5% heat inactivated fetal calf serum and 15% human serum. *Trypanosoma cruzi* trypomastigote forms (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) were cultured in RPMI 1640 medium supplement with 10% fetal bovine serum and 2 mM L-glutamine [7], and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Antiplasmodial activity was determined against the chloroquine- and pyrimethamine-resistant *Plasmodium falciparum* K1 strain. The parasites were maintained by the method of Trager and Jensen [8] in a humidified atmosphere consisting of 4% CO_2, 3% O_2, and 93 % N_2 at 37 °C.

Rat skeletal myoblast cells (L6-cells) and human colon adenocarcinoma cells (HT-29) were seeded in RPMI 1640 medium supplemented with 2 µM L-glutamine, 5.95 g/L HEPES, 2 g/L $NaHCO_3$, and 10% fetal bovine serum as previously reported [1]. The cultures were routinely maintained by weekly passages at 37 °C under a humidified 5% CO_2 atmosphere. Macrophages are harvested by peritoneal wash in 10 mL RPMI (2 µM L-glutamine, 5.95 g/L HEPES, 2 g/L $NaHCO_3$) + 1% of an antibiotic mixture (penicillin G 6 mg/mL [Sigma Aldrich], kanamycin 10 mg/mL [Fluka], fluorocytosin 5 mg/mL [Fluka], and chloramphenicol 1 mg/mL [Fluka] in distilled H_20 + 1% ethanol) from four NMRI mice (RCC Janvier). Each mouse was treated intraperitoneally with 2 mL starch solution (0.2 mg/mL H_20) before harvesting the primary cells. After centrifugation (10 min, 1500 rpm, Rotina 420 R; Hettich Laboratoire), the macrophages were resuspended in RPMI medium + 1% of the antibiotic mixture. This suspension was immediately used for the bioassay.

Evaluation of *in vitro* antiprotozoal activity

Trypanosoma brucei rhodesiense and melarsoprol/pentamidine resistant Trypanosoma brucei rhodesiense: The samples were tested using the Alamar Blue assay to determine the 50% inhibitory concentrations (IC_{50}s) as previously described [5, 9]. Serial threefold dilutions were prepared in 96-well microtiter plates, and 2000 *T. b. rhodesiense* STIB 900 bloodstream forms in 50 µL were added to each well except for the negative controls. Melarsoprol (Arsobal®, purity> 95%; Sanofi-Aventis) and pentamidine (Pentacarinat®, purity> 95%; Sanofi-Aventis) were used as reference drugs. After 70 h of incubation, 10 µL of Alamar blue marker (12.5 mg resazurin (Sigma-Aldrich) dissolved in 100 mL of distilled water) was added, and color change was developed for 2 to 6 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices Cooperation) with an excitation wavelength of 536 nm and an emission wavelength of 588 nm was used to read the plates. The IC_{50} values were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

In vitro testing against Trypanosoma brucei gambiense: The same protocol [9] was used as described above with the following modification: drug dilutions were prepared with 5000 *T. b. gambiense* cells in 50 µl. Pentamidine was used as a reference drug.

In vitro testing against T. cruzi: Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 µL RPMI 1640 medium. After 24 h, the medium was removed and replaced by 100 µL containing 5000 trypomastigote forms of *T. cruzi*. After 48 h, the medium was removed and replaced by 100 µL of fresh medium with the serial threefold drug dilutions. The plates were incubated under a humidified 5% CO_2 atmosphere at 37°C for an additional 96 h. Then chlorophenyl red *β*-D-galactopyranoside agent (CPRG)/Nonident (50 µL) (Sigma-Aldrich) was added to all wells, and a color change was developed within 2 to 6 h. The plates were read photometrically at 540 nm. Data were evaluated and IC_{50} values calculated with Softmax Pro software (Molecular Devices) [7]. Benznidazole (purity > 95%, Sigma-Aldrich) was used as a standard drug.

In vitro testing against P. falciparum: Antiplasmodial activity was determined with the [^{3}H]-hypoxanthine incorporation assay [10]. Chloroquine (purity > 95%, Sigma-Aldrich) and artesunate (purity > 95%, Mepha) were used as standard drugs. Briefly, infected human red blood cells (final parasitemia and hematocrit were 0.3% and 1.25%, respectively) in RPMI 1640 medium were exposed to twofold serial drug dilutions in 96-well micro titer plates. After 48 h of incubation, 50 µL of [^{3}H]-hypoxanthine (0.5 µCi) were added to each well. The plates were incubated for further 24 h before being harvested using a Betaplate cell harvester (Wallac) onto glass-fiber filters and then washed with distilled water. The dried filters were inserted into plastic foils with 10 mL scintillation fluid. The radioactivity was counted with a Betaplate liquid scintillation counter (Wallac) as counts per minute per well at each drug concentration and compared to the untreated controls. IC_{50} values were calculated from sigmoidal inhibition curve.

In vitro cytotoxicity testing

The cytotoxicity assay was performed using the Alamar Blue assay [9] described above with rat skeletal myoblasts (L6-cells) and HT-29 cells seeded in 100 µL RPMI 1640 in 96-well microtiter plates (4000 cells/well, 8000 cells/well, respectively). The macrophages suspension was counted by hemocytometer and seeded in 100 µL RPMI 1640 in 96-well microtiter plates (4000 cells/well). After 24 h, the medium was removed and replaced by 100 µL of fresh RPMI 1640 with or without a serial threefold drug dilution. Podophyllotoxin (purity > 95%, Sigma-Aldrich) was used as the reference drug. After 70 h of incubation under a humidified 5% CO_2 atmosphere, 10 µL of the Alamar blue marker (see above) was added to all wells. The plates were incubated for an additional 2 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices) was used to read the plates using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50}s were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

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CHAPTER 4

Structure-activity relationship study of sesquiterpene lactones and their semi-synthetic amino derivatives as potential antitrypanosomal products

Fourth publication:

Since cynaropicrin was not able to cure the acute sleeping sickness mouse model, a SAR study was set up, which included 18 natural STLs and 16 semi-synthetic STL amines. This small library was tested *in vitro* against *T. b. rhodesiense* and mammalian cancer cells for a better understanding of STLs structural features, which contribute to their activities. The conclusion is that the α-methylene-γ-lactone is necessary for both antitrypanosomal and cytotoxicity effects.

In an attempt to improve CYN's bioavailability, the exocyclic double bond in the lactone ring was masked to obtain a water soluble dimethylamino derivative. Both compounds (original and its prodrug) were orally administered in the acute sleeping sickness mouse model at 50 mg/kg/d for four consecutive days. The *in vivo* toxic effects of the prodrug after oral application was less compared to the origin, but the mean survival time was the same as for the controls [1].

Determination of compounds IC_{50}s against T. b. rhodesiense including their cytotoxicity assessments, organization of vernodalin from Prof. Ohigashi, writing of the manuscript, and preparation of the figure and the tables were my contribution to this publication.

Stefanie Zimmermann

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Structure-Activity-Relationship Study of Sesquiterpene Lactones and Their Semi-synthetic Amino Derivatives as Potential Antitrypanosomal Products

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ABSTRACT

Sesquiterpene lactones (STLs) are natural products, which have potent antitrypanosomal activity *in vitro* and, in the case of cynaropicrin, also reduce parasitemia in the murine model of trypanosomiasis. To explore structure-antitrypanosomal activity-relationships a set of 34 natural and semi-synthetic STLs and amino-STLs was tested *in vitro* against *T. b. rhodesiense* (which causes East African sleeping sickness) and mammalian cancer cell (rat bone myoblast L6 cells). The conclusions are that the α-methylene-γ-lactone is necessary for both antitrypanosomal effects and cytotoxicity. Antitrypanosomal selectivity is facilitated by 2-(hydroxymethyl)acrylate or 3,4-dihydroxy-2-methylenebutylate side chains, and by the presence of cyclopentenone rings. Semi-synthetic STL amines with improved activity over the native STLs were those with morpholino and dimethylamino groups. The dimethylamino analogue of cynaropicrin was prepared and tested orally in the *T. b. rhodesiense* acute mouse model, where it showed reduced toxicity over cynaropicrin, but also reduced antitrypanosomal effects.

INTRODUCTION

Sleeping sickness or Human African Trypanosomiasis (HAT) is a deadly protozoal disease, caused by *Trypanosoma brucei* species, and spread by tsetse flies (*Glossina spp.*). The two human pathogenic subspecies *T. b. rhodesiense* (95% of cases) and *T. b. gambiense* (5%) differ by geographic distributions, clinical pictures, and drugs used for their treatment.[1] Currently there are about 30 000 annual HAT cases, and as many as 30 million live in HAT endemic areas.[2] Despite some recent successes like *nifortimox*-eflornithine combination therapy (NECT),[3] HAT drugs are still insufficient by modern standards, and need to be replaced by drugs that are safer and easier to administer.[4]

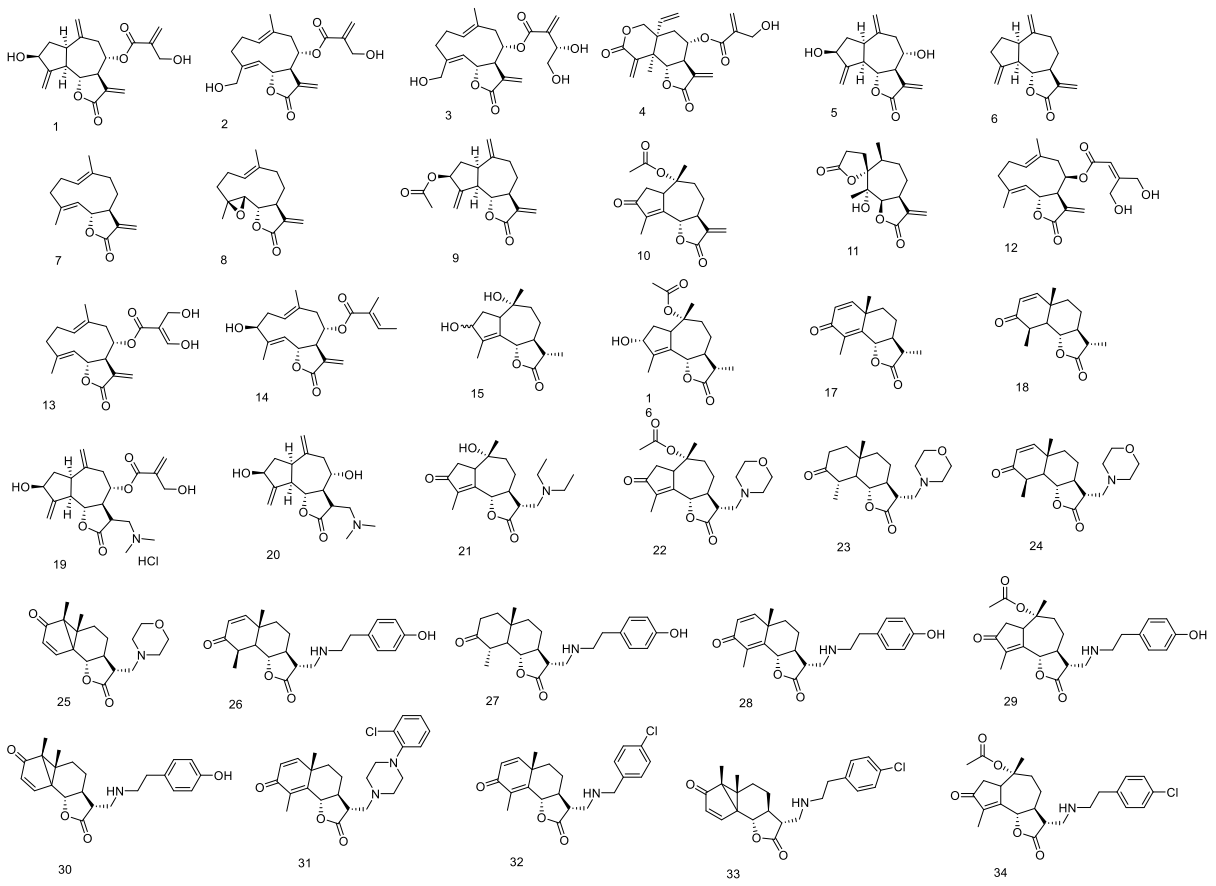
Natural products from plants have been instrumental in developing drugs to treat protozoal diseases like malaria (quinine, artemisinin),[5,6] but currently no natural product based antitrypanosomal drugs is

in use or in late stage development. We recently reported the *in vivo* activity[7] and mode of action[8] of cynaropicrin (**1**) - a sesquiterpene lactone (STL). This was the first reported plant compound with *in vivo* anti-*T. brucei* effects, but more than 883 plant derived compounds have shown antiprotozoal (antitrypanosomal, antiplasmodial, and antileishmanial) effects *in vitro*, of which 87 were STLs.[5,6] STLs are a chemotaxonomic feature of the largest plant family, the Asteraceae,[9] and to date more than 5000 of them are known.[5,10] STLs are a promising compound class for antitrypanosomal drug discovery,[11-13] yet a better understanding of the structural features, which contribute to activity, is expedient.[14,15] This study explores structure activity relationships in a set of 18 natural STLs and 16 semi-synthetic STL – amines against *T. b. rhodesiense* and mammalian cancer cell (L6 cells) *in vitro*. The antitrypanosomal effects of eight of the compounds including **1**, **2**, **5-8**, **10**, and **13** have been reported before [7,11,12], but were included for comparison. Additionally, based on the *in vivo* antitrypanosomal effects of **1** after intraperitoneal application,[7,8] **1** and the dimethylamino derivative **19** were tested *in vivo* in the acute mouse model with oral application. The rationale behind derivative **19** was that masking the α,β-unsaturated enone in the lactone ring would possibly create a prodrug with increased water solubility, improved pharmacokinetic properties, and reduced unspecific binding to biological thiols via Michael reaction of the α-methylene-γ-lactone. Through subsequent bioactivation it would be converted to the parent compound **1** and hence, display its biological activity on the target. A similar approach had been previously successfully applied to several STLs with anticancer activity like helenalin, costunolide, and parthenolide.[16]

RESULTS and DISCUSSION

In vitro activity of compounds **1**-**34** against *T. b. rhodesiense* (STIB 900 strain), cytotoxicity against mammalian cells (rat myoblast L6 cells), as well as selectivity indices (SI; IC_{50} L6 / IC_{50} *T. b. rhodesiense* cells) are shown in Table 1.

Chart 1. Structures of sesquiterpene lactones (STLs) **1-18** and semi-synthetic STL amino derivatives **19-34**.



1 and onopordopicrin (**2**), which both had 2-(hydroxymethyl)acrylate side chains, showed IC_{50}s of 0.3 and 0.4 µM and SIs of 7.8 and 8.2, respectively. Prolongation of the **2**s side chain to a 3,4-dihydroxy-2-methylenebut**y**late as in cnicin (**3**) did not reduce activity (0.4 µM) or selectivity (SI: 10). Compound **5**, which lacked the 2-(hydroxymethyl)acrylate side chain of **1**, had a 16 fold lower antitrypanosomal activity (4.9 µM) and a nine fold lower cytotoxicity (19 µM) as compared to **1**. The antitrypanosomal effects (IC_{50} 4.4 µM) of dehydrocostuslactone (**6**), which lacks the two hydroxyl groups seen in **5**, were similar to **5** but with reduced selectivity (SI 1.9). Costunolide (**7**) was slightly less active (IC_{50} 1.3 µM) than its epoxy derivative parthenolide (**8**) (IC_{50} 0.8 µM). Zaluzanin D (**9**) a 7-acetoxy analogue of **6**, had half the antitrypanosomal activity (11 µM) and reduced selectivity (SI 1.4) when compared to **6**. Compound **10**, which had a cyclopentenone ring, exhibited considerably higher activity (IC_{50} 0.6 µM) against *T. b. rhodesiense* than **9** and **6**. Compounds **12** and **13** (eupatoriopicrin) are 4-hydroxy-3-(hydroxymethyl)but-2-enate (**12**) and (E)-3-hydroxy-2-(hydroxymethyl)acrylate (**13**)

derivatives of **7**. Their IC_{50}s of 0.9 (**12**) and 1.2 μM (**13**) were similar to that of the parent compound **7** (IC_{50} 1.3 μM; SI 5.9), but their selectivity indices were lower (SIs of 2.5 (**12**) and 1.3 (**13**), respectively). Compound **14**, a 2-methylbut-2-enate STL, was also less active and selective than the 2-(hydroxymethyl)acrylate STLs **1**, **2**, and **3**. Compounds **15-18**, which lack of an exocyclic methylene group in the lactone ring, showed reduced antitrypanosomal activities (IC_{50}s > 12 μM) and a total loss of their cytotoxicities.

The antitrypanosomal activity of the semi-synthetic dimethylamino derivative **19** (IC_{50} 0.5 μM) was moderately higher than that of the parent compound **1** (0.3 μM), whereas cytotoxicity was slightly decreased (SI 10.8 versus 7.8). Likewise derivative **20** was compared to its parent compound **5**. It showed slightly higher potency against *T. b. rhodesiense* (IC_{50} 3.6 μM for **20**, and 4.9 μM for **5**), but also higher cytotoxicity (SI 2.5 and 3.9, respectively). Compound **21** (IC_{50} 4.2 μM), a diethylamide derivative of **15**, was markedly more active than **15** (IC_{50} 55 μM). The STL derivatives **22-25** had morpholino groups. The adduct **22** (IC_{50} 0.7 μM) was as potent as parent compound **10** (IC_{50} 0.7 μM), with slightly increased selectivity (SIs of 10.3 and 7.6, respectively). Compounds **23** and **24** showed little cytotoxicity (IC_{50}s 65.6 μM and 31.3 μM, respectively), but **24** had much higher antitrypanosomal activity (IC_{50} 2.4 μM) than **23** (IC_{50} 11.8 μM). Compound **25** had a similar (IC_{50} 2.6 μM) activity against *T. b. rhodesiense* as **24**.

The STL 4-(2-aminoethyl)phenols **26-30** showed low cytotoxicities (IC_{50}s 22.1 μM to > 236 μM), and **29** had the highest antitrypanosomal activity (IC_{50} 6.6 μM). The STL 1-(2-chlorophenyl)piperazine **31** and the 2-(4-chlorophenyl)ethanamines **32-34** all showed low antitrypanosomal activity (IC_{50}s >5 μM) and cytotoxicity (IC_{50}s >20 μM).

Table 1. *In vitro* activity of compounds against STIB 900 strain and L6 cells

Compound	*T. brucei* STIB 900 IC_{50} (µM)[a]	L6 cells IC_{50}(µM)[a]	SI[b]
1	0.3 ± 0.001	2.2 ± 0.3	7.8
2	0.4 ± 0.01	3.1 ± 1.1	8.2
3	0.4 ± 0.1	4.2 ± 0.9	10
4	0.2 ± 0.01	0.6 ± 0.02	3
5	4.9 ± 0.34	19.2 ± 3.2	3.9
6	4.4 ± 1.2	8.3 ± 1.9	1.9
7	1.3 ± 0.4	7.7 ± 1.3	5.9
8	0.8 ± 0.5	5.2 ± 0.9	6.5
9	10.8[c]	15.6	1.4
10	0.6 ± 0.2	4.3 ± 0.5	7.6
11	5.8 ± 0.7	6.9 ±1.8	1.2
12	0.9 ± 0.2	2.2 ± 0.1	2.5
13	1.2 ± 0.2	1.6 ± 0.1	1.3
14	3.1 ± 0.3	10.5 ± 0.1	3.4
15	54.7 ± 8.0	353.2 ± 4.0	6.4
16	45.7 ± 5.0	> 292.2	> 6.4
17	41.5 ± 0.8	> 365.9	> 8.8
18	12.9 ± 2.4	34.0 ± 1.5	2.6
19	0.5 ± 0.003	5.2 ± 1.3	10.4
20	3.6 ± 1.0	8.6 ± 1.3	2.5
21	4.2 ± 0.8	9.4 ± 2.2	2.2
22	0.7 ± 0.1	7.4 ± 0.8	10.3
23	11.8 ± 2.7	65.6 ± 9.7	5.6
24	2.4 ± 0.7	31.3 ± 1.4	13.3
25	2.6 ± 0.5	9.9 ± 1.6	3.8
26	6.7 ± 1.3	> 236.2	> 35.0
27	13.0 ± 1.4	45.6 ± 3.2	4
28	13.4 ± 1.1	87.9 ± 2.2	6.5
29	6.6 ± 0.7	22.1 ± 4.0	3.3
30	9.9 ± 1.6	31.8 ± 1.3	3.2
31	7.0 ± 2.2	21.8 ± 2.7	3.1
32	10.6 ± 1.5	34.8 ± 1.2	3.4
33	10.2 ± 3.2	27.6 ± 6.7	2.7
34	5.4 ± 1.1	22.5 ± 1.6	4.2
Melarsoprol[d]	0.01 ± 0.01		
Podophyllotoxin[e]		0.02 ± 0.01	

[a] Average of three independent assays. [b] Selectivity Index (SI): IC_{50} against L6 cells divided by IC_{50} against STIB 900 strain. [c] tested once. [d] positive control for STIB 900 assay. [e] positive control for cytotoxicity assay.

We recently reported *in vivo* antitrypanosomal effects of **1**.[7,8] Upon intraperitoneal administration, the parasitemia was decreased over several days, but the compound was not able to cure the mice when they were treated with 10 mg/kg/b.i.d. for four consecutive days. In an attempt to improve the bioavailability, the exocyclic double bond in the lactone ring was masked to obtain water soluble dimethylamino derivative **19**. Compounds **1** and **19** were orally administered in the acute sleeping sickness mouse model. Four mice, each treated with 50 mg/kg body weight/day of **1**, showed reduced parasitemia on day 7 after infection. However, the animals were euthanized on day 10 postinfection due to obvious signs of cytotoxicity of the compound. Mice treated with compound **19** exhibited less signs of toxicity. However, the compound showed no *in vivo* efficacy, since the mean survival time was the same as for the control (Table 2).

Table 2. Activity of compound **1** and **19** in the STIB 900 mouse model of trypanosomiasis.

Compound	RA[a]	dose (mg/kg)	survival (days)[b]
1	po	4 x 50	9.5
19	po	4 x 50	10
	po	4 x 25	8.5

[a] RA, route of administration: oral (po). [b]Average days of survival of all mice; untreated controls euthanized at day 10 postinfection.

This structure activity relationship (SAR)-study showed the STL 2-(hydroxymethyl)acrylates **1**, **2**, and **4** alongside the STL 3,4-dihydroxy-2-methylenebutylate **3** are the most active and selective STLs against *T. b. rhodesiense*. These compounds have two active α,β-unsaturated enone moieties in common of which one methylene group is at the lacton ring and the other exocyclic double bond is at the side chain. Compound **10** has no side chain with enone function but does possess a cyclopentenone group, which can serve as the additional reactive enone. In fact a third reactive α,β-unsaturated group found in vernodalin (**4**) is expressed in slightly better potency, but revealed higher toxicity than **1**. These results are supported by decreased antitrypanosomal and cytotoxicity IC_{50}s of corresponding STLs lacking side chains (**5**, **6**), not having reactive α,β-enone functions in their side chains (**12**, **13,**

and **14**), and loss of the reactive terminal CH_2 in both the lactone ring and the side. The results are in accordance with findings by others.[16,17] Schmidt et al. (2009) showed in a SAR study with 40 STLs against the same structural features determined both antiprotozoal and cytotoxic activity α,β-unsaturated structural elements.[14] Many bioactivities in STLs have been attributed to a Michael addition of the methylene-γ-lactone motif to biological thiols.[18] Recent findings on the molecular interactions of the two α,β-unsaturated nucleophilic enone groups at C13 and C3´ in **1** with trypanothione and glutathione in trypanosomes via a Michael addition show the long presumed STLs mode of action, alongside inhibition of ornithine decarboxylase.[8] The same mode of action can be expected for **2**, **3**, and **4**. Regarding the 14 semisynthetic tested STL amines it was observed that the addition of morpholine goup and dimethylamine groups maintained or even enhanced the activity and selectivity of their amino STL derivatives, whereas, 4-(2-aminoethyl)phenol groups, 2-(4-chlorophenyl)ethanaminate groups or 1-(2-chlorophenyl)piperazine groups were not compared to their parent compounds.

The *in viv*o toxic effects of **19** in the *T. brucei rhodesiense* acute mouse model after oral application were reduced compared to **1**, but the antitrypanosomal effects were too. Further antitrypanosomal *in vivo* studies with other orally applied STL amino derivatives, are needed to demonstrate if the use of amino STLs as prodrugs is a reasonable approach to improving STLs suitability as antitrypanosomal drugs.

EXPERIMENTAL SECTION

Sample Preparation for Biological Testing.

Compounds were dissolved in DMSO (10 mg/mL) and stored at -20 °C until testing. Fresh dilutions in medium were prepared for each biotest. Final test concentrations did not exceed a 1%. DMSO and assays were done at least three times independently. The purity of all compounds was > 95% if not stated otherwise.

***Trypanosoma brucei rhodesiense* (STIB 900 Strain) Bioassay.**

Evaluation of *in vitro* antiprotozoal activity against *T. b. rhodesiense* was done using the Alamar Blue assay to determine IC_{50}s as previously described.[19] Serial threefold dilution were prepared in 96-well micro titer plates and 4000 *T. b. rhodesiense* STIB 900 bloodstream forms in 50 µL were added to each well except for the negative controls. Melarsoprol (Arsobal®, purity > 95%, Sanofi-Aventis, Meyrin, Switzerland) was used as reference drugs. After 70 h of incubation 10 µL of Alamar blue marker (12.5 mg resazurin (Sigma-Aldrich, Buchs, Switzerland) dissolved in 100 mL of distilled water) was added, and color change was developed for 2 to 6 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices Cooperation, Sunnyvale, CA) with an excitation wavelength of 536 nm and an emission wavelength of 588 nm was used to read the plates. The IC_{50} values were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

Rat Myoblast Cell L6-Cytotoxicity Assay.

The cytotoxicity assay was performed using the Alamar Blue assay described above with rat skeletal myoblasts (L6-cells) seeded in 100 µL RPMI 1640 in 96-well micro titer plates. After 24 h the medium was removed and replaced by 100 µL of fresh RPMI 1640 with serial threefold drug dilution. Podophyllotoxin (purity > 95%, Sigma-Aldrich) was used as a reference drug. After 70 h of incubation under a humidified 5% CO_2 atmosphere, 10 µL of the Alamar blue marker (see above) was added to all wells. The plates were incubated for an additional 2 h. A Spectramax Gemini XS micro plate fluorescence reader (Molecular Devices) was used to read the plates using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50}s were calculated from the sigmoidal growth inhibition curves using Softmax Pro software (Molecular Devices).

Acute Mouse Sleeping Sickness Model.

This model mimics the first stage of the human African trypanosmiasis. Adult female NMRI mice were purchased from Janvier (St. Berthevin, France). They weighed between 20 and 25 g at the beginning of the study and were kept under standard conditions in macrolon type III cages with food

pellets and water *ad libitum* at 22 °C and 60-70% humidity. All protocols and procedures used in this study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland (authorization N° 739; 11.12.2009). The samples were first dissolved in 100% DMSO followed by addition of distilled H_2O to a final DMSO concentration of 10%. For the establishment of the *in vivo* antitrypanosomal activity, the mice were infected intraperitoneally with 1 x 10^4 STIB900 bloodstream forms. Experimental groups of four mice were treated orally once a day on four consecutive days from day 3 to day 6 post infection. A control group of four mice was infected, but remained untreated. The determination of the parasitemia was done on day 7 post infection. Six µL of tail blood were diluted in 24 µL sodium citrate (3.2%), whereby the first µL was discarded to obtain circulating blood. Five µL of this mixture were transferred to a glass slide and covered with an 18 x 18 mm cover slide. The sample was examined under a light microscope (200-fold magnification) and parasites were counted in 3 of the 16 squares of the grid.

Test compounds

Cynaropicrin (**1**) was isolated from artichoke leaves as previously reported.[7] Compound **5** was prepared by mild alkaline hydrolysis of **1** as described by Zimmermann et al.[7] Zaluzanin D (**9**) and dehydrocostuslactone (**6**), were isolated from *Saussurea costus* as referenced.[11] Compounds **10**[20] and **16**[21] were synthesized according to literature methods. Vernodalin (**4**) was provided from Prof. Hajime Ohigashi, Kyoto University, Japan. Psilostachyin A (**11**) was kindly supplied by Dr. Wolfgang Schühly, University of Graz, Austria. Onopordopicrin (**2**) was isolated from *Arctium nemorosum.*[12] Nobilin (**14**) was kindly supplied by Prof. Imanidis from the University Applied Sciences and Arts Northwestern Switzerland. Compound **12** was from Prof. Merfort,University of Freiburg, Germany. Cnicin (**3**) was isolated from *Cnidus benedictus* L[22]. Eupatoriopicrin (**13**), costunolide (**7**), and parthenolide (**8**) were isolated from *Saussurea costus*.[11] Santonin (**17**) was purchased from Fluka Chemie (Buchs, Switzerland, > 98% purity). Compounds **18**, **21**-**32**, and **34** were synthesized as detailed elsewhere.[20, 23]

General Experimental Information.

NMR spectra were run on a 400 MHz Varian INOVA instrument. Samples were referenced against chloroform at 77.00 ppm for ^{13}C and against tetramethylsilane at 0.00 ppm for ^{1}H. High resolution mass spectra were recorded on a Waters SYNAPT G1 HDMS mass spectrometer operated in electrospray mode. Leucine enkephalin (50 pg/ml) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDa. Melting points were determined using a Mettler FP62 capillary melting point apparatus and are uncorrected. All reagents were of reagent grade purchased from Sigma-Aldrich (Schnelldorf, Germany) and were used without any further purification. Solvents used for chromatography or extractions were distilled prior to use. Thin-layer chromatography was carried out using pre-coated aluminum-backed plates (Merck Silica Gel 60 F_{254}). Column chromatography was performed on Fluka silica gel 60 (70–230 mesh). Dry solvents were purified as described by Perrin and Armarego.[24] All starting materials were obtained commercially and used without further purification.

((3S,3aS,6R,9bS)-6,8-dihydroxy-3,6,9-trimethyl-3,3a,4,5,6,6a,7,8-octahydroazuleno[4,5-b]furan-2(9bH)-one) (15).

A solution of *O*-acetylisophotosantonin (1.475 g, 4.813 mmol) in MeOH (49 mL) at 0 ºC was treated with $NaBH_4$ (0.293 g, 7.750 mmol) carefully. The reaction was left at 0 ºC for 3 h, then left to warm to room temperature overnight. The mixture was extracted from saturated aqueous NH_4CL (50 mL) with EtOAc (3 x 50 mL), the extracts pooled and dried ($MgSO_4$). The dried filtrate was concentrated to a tacky white foam, then dissolved in EtOH (16 mL). 5% aqueous KOH (150 mL) was added and the mixture stirred for 18 h at rt. The mixture was acidified to pH < 2 with 18% aqueous HCL, stirred for 30 min, extracted with EtOAc (3 x 50 mL) and washed with saturated aqueous K_2CO_3. Concentration yielded a yellow solid, which was recrystallized (EtOAc/hexane) to a white amorphous powder (0.313 g, 36%). NMR showed an approximately 2.2:1 mixture of secondary alcohols had been isolated. δ_H (400 MHz, $CDCl_3$ + CD_3OD) 4.73 (1H, d, *J* 11.0), 4.64 (0.4H, d, *J* 11.0), 4.54 (0.4H, d, *J* 7.1), 4.49 (1H, t, *J* 6.8), 2.90 (1H, br td, *J* 1.9, 7.5), 2.45 (1H, dt, *J* 8.0 and 13.8), 2.34 – 2.12 (2H, m), 2.03 – 1.91 (4H, m), 1.91 – 1.79 (5H, m), 1.65 (1H, dd, *J* 2.3 and 16.0), 1.61 (1H, td, *J* 6.7 and 13.7), 1.46 –

1.30 (1.5H, m), 1.22 (1H, d, *J* 6.9), 1.22 (3H, d, *J* 6.9), 1.03 (3H, s), 0.91 (1H, s); δ_C (101MHz, $CDCl_3$ + CD_3OD) 178.82, 178.71, 143.96, 143.79, 133.32, 131.28, 82.04, 81.71, 79.59, 76.97, 74.40, 74.23, 55.57, 54.31, 48.96, 48.92, 44.87, 44.27, 41.51, 41.34, 34.81, 34.36, 25.41, 25.26, 20.88, 20.56, 13.23, 12.23, 12.20; HRMS (ESI) calculated $C_{15}H_{21}O_3$ 249.1491, found 249.1423 (MH^+ - H_2O); and calculated $C_{15}H_{21}O_4$ 265.1440, found 265.1392 (MH^+ - H_2).

(3R,3aR,4S,6aR,8S,9aR,9bR)-3-((dimethylamino)methyl)-8-hydroxy-6,9-dimethylene-2-oxododecahydroazuleno[4,5-b]furan-4-yl 2-(hydroxymethyl)acrylate (19).

To a cold solution of **1** (0.50 g; 1.44 mmol) in absolute EtOH (15 mL), dimethylamine, (0.72 mL, 2.0 M solution in MeOH) was added under argon atmosphere. The solution was stirred at 5 ºC for 5 h, then concentrated under reduced pressure and recrystallized from acetone/Et_2O. The amino adduct (0.222 g; 0.566 mmol) was then dissolved in MeOH (5 mL) and a solution of HCL (0.45 mL; 1.25 N solution in MeOH) was added dropwise. After evaporation of the solvent the compound **19** was recovered as a yellow solid (0.242 g; 40%). ^{1}H NMR (500 MHz, CD_3OD): δ 6.28 (s, 1H), 5.97 (s, 1H), 5.31 (m, 2H), 5.15 (m, 2H), 5.00 (s, 1H), 4.50 (dt, *J* 8.0, 2.0 Hz, 1H), 4.44 (m, 1H), 4.30 (s, 2H), 3.62 (m, 1H), 3.50-3.42 (m, 2H), 3.03-2.91 (m, 8H), 2.83 (m, 1H), 2.72 (m, 1H), 2.33 (dd, *J* 13.4 and 7.0 Hz, 1H), 2.26-2.16 (m, 1H), 1.77-1.68 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 177.55, 166.72, 154.17, 143.95, 141.84, 127.82, 117.58, 111.28, 81.77, 77.26, 73.82, 61.98, 58.34, 50.74, 49.85, 44.98, 44.77, 42.02, 40.54, 39.51. HRMS (ESI) calculated for $C_{21}H_{29}NO_6$ $[M+H]^+$, 392.2067; found 392.2062.

(3R,3aR,4S,6aR,8S,9aR,9bR)-3-((dimethylamino)methyl)-4,8-dihydroxy-6,9-dimethylenedecahydroazuleno[4,5-b]furan-2(9bH)-one (20).

To a cold solution of **5** (0.050 g; 0.191 mmol) in absolute EtOH (5 mL), dimethylamine, (0.1 mL, 2.0 M solution in methanol) was added under argon atmosphere. The solution was stirred at 5 ºC for 5 h and then the mixture was concentrated under reduced pressure. The crude residue was then purified by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1) to afford compound **20** as a yellow oil

(0.052 g; 88 %). ^{1}H NMR (500 MHz, CD_3OD): δ 5.32 (br s, 1H), 5.28 (br s, 1H), 5.05 (br s, 1H), 5.00 (br s, 1H), 4.48 (tt, *J* 10.6, 7.3 and 2.6 Hz, 1H), 4.16 (dd-app. t , *J* 9.7 Hz, 1H), 3.67 (ddd, *J* 9.0, 7.3 and 5.0 Hz, 1H), 3.02-2.80 (m, 4H), 2.73-2.64 (m, 2H), 2.40 (s, 6H), 2.26-2.14 (m, 3H), 1.70 (ddd, *J* 12.8, 9.7 and 8.8 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ 177.71, 154.33, 145.14, 116.01, 111.70, 80.53, 74.00, 60.81, 58.34, 50.53, 46.44, 44.89, 44.82, 43.08, 39.49. HRMS (ESI) calculated for $C_{17}H_{25}NO_4$ $[M+H]^+$, 308.1856; found 308.1862.

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ABBREVIATIONS

STL, sesquiterpene lactones; HAT, human African trypanosomiasis; NECT, nifurtimox-eflornithine combination therapy; SAR, structure activity relationships

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CHAPTER 5

Mode of action of cynaropicrin

Fifth publication:

Trypanosomatids have a unique trypanothione-based redox metabolism to deal with oxidative stress, which replaces the nearly ubiquitous GSH redox system. Because the trypanothione redox system exclusively occurs in trypanosomatids it represents several promising targets such as direct interference with GSH and $T(SH)_2$, trypanothione reductase, trypanothione synthetase, and ornithine decarboxylase.

This publication demonstrated that the uptake of CYN lead to a rapid and complete depletion of GSH and $T(SH)_2$ within 5 min. in the trypanosomes. This action was based on the formation of CYN-thiol adducts by Michael-addition with CYN's reactive exocyclic α,β-unsaturated moieties and GSH and $T(SH)_2$. Irreversible phenotypic changes of the trypanosomes to a stumpy-like form in cell deterioration and death were observed. Additionally, LC-MS/MS ornithine quantification studies indicated that CYN is a potent ODC inhibitor.

This puplication proves the longstanding theory that STLs effect intracellular thiol levels in general and $T(SH)_2$ in particular [1].

Preparation and structure elucidation of $T(S\text{-}CYN)_2$ and GS-CYN, development of intra,- and extracellular extraction protocol, stability test for CYN-thiol peptide adducts, extraction control analysis, quantitative analysis of CYN, $T(SH)_2$, GSH, GS-CYN, $T(S\text{-}CYN)_2$, and ornithine from intact T. b. rhodesiense cells and from the extracellular milieu by UHPLC-MS/MS, investigation of protein-binding of CYN during the extraction of the extracellular milieu, writing of the manuscript, and preparation of all figures and tables.

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Cynaropicrin targets the trypanothione redox system in *Trypanosoma brucei*

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Abstract

In mice cynaropicrin (CYN) potently inhibits the proliferation of *Trypanosoma brucei* - the causative agent of Human African Trypanosomiasis - by a so far unknown mechanism. We hypothesized that CYNs α,β-unsaturated methylene moieties act as Michael acceptors for glutathione (GSH) and trypanothione ($T(SH)_2$), the main low molecular mass thiols essential for unique redox metabolism of these parasites. The analysis of this putative mechanism and the effects of CYN on enzymes of the $T(SH)_2$ redox metabolism including trypanothione reductase, trypanothione synthetase, glutathione-S-transferase, and ornithine decarboxylase are shown. A two step extraction protocol with subsequent UPLC-MS/MS analysis was established to quantify intra-cellular CYN, $T(SH)_2$, GSH, as well as GS-CYN and $T(S\text{-}CYN)_2$ adducts in intact *T. b. rhodesiense* cells. Within minutes of exposure to CYN, the cellular GSH and $T(SH)_2$ pools were entirely depleted, and the parasites entered an apoptotic stage and died. CYN also showed inhibition of the ornithine decarboxylase similar to the positive control eflornithine. Significant interactions with the other enzymes involved in the $T(SH)_2$ redox metabolism were not observed. Alongside many other biological activities sesquiterpene lactones including CYN have shown antitrypanosomal effects, which have been postulated to be linked to formation of Michael adducts with cellular nucleophiles. Here the interaction of CYN with biological thiols in a cellular system in general, and with trypanosomal $T(SH)_2$ redox metabolism in particular, thus offering a molecular explanation for the antitrypanosomal activity is demonstrated. At the same time, the study provides a novel extraction and analysis protocol for components of the trypanosomal thiol metabolism.

Keywords: Sesquiterpene lactone, *Trypanosoma brucei*, trypanothione, drug target, HPLC-MS/MS

1. Introduction

Human African Trypanosomiasis (HAT) is a deadly parasitic disease, which is caused by *Trypanosoma brucei* sub-species, and spread by the bite of the tsetse fly (*Glossina spp.*). As there is no vaccination chemotherapy remains the principal control of HAT. Severe side effects, treatment failures and complex administration schemes urgently demand for safer orally administerable drugs [1].

Several pathways in trypanosomes may provide suitable targets for new drugs including ergosterol- and purin biosynthesis, various kinases, farnesyl transferase, proteases, pyrimidine biosynthesis, compartmentalized glycolysis, and finally trypanothione-based redox metabolism [2] (**Fig. 6**), with which a reducing intracellular milieu is maintained [3,4]. Because the trypanothione redox system is unique to trypanosomatids it represents several promising drug targets such as trypanothione synthetase (TryS), trypanothione reductase (TR), spermidine synthase (SpS), and ornithine decarboxylase (ODC), or by direct interaction with glutathione (GSH) and trypanothione ($T(SH)_2$) (**Fig. 6**) [5].

We recently reported that cynaropicrin (CYN), a sesquiterpene lactone (STL) found in artichokes (*Cynara scolymus* L.) and some species of cornflowers (*Centaurea spp.*), inhibits the proliferation of *T. b. rhodesiense* in the acute mouse model [6]. CYN is the so far only plant compound demonstrated to have in vivo anti *T. b. rhodesiense* activity. Numerous other STLs have, however, shown antitrypanosomal effects in vitro [7]. Schmidt et al. supplied two excellent reviews of antiprotozoal in vitro effects of 883 plant derived natural products including 83 STLs [7,8]. The authors showed in a QSAR study of 40 STLs, that the antitrypanosomal activity was linked to the presence of the α-methylene-γ-lactone group [9,10]. Numerous biological activities by STLs have been attributed to the covalent binding of the reactive α-methylene-γ-lactone to sulfhydryl groups in biomolecules like L-cysteine and GSH via a nucleophilic Michael addition [11-13].

Considering trypanosomes depend on their trypanothione-based redox system as a sole means of detoxification [14], and CYN contains reactive exocyclic α,β-unsaturated keto moieties (**Fig. 1**) [6], it was assumed that CYN may interact with GSH and/or $T(SH)_2$.

Here efficient extraction protocols for CYN, the thiol peptides $T(SH)_2$ and GSH, as well as GS-CYN and $T(S\text{-}CYN)_2$ adducts from *T. b. rhodesiense* parasites were developed and validated, and the use of ultra high performance liquid chromatography separation methods combined with tandem mass spectrometry methods (UPLC-MS/MS) to quantify these in part per billion (ppb) concentrations are described. Furthermore, the direct effects of CYN on various enzymes involved in $T(SH)_2$ redox metabolism, TryS, TR, and ODC were assessed.

2. Material and methods

2.1. Solvents and reagents

CYN was isolated as previously described [6]. GSH and forskolin were obtained from LC laboratories (Woburn, USA); γ-Glu-Ala-Gly (EAG) was synthesized by GeneCust (Dudelange, Luxembourg); $T(SH)_2$ was synthesized as previously described [15]; dithiothreitol (DTT) and bovine serum albumin (BSA) were from Sigma-Aldrich (Switzerland); H_2O was obtained from an EASYpure II water purification system (Barnstead; Dubuque, IA, USA); acetonitrile, methanol, and formic acid (FA) were all UPLC-MS grade from BioSolve (Valkenswaard, Netherlands); N_2 was produced with a generator (Schmidlin Labor + Service AG, Neuheim, Switzerland); argon was from Carbagas (Basel, Switzerland); sodium phosphate (Na_2HPO_4) was from Fluka Chemika; NaCl from Scharlau (Barcelona, Spain), glucose monohydrate from Biochemika, Applichem (Darmstadt, Germany); DFMO was a gift of Dr. Cyrus Bacchi, (Pace University, New York, USA); and DMSO-d_6 was from Armar Chemicals (Döttingen, Switzerland).

2.2. Preparation and structure elucidation of T(S-CYN)$_2$ and GS-CYN

CYN (45 µM) was incubated with either T(SH)$_2$ or GSH (both 450 µM) in H_2O (+0.1 % FA) supplemented with 250 µM DTT, for 10 min at room temperature (rt). Ten µL of the reaction solution were analyzed by HPLC-MS using an Agilent series 1100 HPLC system (Agilent, Heilbronn, Germany), coupled to an Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Separation conditions: A SunFire RP-18 column (3.5 µm, 3 x 150 mm, Waters GmbH, Eschborn, Germany) at rt was used. The mobile phase consisted of A: H_2O + 0.1% FA, and B: acetonitrile + 0.1% FA. The pump program was 10%-100% B in 20 min, and 100% B for 5 min; the flow rate was 0.5 mL/min The CYN-thiol peptide adducts eluted at retention time (^{t}R) 2.1 min (T(S-CYN)$_2$) and ^{t}R 2.4 min (GS-CYN), respectively. They were collected, dried under N_2 (Thermolyne Dri-Bath, Ismatec SA, Zürich, Switzerland), and subjected to ^{1}H NMR measurements in 70% H_2O and 30% DMSO-d_6 using a Bruker Avance III^{TM} 500 MHz spectrometer (Bruker, Fällanden, Switzerland) [16].

2.3. Quantitative analysis of CYN, T(SH)$_2$, GSH, GS-CYN, and T(S-CYN)$_2$, from intact T. b. rhodesiense cells by UHPLC-MS/MS

Ultra high performance liquid chromatography (UHPLC-MS/MS): An Acquity UHPLC (Waters Corp., Milford, USA) coupled to an Acquity tandem quadrupole MS detector (TQD) was used with cooled autosampler set to 10 °C protected from light and a column heater set to 45 °C. Separation conditions: UHPLC HSS T3 column (1.8 µm, 100 mm x 2.1 mm, Waters Corp., Milford, USA), H_2O (+0.1% FA) 100 - 0% in 4 min, 100% acetonitrile (+0.1% FA) for 1 min; the flow rate was 0.5 mL/min. Data were acquired with MassLynx V4.1 software and processed for quantification with QuanLynx V4.1 (Waters Corp., Milford, USA) in positive ionization mode (ESI$^+$), with argon as collision gas. MS/MS parameters were determined automatically by Waters IntelliStart software and optimized manually afterwards. The source temperature was 150 °C and desolvation temperatures were 300 – 400 °C. Table 1 summarizes the MS/MS parameters [17].

2.4. Quantitative analysis of ornithine from intact T. b. rhodesiense cells by UHPLC-MS/MS

UHPLC was performed on an Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA) series instrument equipped with a binary pump (G4220A), an autosampler (G4226A) regulated at 10°C (G1330B), a thermostatted column compartment (G1316A) at 80°C and a Flexcube (G4227A). Separation conditions: An Acquity UHPLC BEH amide column, 50 x 2.1 mm, 1.7 µm (Waters Corp., Milford, MA, USA) was used, the flow rate was 0.5 mL/min, H_2O (+0.1% FA) 95 - 5% in 2.5 min, 100% acetonitrile (+0.1% FA) for 0.5 min. Tandem mass spectrometry analysis was performed on an Agilent Technologies 6430 Triple Quadrupole MS/MS system (Agilent Technologies, Santa Clara, CA), with a MassHunter software B.05.00 workstation. ESI^+ mode was used for ornithine with the following settings: capillary voltage 4000 V, source temperature 350°C, Electron-Multipler Voltage 500 V, drying gas (pure nitrogen) flow 13 L/min, and nebulization pressure of 60 psi (Table 1).

2.5. Stock solutions of standards

Stock solutions (SS, 1 mg/mL) of GSH and $T(SH)_2$ were prepared in H_2O (+0.1% FA) supplemented with a 20 fold excess of DTT. Ornithine SS (1 mg/mL) was solved in H_2O (+0.1% FA). CYN was dissolved in DMSO, and $T(S\text{-}CYN)_2$ and GS-CYN were prepared in H_2O:DMSO mixtures (70:30, v/v) with further dilution in H_2O (+0.1% FA) to contain < 1% DMSO in the bioassays. SSs were prepared freshly on a daily bases.

2.6. Stability test for CYN-thiol peptide adducts

The stabilities of the SSs of CYN, GSH and the GS-CYN-monoadduct were monitored directly before and after 72 h of storage at rt unprotected from light. Generally, good stabilities were observed for all compounds. At time point zero and after 72 h storage, the concentrations of CYN, GSH, GS-CYN-monoadduct differed by less than 5%, when analyzed by UPLC-MS/MS (data not shown).

2.7. Quantification of CYN, $T(SH)_2$, GSH, GS-CYN, $T(S\text{-}CYN)_2$, and ornithine in T. b. rhodesiense

T. b. rhodesiense (STIB 900) bloodstream forms were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with 0.2 mM 2-mercaptoethanol as described by Baltz et al. [18] with the following modifications: 1 mM sodium pyruvate, 0.5 mM hypoxanthine, and 15% heat-inactivated horse serum.

2.7.1. Preparation of cell lysates for the analysis of cell contents

Trypanosome cultures were grown to a density of 2.0 x 10^6 cells/mL in 50 mL flasks and CYN was added (resulting in 50 µM; final DMSO concentration < 1%). After incubation for 0, 5, 10, 20, 30, and 40 min, the samples were transferred into 50 mL Falcon tubes (Eppendorf, Germany) and centrifuged for 5 min at 3500 rpm, rt (Rotina 420 R, Hettich Zentrifugen, Bäch, Switzerland). The cell pellets were washed twice with 1.0 mL sodium phosphate buffer, pH 8.0 (60 mM Na_2HPO_4, 44 mM NaCl, 50 mM glucose monohydrate) and lysed by adding 0.5 mL H_2O (+0.1% FA). After centrifugation for 2 min at 13'200 rpm, rt (Eppendorf Centrifuge, 5415, Switzerland) the lysates were transferred into 96 deep-well plates. Thiol peptides (GSH and $T(SH)_2$), CYN, GS-CYN, $T(S\text{-}CYN)_2$, and ornithine were then immediately quantified by UHPLC-MS/MS. This was done for at least three independent experiments. To calculate the intracellular concentration of analytes in trypanosomes, the concentration in the UHPLC-MS/MS samples was multiplied by the dilution factor of the pellets during lysis. The pellets volume was calculated as the cell density determined in the culture before centrifugation times the volume of a trypanosome (58 femtolitres) [14].

2.7.2. Analysis of the extracellular milieu

After centrifugation of the 50 mL STIB 900 culture in the Falcon tubes an aliquot of 200 µL supernatant was extracted with 150 µL BSA solution (60 g/L) and 1.0 mL ice cold acetonitrile. The samples were shaken for 10 min at 1400 rpm, rt (thermomixer compact, Eppendorf, Switzerland) and centrifuged for 20 min at 1200 rpm, rt (centrifuge mini Spin plus,

Eppendorf, Switzerland). The supernatants were transferred to 96 deep-well plates (Eppendorf, Germany). GSH and $T(SH)_2$, CYN, GS-CYN, and $T(S\text{-}CYN)_2$ were immediately quantified by UHPLC-MS/MS.

2.7.3. Extraction control

Forskolin was used as internal standard (IS) for CYN. EAG, a synthetic GSH homologue, where the cysteine is replaced by alanine to prevent adduct formation with CYN, served as IS for GSH and $T(SH)_2$ (**Fig. 1**). Either forskolin or EAG (SS 1 mg/ml) was added to the 0.5 mL extraction solutions. For further sample preparation see section 2.7.1. Additionally, forskolin was used as a control for the efficiency of the extraction of the supernatant (see 2.7.2). Both the cell extraction controls and the supernatant extraction controls were analyzed in three independent experiments. Recovery factors were calculated in percentage and included in the quantification of the thiol-adducts in vitro. MS/MS parameters for standards are shown in Table 1.

2.7.4. Investigation of protein-binding of CYN during the extraction of the supernatant

CYN was quantified in the supernatant using a modified protocol: 50 mL medium without parasites was exposed to 50 µM CYN. Sample preparation and UHPLC-MS/MS analysis were done three times independently as in 2.7.2., and percentages of recovery were included into the thiol-adduct quantification study. Quantification of 50 µM in 50 mL medium yielded 21.7 ± 0.2 µM free CYN, meaning the total loss during the supernatant extraction step was 56.6%. Albumin was not used in the cell extraction protocol, which is why this experiment was not done for that setup.

2.8. Enzymatic assays

2.8.1. T. cruzi trypanothione reductase (TcTR)

Reversible inhibition of TcTR was studied as described by Jockers-Scherübl [19]. The assay contained in a total volume of 1 mL: 40 mM Hepes, 1 mM EDTA; pH 7.5, 100 µM

NADPH, and 200 µM CYN (4 mM stock solution in DMSO) or the respective amount of DMSO and 2 to 4 nM TcTR. The reaction was started by adding 96 µM TS_2 and the absorption decrease at 340 nm was followed at 25 °C. For detecting putative irreversible inhibition, 1 µM TR was incubated at 25 °C with 100 or 200 µM of CYN in the presence and absence of 625 µM NADPH. After 0.5, 5, 20, 25, 60, 120, 180, 183, 240 min, a 3 µL aliquot was removed and the remaining activity was measured in a standard assay.

2.8.2. T. brucei trypanothione synthetase (TbTryS)

TbTryS activity was measured at 25 °C by coupling the ATP hydrolysis to NADH consumption as previously described [20]. The reaction mixture contained in a total volume of 1 mL of 100 mM Hepes, pH 8.0, 0.5 mM EDTA, 10 mM $MgCl_2$, 200 µM NADH, 1 mM phosphoenolpyruvate, 2 U pyruvate kinase, 2 U L-lactate dehydrogenase, about 350 nM TbTryS, 200 µM CYN (stock solution 4 mM in DMSO) or 50 µL DMSO, 2.5 mM ATP, and 0.1 mM GSH. The reaction was started by adding 8 mM spermidine. To test for covalent inactivation, 6.9 µM TryS was preincubated with 200 µM CYN at 25 °C. After 0, 25, 60, 120, 180, 240, and 300 min, a 50 µL aliquot was removed and the remaining activity measured in a standard assay.

2.8.4. T. b. rhodesiense ornithine decarboxylase (ODC) in STIB900 cells

Ornithine levels were monitored from 2 x 10^6 *T. b. rhodesiense* (STIB900) cells (see 2.7.1.) after 20 min of exposure to 50 µM CYN by UHPLC-MS/MS. In parallel, experiments with the positive control DFMO (50 µM) were done (see **Table 1**).

3. Results

The working hypothesis for this study was that CYN shows trypanocidal activity, because it binds to the thiol moieties of $T(SH)_2$ and GSH, thus affecting the redox homeostasis of the parasites. To prove this it was first necessary to synthesize the reference compounds GS-CYN and $T(S\text{-}CYN)_2$, and confirm their structures (see 2.2.). CYN was mixed with either $T(SH)_2$ or GSH and the reaction solution was subjected to HPLC-MS. In the case

of $T(SH)_2$, the chromatogram (in positive ESI mode) showed the educts plus one major peak (^tR: 2.45 min) with a mass of 1070.4 *m/z* $[M+H]^+$, and its corresponding double charged ion of 535.8 *m/z* $[M/2+H]^+$. This indicated that CYN had formed an adduct with $T(SH)_2$. The LC-MS analysis of the GS-CYN reaction solution (in positive ESI mode) showed two main peaks in addition to the educts. The MS spectrum of the first peak (^tR: 5.20 min) showed a mass of 654.1 *m/z* $[M+H]^+$, indicating a GS-CYN monoadduct. The second peak (^tR: 4.10 min), whith an 80 times lower intensity, showed a mass of 961.2 *m/z* $[M+H]^+$ and a double charged ion 481.2 *m/z* $[M/2+H]^+$, indicating a bisadduct of two GSHs with one CYN. The GS-CYN and $T(S\text{-}CYN)_2$ adducts were isolated by HPLC, dried under N_2, and their structures elucidated by ^1H NMR (**Fig. 2** and **3**). The spectrum of $T(S\text{-}CYN)_2$, when compared to those of CYN and $T(SH)_2$, showed that the proton signals at exocyclic double bonds at position 13 (5.8 and 6.2 ppm, both d), and 3' (5.5 and 6.2 ppm; both d) had disappeared (**Fig. 1**). This confirmed that the isolated product was a bisadduct in which CYN had reacted with both thiol groups of $T(SH)_2$ via a Michael addition on the positions C13 and C3' (**Fig. 2**). Because $T(SH)_2$ consists of two GSH units with a spermidine - an unsymmetrical molecule - as linker, there are two possible isomers of the $T(S\text{-}CYN)_2$ bisadduct linked at C13 and C3', which could not be differentiated here. The ^1H spectrum of the GS-CYN adduct lacks the proton signals of the exocyclic double bond at the lactone ring, indicating that GSH had reacted with this exocyclic methylene group (**Fig. 3**).

The next step was to verify that these CYN-thiol adducts are actually formed in the living parasites. The parasites were treated with 50 µM CYN, harvested, lysed and analyzed at different time points by UHPLC-MS/MS. After 5 min, an extracellular concentration of 35 µM CYN, and intracellular concentrations of 23 ± 5 µM CYN, 42 ± 17 µM GS-CYN-monoadduct, and 49 ± 18 µM $T(S\text{-}CYN)_2$-bisadduct were measured. A complete depletion of the parasites intracellular reduced free thiol pool, which had been 104 ± 28 µM GSH and 729 ± 103 µM $T(SH)_2$ at time point zero (**Fig. 4**) was observed. Over 40 min of exposure to CYN the concentration of the CYN-thiol adducts gradually decreased to concentrations below the LLOQ (**Table 1**). Concentrations of $T(SH)_2$, GSH, GS-CYN, and $T(S\text{-}CYN)_2$ measured in the

extracellular milieu were all below LLOD. The depletion of intracellular GSH and $T(SH)_2$ was irreversible, which was shown by washing the cells after exposure to 50 µM CYN for 5, 10, 20, 30, and 40 min and replacing the supernatant with fresh medium, where it was observed, that the trypanosomes were not able to recover, and inevitably died (data not shown).

In parallel to the UHPLC-MS/MS analysis, the CYN treated trypanosomes were observed under a light microscope (Leitz, Wetzlar, Germany). After 0, 5, 10, 20, 30 and 40 min of incubation, the parasites were stained with Giemsa (Merck, Darmstadt, Switzerland), and phenotypes were evaluated. The trypanosomes, which at time point zero had shown the regular slender form, transformed within 5-10 minutes of exposure to CYN into an intermediate form, and finally into a stumpy-like form, and died. The stumpy-like form is indicative of an apoptosis-like behavior (**Fig. 4**) [21].

As shown above, there was no detectable free GSH and $T(SH)_2$ left in the cells after 5 min of incubation with 50 µM CYN. The thiols bound in the GS-CYN and $T(S\text{-}CYN)_2$ adducts accounted for 15% (GSH) and 4% ($T(SH)_2$) of reduced thiols present at time point zero. Therefore, the binding of CYN to GSH and $T(SH)_2$ could not entirely explain the complete depletion of the intracellular thiol pool, and it seemed appropriate to determine a putative interaction of CYN with enzymes involved in the formation and regeneration of $T(SH)_2$. For this reason, the effect of CYN on ODC, TryS, and TR was studied.

In *T. b. rhodesiense* cells treated for 20 min with 50 µM CYN the intracellular concentration of ornithine had increased twelve-fold from 4 ± 1 µM to 50 ± 21 µM. DFMO treated cells showed concentrations of 48 ± 13 µM after 20 min. It can thus be concluded that CYN was a similar strong ODC inhibitor than the positive control DFMO, which is used as a drug to treat late stage *T. b. gambiense* HAT [5].

In the case of TryS, 200 µM CYN was added to a standard assay and there was no significant inhibition of the enzymes activities (data not shown). To study whether irreversible binding occurred, TryS and TR were pre-incubated with CYN for up to 240 min, and the remaining activity was measured in the standard assay (**Fig. 5**). After 240 min of

preincubation the activity of TryS was decreased by 51% (**Fig. 5A**). Also in the case of TR, incubation with 100 and 200 µM CYN did not strongly affect the enzymes activity (Fig. 5B). It was thus demonstrated, that CYN only weakly inactivates TryS and TR at very high concentrations. It is therefore unlikely that TryS or TR are main molecular targets for CYN. The TryS assay was done without DDT to rule out the influence of a Michael adduction of CYN to DDT occurring (**Fig. 5A**).

In summary, it was shown that 50 µM CYN completely depletes intracellular GSH and $T(SH)_2$ pools in *T. brucei* STIB900, via covalent binding of CYN to free sulfhydryl groups of GSH and $T(SH)_2$ via a Michael addition, and by inhibition of ODC. These effects combined sufficiently explain the potent antitrypanosomal effects of CYN (**Fig. 6**).

Discussion

It is shown, that CYN binds to GSH and $T(SH)_2$ in intact *T. b. rhodesiense*. The notion that STLs reactive enone groups might act as Michael acceptors for sulfhydryl groups in biomolecules, has been described before. Kupchan and co-workers in 1970 [11] showed that elephantopin, eupatundin, and vernolepin bind covalently via a Michael addition of the α-methylene-γ-lactone group to the sulfhydryl group of L-cysteine. With an excess of L-cysteine, elephantopin formed a bisadduct with two L-cysteines through a second addition to the other reactive enone moiety. The elephantopin-cysteine adducts were isolated and structurally elucidated with ^{1}H-NMR. Schmidt et al. showed that the antitrypanosomal STL helenalin [22] formed 2-monoadducts and 2,13-bisadducts with both GSH and cysteine in a cell free assay [23]. These adducts too were isolated and structurally elucidated by ^{1}H-NMR. Fairlamb et al. [24] showed that melarsoprol, an organo-arsenic drug used to treat 2nd stage *T. b. rhodesiense* HAT, can form a melarsenoxide-trypanothione-complex in vitro, which might represent the mode of action of melarsoprol.

The experimental data proves for the first time that the formation of STL-thiol adducts indeed takes place in intact cells, and thus could explain the in vitro activity observed for many STLs [7], and the in vivo effects of CYN [6]. All extraction parameters were carefully

monitored to ensure adducts were generated in the cells and not extraction artefacts. The formation of adducts and depletion of thiols were monitored in a time-dependent manner. The uptake of CYN into the cells was efficient, as after 5 min of exposure to 50 µM CYN the extracellular CYN concentration was 35 µM, and the total intracellular CYN amounted to 214 µM (as free CYN, GS-CYN, and $T(S\text{-}CYN)_2$).

Established quantitative HPLC methods for studying cellular GSH and $T(SH)_2$ use fluorescent dyes such as monobromobimane or Ellman's reagent for thiol derivatization and fluorescence detection [25,26]. In the methodology presented, the complex extraction and derivatization steps [24,27] are replaced by a simple two-step extraction with water, and subsequent direct analysis by UPLC-MS/MS [28]. The advantages of analyzing $T(SH)_2$ and GSH with UHPLC-MS/MS compared to HPLC methods using derivatization, lie in the direct detection of thiols/ and thiol adducts with far superior sensitivity in the ppb range. The applicability of these extraction and quantification protocols is not limited to trypanosomes, but could be applied for studying biological thiols and thiol derivatives in other cellular systems.

In summary, it was shown that the uptake of CYN leads to a rapid and complete depletion of GSH and $T(SH)_2$, due to the formation of CYN-thiol adducts and the inhibition of ODC. This results in irreversible phenotypic changes of the trypanosomes to a stumpy-like form in cell deterioration, and death. Exemplified by CYN this study proves the longstanding theory that STLs effect intracellular thiol levels in general and $T(SH)_2$ in particular.

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Legends for figures

Fig.1. Structures of the sesquiterpene lactone cynaropicrin (CYN), the parasite main low molecular mass thiols glutathione (GSH) and trypanothione ($T(SH)_2$) and the internal standards forskolin and EAG.

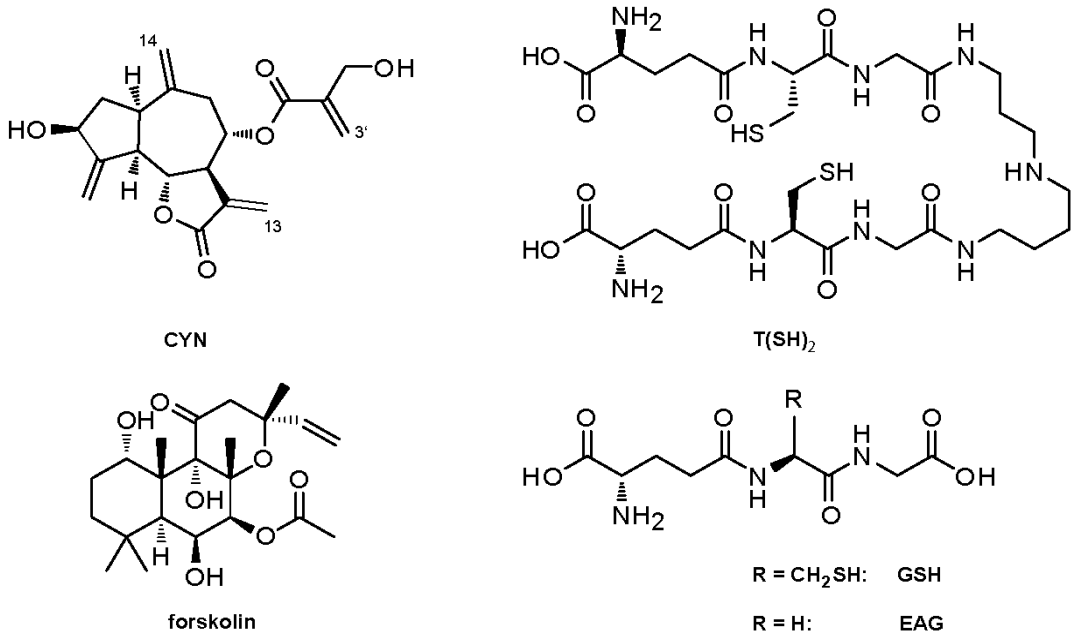

Fig.2. ^1H NMR spectrum of $T(S\text{-}CYN)_2$ bisadduct.

The ^1H NMR spectra of the $T(S\text{-}CYN)_2$ bisadduct, $T(SH)_2$ and CYN recorded in 70% H_2O and 30% DMSO-d_6. The exocyclic proton signals at position 13 (red color) and position 3' (blue color) of CYN are absent from the adduct. The structural formula represents one of two possible isomers.

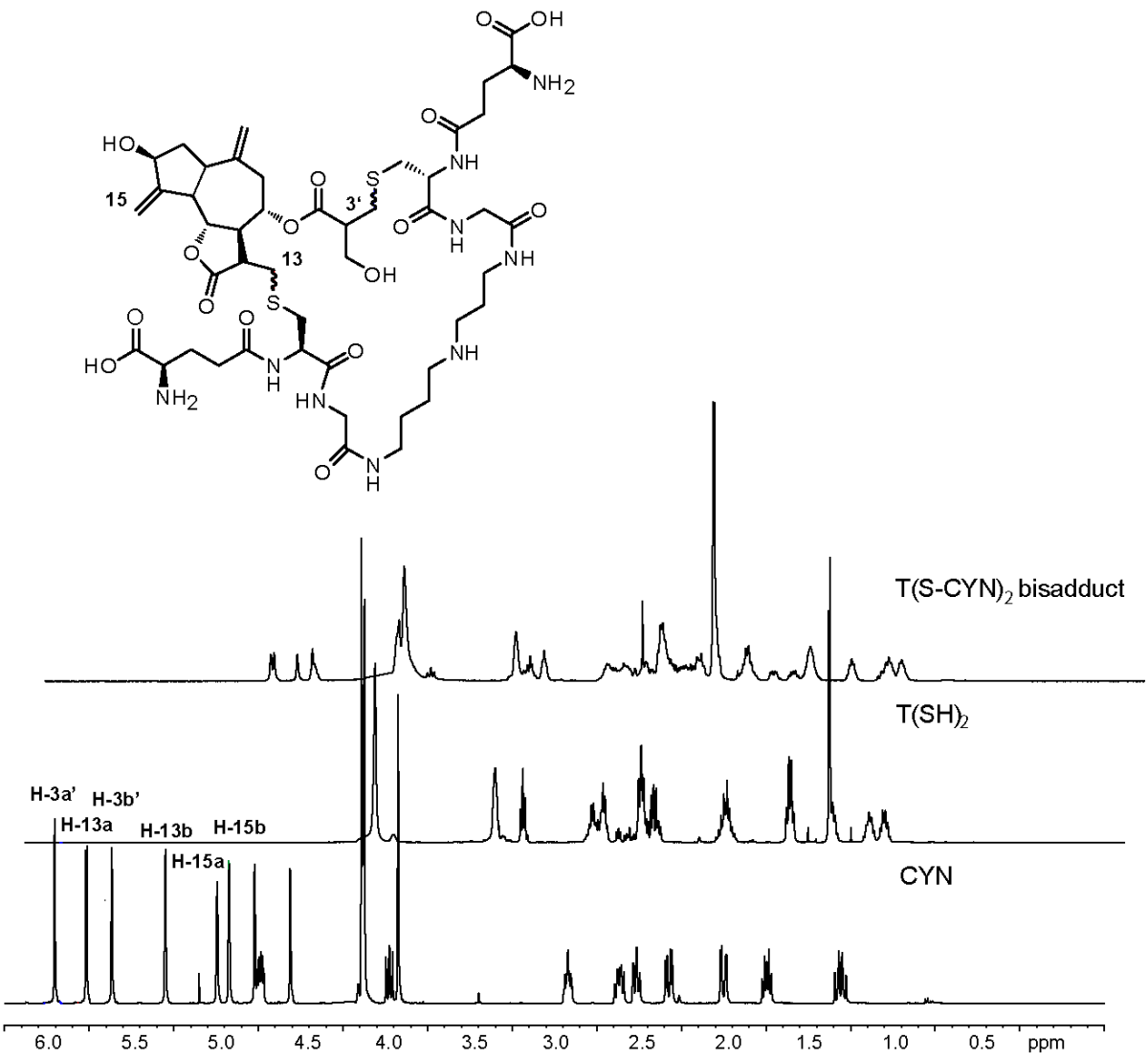

Fig.3. ^{1}H NMR spectrum of GS-CYN monoadduct.

The ^{1}H NMR spectra of the GS-CYN adduct and CYN measured in 70% H_2O and 30% DMSO-d_6. The exocyclic methylene signals at position 13 of CYN do not appear in the adduct. This correlates to a GS-CYN monoadduct, where the GSH is bound to the methylene moiety of the lactone.

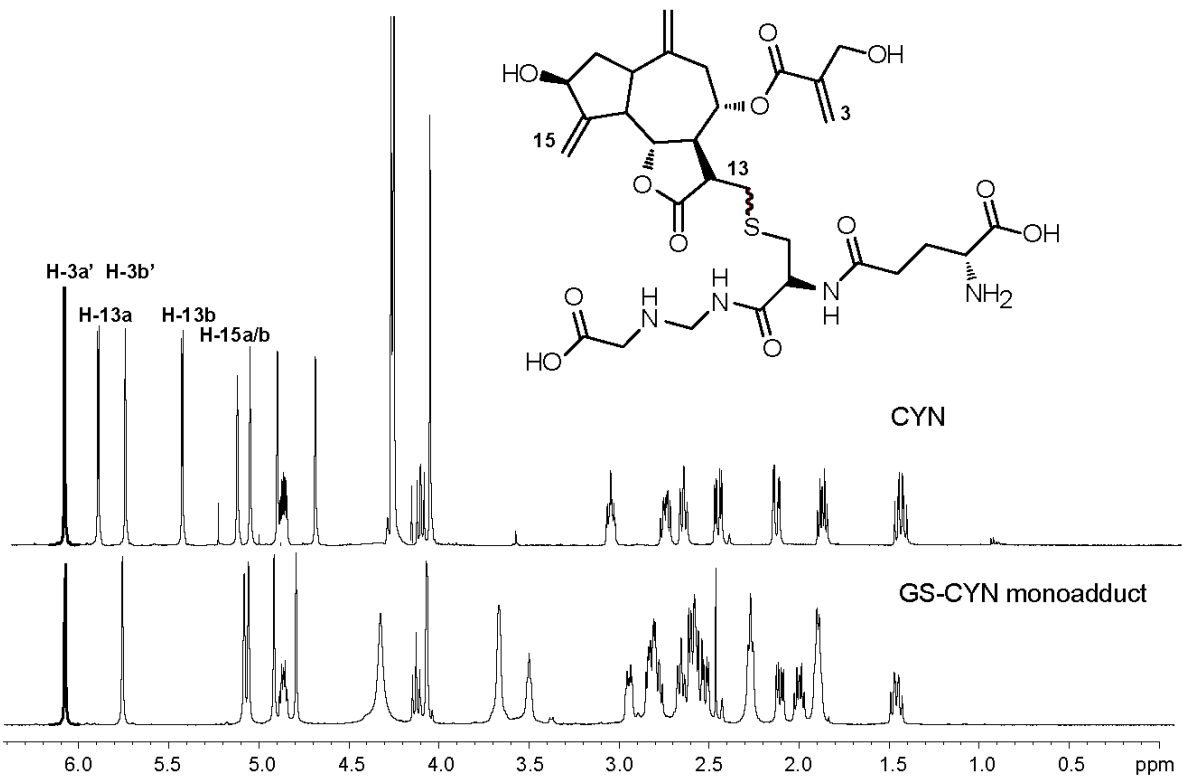

Fig. 4. Quantification of GSH, $T(SH)_2$, and the respective GS-CYN, and $T(S\text{-}CYN)_2$.

Intracellular concentrations were determined for thiol peptides GSH and $T(SH)_2$, CYN-thiol adducts, and the extracellular concentration of CYN after exposure to 50 µM CYN for 0, 5, 10, 20, 30, and 40 min. Quantification was done with at least three independent experiments. The phenotypes of the trypanosomes were evaluated at each time point by Giemsa staining and light microscopy. Phenotype abbreviations: sl: slender; im: intermediate; s: stumpy-like.

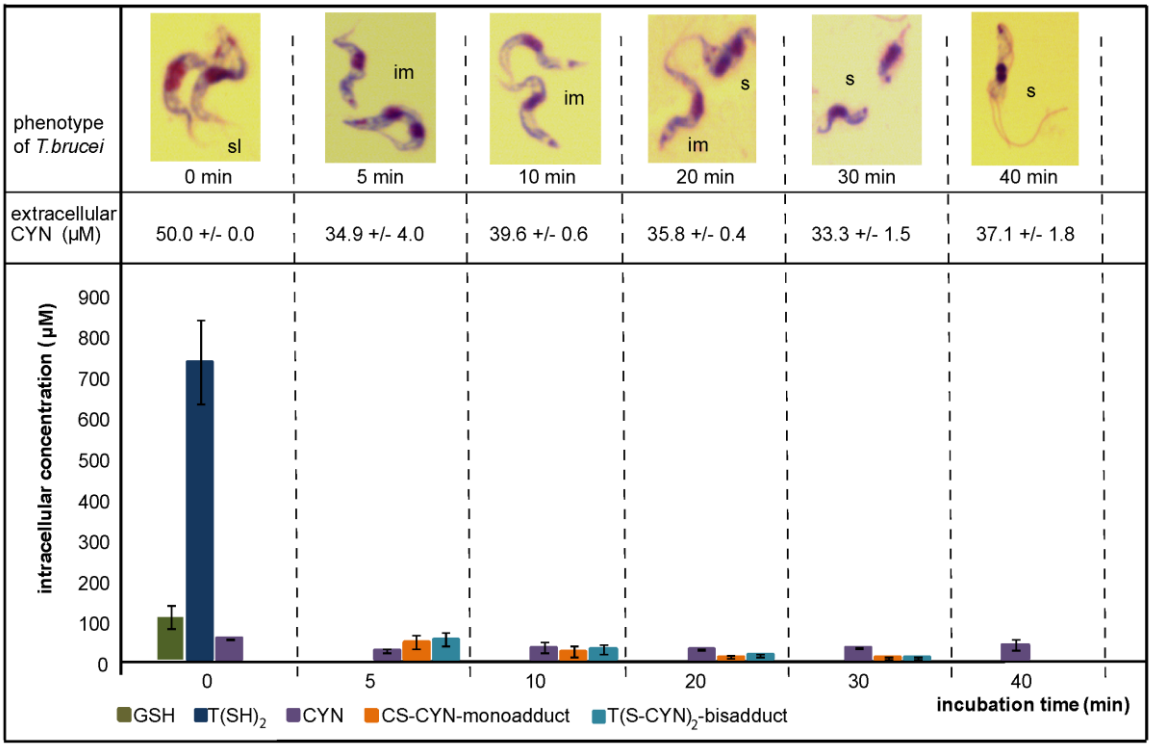

Fig. 5. Time dependent inactivation of TcTR and TbTrys by CYN.

A) TbTryS was pre-incubated with 200 µM CYN and the remaining activity was measured after different time points **B)** TR was pre-incubated with 100 µM or 200 µM CYN in the presence of NADPH. Control minutes contained TR and NADPH or TR and CYN, respectively.

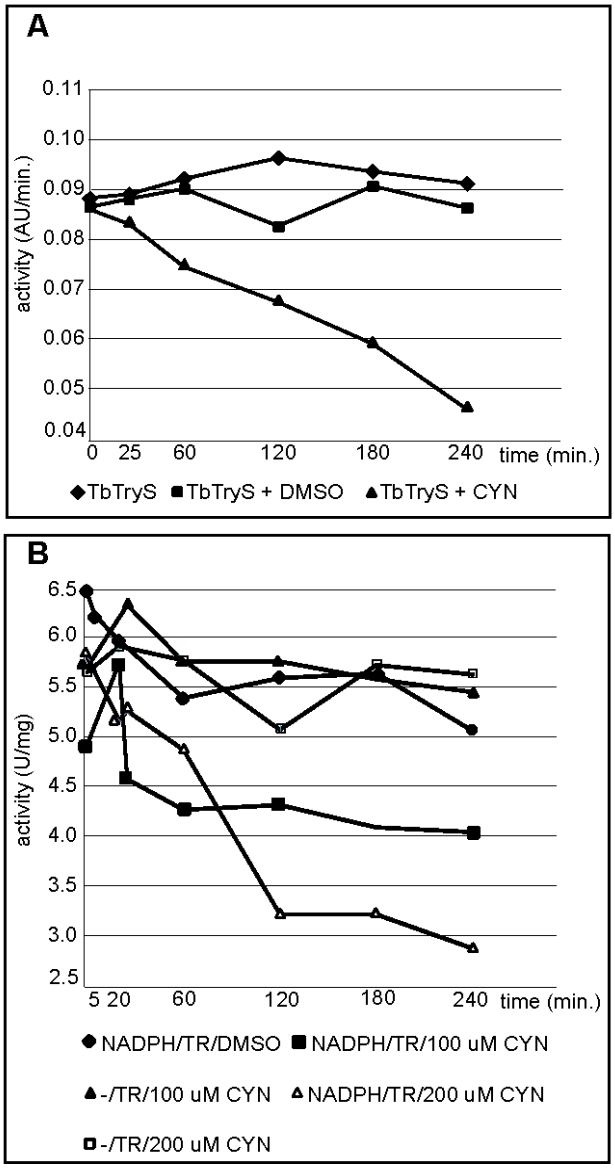

Fig. 6. Overview of potential targets for CYN in the thiol redox metabolism of African trypanosomes.

The Figure presents the biosynthesis of GSH and $T(SH)_2$ in African trypanosomes, and shows the direct effects of CYN in different steps of thiol redox metabolism. $T(SH)_2$ is synthesized by trypanothione synthetase (TryS) from two molecules of GSH that are linked by a spermidine bridge. The spermidine is delivered by spermidine synthase (SpS) from putrescine, which in turn is derived from ornithine by ODC. $T(SH)_2$ is maintained in its reduced state by trypanothione reductase (TR). **Glu:** glutamate; **Cys:** cysteine; **GSH1:** γ-glutamylcysteine synthetase 1; **GSH2:** γ- glutamylcysteine synthetase 2; **GSH:** glutathione; **CYN:** cynaropicrin; **ODC:** ornithine decarboxylase: **SpS:** spermidine synthase; **dSAM:** S-

adenosyl-L-methionine; **Sp:** spermidine; **TryS:** trypanothione synthetase; **$T(SH)_2$:** reduced disulfide trypanothione; **TR:** trypanothione reductase; **TS_2:** disulfide trypanothione.

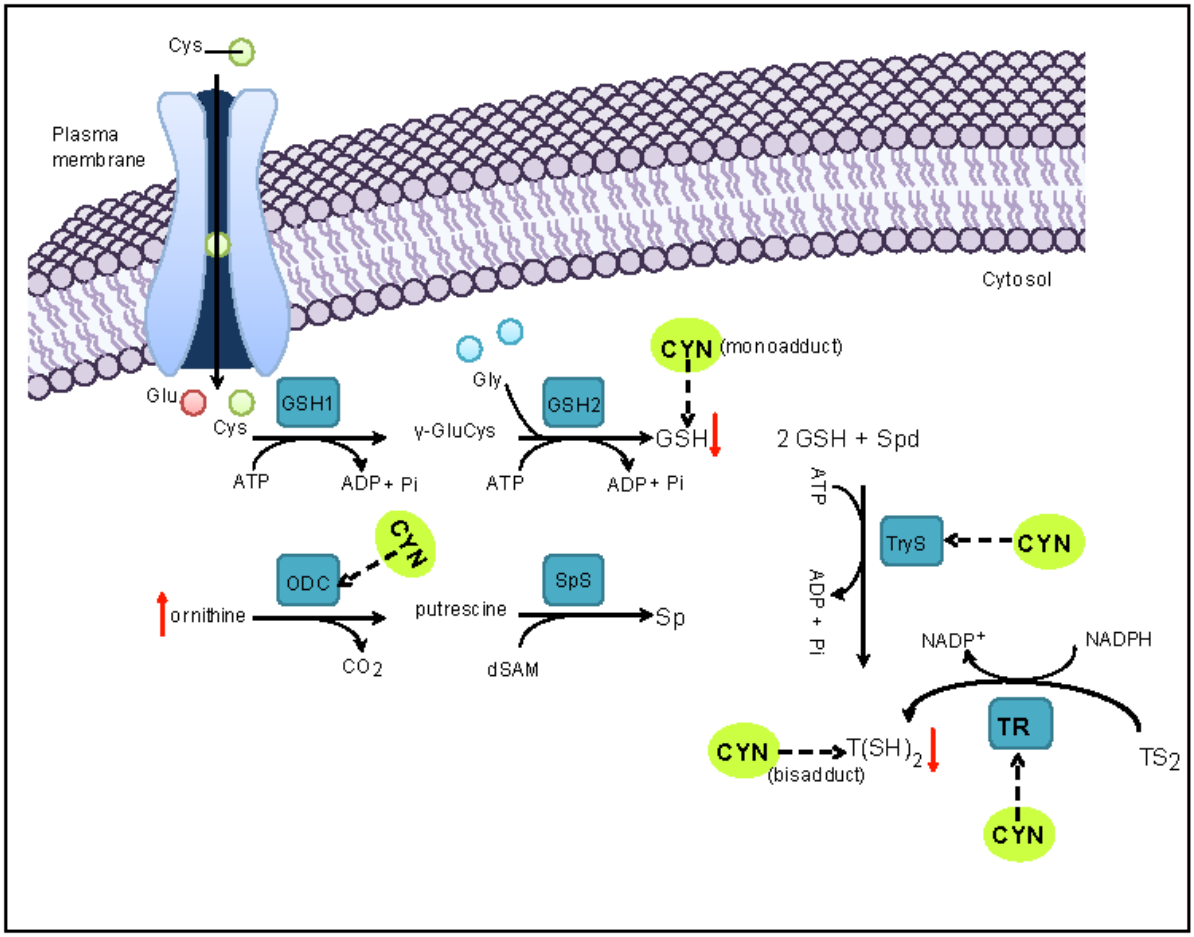

Table 1

Summary of MS/MS parameters.

Compound	MRM transitions	$^{t}R^{a}$ (min)	CV (V)	CE (eV)	LOD (ng/mL)	LLOQ (ng/mL)
CYN	346.7 > 227.02	3.0	32	12	10	156.25
GSH	308.0 > 179.12	1.0	38	10	10	62.5
$T(SH)_2$	362.3 > 233.40	2.1	20	20	25	156.25
$T(S\text{-}CYN)_2$-bisadduct	535.3 > 471.39	2.1	39	12	25	93.75
GS-CYN-monoadduct	654.3 > 524.93	2.4	37	10	50	78.125
Forskolin	411.0 > 375.20	3.8	20	8	n.d.[b]	n.d.[b]
EAG	276.1 > 147.00	0.8	38	10	n.d.[b]	n.d.[b]
Ornithine	133.1 > 115.9	1.9	68	9	0.5	7.82

[a] ^{t}R = retention time

[b] n.d. = not determined

[c] LOD = Limit Of Detection (signal to noise ≥ 3)

[d] LLOQ = Lower Limit Of Quantification

CHAPTER 6

General discussion and outlook

6.1. General discussion

The typical first step in the process of discovering active compounds against tropical diseases has been HTS campaigns of large libraries. Our lead discovery focus relies on identifying compounds from natural sources, which are unique regarding their chemical diversity, because they have been created by evolution to interact with biological targets. Therefore, NPs may show advantages in drug discovery over synthetic compounds. Over the last seven years our group collected and tested 724 plants from two out of six floristic realms (holarctic and palaeotropical). Additionally, we established a focused library with 64 fungi, which were collected in Austria. Fungi remain a relatively poorly studied source of NPs in modern drug discovery, especially in antiprotozoal drug discovery despite their species richness. With up to 5 million species [1,2] fungi outnumber higher plants by at least 16:1. The plants and fungi were extracted resulting in 2151 extracts, which were tested against living *T. b. rhodesiense*, *P. falciparum*, *L. donovani*, and *T. cruzi* [3-6].

Among these extracts were 177 produced from traditional used antimalarial Iranian plants and from plants, which were reported in European Rennaisance herbal books as antimalarial remedies [4,7,8-15]. This leads to the question if extracts from traditionally used plants show higher “hit rates” than randomly selected plants. Gyllenhall by himself exactly asked this question and showed by analyzing screening results from ethno-medical used plants versus randomly selected ones, that traditionally used plants indeed had higher “hit rates” and that may thus be better sources for finding active compounds [16]. In our antiplasmodial drug discovery screening we observed the same results: When the number of hits (defined as more than 50% inhibition at 4.8 µg/mL) from extracts from traditionally used antimalarial remedies were compared to those from randomly selected plants we observed a five times higher “hit rate” for the ethno-medically used ones (19.7%) over the randomly selected ones (4.5%). In our case the plants were documented as antimalarial remedies in eight original herbals from 16th and 17th century herbals (Bock, Fuchs, Matthiolus, Lonicerus, Brunfels, Zwinger, Tabernaemontanus) from the European Renaissance epoch [7] and as Iranian traditional used remedies reported in the books of Biruni, Hooper, Field, Razi, Zargar [17-20]. The written documented uses, makes our selection transparent in comparison to plants used in purely oral traditions.

In our screening campaign against antiparasitic parasites 2.7% of the plant extracts showed activity of more than 50% at 0.81 µg/mL. Those hits were studied using our HPLC-based activity profiling leading to 110 isolated compounds of which 13 inhibited *T. b. rhodesiense* and 3 inhibited *P. falciparum* below 0.5 µM. Among those active antiprotozoal agents 2 showed poor selectivity with IC_{50} below 0.5 µM against rat myoblast cells (Figure 6).

DNDi, a not for profit research and collaboration body, dedicated to providing novel drugs for neglected diseases, recently reported the results from an HTS campaign that used the same parasite strains and bioassays to identify new drug candidates against human African trypanosomiasis. It was shown that from the tested 87'296 small molecules none had an activity below 0.5 µM and that only three of the tested compounds showed antitrypanosomal activity between 0.6 and 0.9 µM [21]. This clearly indicates that our antiprotozoal drug discovery approach using the diversity of NPs and also the ethno-medical selection of plants may deliver more actives than the research of well-connected and financial more blessed organizations like DNDi and their partners.

The results of this thesis has shown STLs to be among the most promising classes of compounds against *T. b. rhodesiense* as they provide more than the half of the active NPs reported here. This is in accordance with other authorities like Schmidt who recently reported 883 plant derived compounds showing antiprotozoal effects *in vitro* of which 87 were STLs [22,23]. Many *in vitro* bioactivities in STLs have been described, but CYN reported here, was the first plant NP with *in vivo* activity against *T. b. rhodesiense*. However, CYN was not able to cure *T. b. rhodesiense* infected mice, but merely reduced the parasitemia. In cooperation with Prof. Usuki's group from Sophia University in Tokyo we successfully synthesized semi-synthetic derivatives of CYN to gain a better understanding of the structural features, which contribute to antitrypanosomal activity. The results of the STL SAR-study demonstrated that antitrypanosomal and cytotoxic effect depended on bifunctional α,β-unsaturated exocyclic methylene groups such as those found in CYN and in the most active compounds tested in this study [24]. These findings were in agreement with Schmidt's QSAR study of 40 STLs against protozoal parasites [25].

Many bioactivities of STLs have been attributed to a nucleophilic Michael addition of the α-methylene-γ-lactone motif to biological thiols [26]. Kupchan and co-workers in 1970 [27] showed that elephantopin, eupatundin, and vernolepin bind covalently via a Michael addition of the α-methylene-γ-lactone group to the sulfhydryl group of L-cysteine. With an excess of L-cysteine, elephantopin formed a bisadduct with two L-cysteines through a second addition to the other reactive enone moiety. Schmidt et al. showed that the antitrypanosomal pseudoguajanolide STL helenalin formed 2-monoadducts and 2,13-bisadducts with both GSH and cysteine in a cell free assay [28]. The experimental data in my

fifth publication proves for the first time that the formation of STL-thiol adducts indeed takes place in intact cells and thus could explain the *in vitro* activity observed for many STLs and the *in vivo* effects of CYN [11,29]. For this study I established a quantitative UHPLC-based MS/MS method, which has the advantage compared to known thiol analysis with HPLC methods using derivatization and fluorescent detection [30] that free thiols and thiol adducts can be directly detected in a far superior sensitivity. The reported complex extraction model and derivatization steps were replaced by a simple two-step protocol with water in 96-well format, which allowed directly and rapid analysis of the samples. This method therefore represents a significant improvement over existing assays. Their applicability is not limited to trypanosomes, but could be applied for studying biological thiols and thiol derivatives in other cellular systems.

All of the current used drugs against HAT stage 1 and 2 except for DFMO, which inhibits ODC, elicit their antitrypanosomal effects by yet unknown or else non specific modes of actions: Alsford reported that lysosomal functions is a central role in suramins mode of action and combination studies with DFMO resulted in linkage to spermidine synthesis. Fairlamb discussed suramin's trypanocidal effects as well and concluded that interference with two key enzymes involved in glycolysis play an important role. Pentamidine's mode of trypanocidal action may collapse the mitochondrial membrane potential, because of its millimolar accumulation in the mitochondrion. Melarsoprol, the only available drug for second stage *T. b. rhodesiense* HAT, forms a stable complex with $T(SH)_2$, but whether it is a part of the mode of action or metabolism, which might be responsible for its toxicity, remains unclear This summary of proposed mode of action of the current available drugs against HAT shows that still only DFMO behavior in the trypanosomes is completely known [31]. Therefore, the studies on CYN's mode of action reported here are valuable and unique.

CYN's failure to cure *T. b. rhodesiense* infected mice led us to try to improve its PK profile by masking the α,β-unsaturated exocyclic double bond at the lactone ring. The rational for this being that masking of the reactive α,β-unsaturated enone group in the lactone ring with an amine would create a prodrug with improved PKs, increased aqueous solubility, and reduced unspecific binding to biological thiols via Michael-addition of the α-methylene-γ-lactone. Through subsequent bioactivation (likely in the liver) the prodrug would be converted back to CYN and would display its biological activity on the target. This approach had been previously successfully applied to several STLs with anticancer activity like helenalin, costunolide, and parthenolide [32]. Sadly though, the lead optimization to improve CYN's bioavailability did not reward any better antitrypanosomal *in vivo* efficacy after oral application. Further PK studies to determine the prodrugs half-life time and plasma concentration would be desirable. Another issue, which needs further investigation, is the

protein-binding by STL in blood via nucleophilic Michael-addition. In general, antitrypanosomal *in vivo* studies with other orally applied STL amino derivatives are needed to demonstrate if the use of amino STLs as prodrugs is a reasonable approach to improving STLs suitability as antitrypanosomal drugs.

6.2. Outlook

The five publications in my PhD thesis covers the early phases of drug discovery for tropical diseases using plants and fungi as potential source (Figure 6): The first and the second publication describes two antiprotozoal HTS campaigns to find potential active extracts (Figure 6; step 1 [4,5]). Our classical HPLC-based activity profiling approach to identify the active ingredient in the extract (Figure 6, step 2 [33]) led to the discovery of CYN, the first NP, which showed *in vivo* activity in the acute sleeping sickness mouse model (Figure 6; step 3 and 4 [11]). The SAR-study of STLs, reported in the fourth publication [24], demonstrated that STLs antitrypanosomal *in vitro* activity is related to their α,β-unsaturated exocyclic doublebond at the lactone and a second active enone moiety such as those found in CYN. Those chemical groups function as Michael acceptors and leading to thiol-adducts in the trypanosomes, thus entered an apoptotic stage and died (Figure 6, step 5 [29]). This mode of action may present a drug discovery model in general and an antitrypanosomal lead discovery approach in particular.

Nevertheless, CYN was not able to cure *T. b. rhodesiense* infected mice and our attempt to increase its PK properties by masking its α,β-unsaturated exocyclic doublebond at the lactone did not enhance its efficacy in mice [24]. My personal opinion is that CYN will be degratuated fast through the first liver passage by phase I epoxidation at the double bonds, which would lead to toxic epoxides, which then may be metabolized by hydrolases to secondary alcohol groups or more likely by glutathion-s-transferase to glutathione-derivatives. Based on the second proposed metabolic path CYNs *in vivo* toxicity can be explained. Then, once the GSH pool is depleted and it therefore can no longer function as protector, the epoxides action will be toxic. These phase I reactions followed by phase II metabolism steps, which is mostly O-glucuronidation, may lead to the STLs early degradation in the first liver passage that might explained why the animals were not cured with CYN. The proposed hypothecally metabolism of CYN is described in Figure 7.

Further PK studies and metabolism CYP450 tests are needed to study CYNs bioavailability and to demonstrate if the use of amino STLs as prodrugs and STLs in general are reasonable antitrypanosomal clinical drug candidates (Figure 6; step 6).

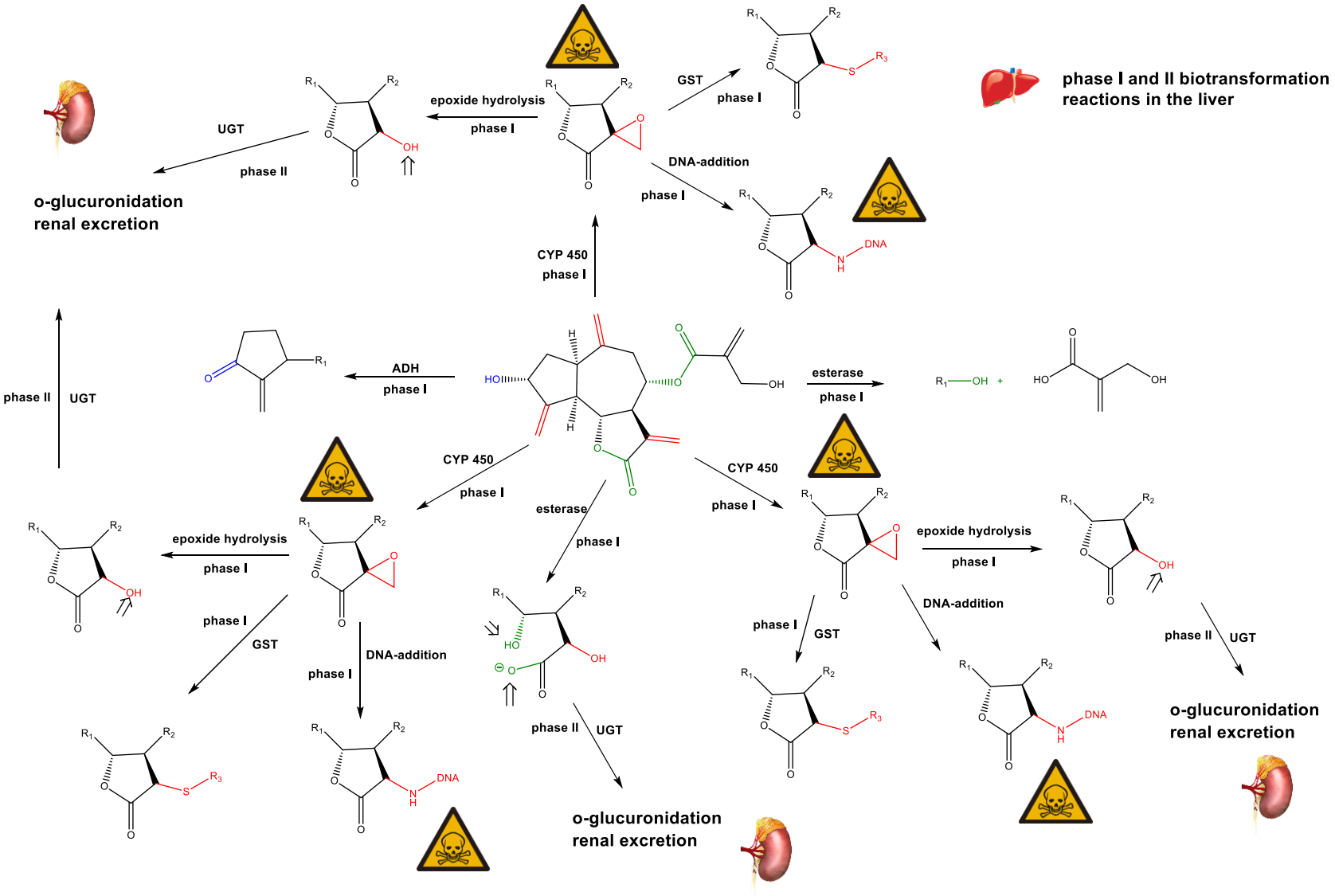

Figure 7. Proposed hypothecally metabolism and excretion pathways of cynaropicrin. **GST**; glutathione-S-transferase; **UGT**; UDP-glucuronosyl transferase

6.3. References

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“ Wer wirklich Chemotherapie treiben will, der wird sich ohne weiteres klar zu machen haben, dass die Auffindung irgend einer Substanz, die gegen gewisse Infektionen eine Wirkung ausübt, immer Sache des Zufalls sein wird; er wird auch sicher nicht erwarten, dass ihm gleich auf ersten Anhieb eine optimale Substanz zufliegen wird, sondern er wird vielmehr zufrieden sein, wenn er überhaupt Stoffe von einer deutlichen, wenn auch beschränkten Wirkungskraft findet.“

Paul Ehrlich 1907

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Curriculum vitae

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Training

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01-02/2012	Research stay at the Biochemical Research Center, Heidelberg, Prof. Luise Krauth-Siegel
10/2005-11/2009	Undergraduate studies in Pharmaceutical Sciences at the University of Basel, Switzerland **Master thesis** titled: “Discovery of new antitrypanosomal leads from *Centaurea salmantica* using HPLC activity profiling at the Institute of Pharmaceutical Biology, University Basel, supervisor Dr. M. Adams.

Teaching experience

2010-2012	Laboratory assistant and lecturer in the practical course Pharmaceutical Biology: microscopy of medicinal plants for Pharmacy students, 14 weeks/year ~ 90 students
2012	Supervising practical part of the lecture Systematik der Arznei- und Giftpflanzen, 14 weeks/semester ~ 130 students

Supervision of master theses

2012	Semira Thomi: Isolation and structure elucidation of antiplasmodial compounds from *Dictamnus albus* L. *Hyssopus officinalis* L., *Arctium nemorosum* Lej.

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13. Moradi-Afrapoli F, Yassa N, **Zimmermann S**, Saeidnia S, Hadjiakhoondia A, et al. (2012) Cinnamoylphenethyl amides from *Polygonum hyrcanicum* possess anti-trypanosomal activity. Nat Prod Commun 7: 753-755.

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Posters

2010

Zimmermann S., Adams M., Brun R., Hamburger M. Antitrypanosomal activity of cynaropicrin isolated from *Centaurea salmantica*. 3th Annual Research Meeting. Basel, Switzerland, January 2010.

Zimmermann S., Adams M., Julianti T., Hata Y., Brun R., and Hamburger M. HPLC- based activity profiling for new antiparasitic leads: *In vitro* and *in vivo* antitrypanosomal activity of cynaropicrin. 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Berlin, Germany, August 2010.

Zimmermann S., Adams M, T. Julianti, Y. Hata, R. Brun, M. Hamburger. Cynaropicrin is active against sleeping sickness. 3th Swiss Pharma Science Day. Bern, Switzerland, September 2010.

2011

Zimmermann S., Adams M, R. Brun, M. Hamburger. Cynaropicrin is active in the African sleeping sickness mouse model. 4th Annual research meeting, Basel, Switzerland, February, 2011.

Zimmermann S., Oufir M, Adams M, Brun R., Hamburger M. A quantitative LC-tandem mass spectrometry method for the analysis of thiol peptides in kinetoplastids. 4th Swiss Pharma Science Day, Bern, Switzerland, August, 2011.

Zimmermann S, Adams M, Brun R, Hamburger M. Cynaropicrin is active in the African sleeping sickness mouse model. 60th Congress of American Society of Tropical Medicine and Hygiene (ASTMH). Philadelphia, USA, December, 2011.

2012

Zimmermann S, Adams M, Oufir M, Brun R, Hamburger M. Quantitative LC-MS/MS analysis of cynaropicrin thiol peptide adducts in *Trypanosoma brucei*. 5th Annual Research Meeting. Basel, Switzerland, February, 2011.

Zimmermann S, Adams M, Ebrahimi SN, Brun R, Hamburger M. Searching for antitrypanosomal and antiplasmodial natural products from plants and fungi. 5th Swiss Pharma Science Day, Bern, Switzerland, August, 2012

Zimmermann S, Adams M, Ebrahimi SN, Hata Y, Brun R, Hamburger M. Searching for antitrypanosomal and antiplasmodial natural products from plants and fungi. Emerging Paradigms in Anti-Infective Drug Design Symposium, London, UK, September, 2012.

Short Lectures

HPLC-based activity profiling for new antiparasitic leads – *in vitro and in vivo* antitrypanosomal activity of cynaropicrin, 58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Young Researcher Workshop, Berlin, Germany, August 2010.

Cynaropicrin: the first natural product with *in vivo* activity against *Trypanosoma brucei rhodesiense*, 60th Congress of American Society of Tropical Medicine and Hygiene (ASTMH). Young Investigator Award, Philadelphia, USA, December, 2011.

Cynaropicrin targets the trypanothione redox system in *T. brucei*. 30th Annual Research Trypanosomatid Meeting, Leysin, Switzerland, January 2013.

Awards

Young Investigator Award

58th International Congress and Annual Meeting of the Society for Medicinal Plant and Product Research.
Aug 28 – Sep 2 Berlin, 2010, Germany

1th prize poster Award

3th Swiss Pharma Day
Aug 3, Bern, 2012, Switzerland

ASTMH Young Investigator Award

60th Congress of American Society of Tropical Medicine and Hygiene (ASTMH).
Dec 4-8, 2011, Philadelphia, USA