Pharmacological investigations on current and new drugs for treatment of human African trypanosomiasis

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Dekan

The ancient dragon of Africa the trypanosome is firmly entrenched. Like Tolkien's Smaugh, it sometimes slumbers and smoulders quietly for long periods but at any moment it may awaken and cause widespread loss of life to man and his domestic animals (Maegraith, 1970).

Summary

Trypanosomiasis remains a major threat to humans in sub-Saharan Africa with 55-60 million people in 36 countries at risk of infection with *T.b. gambiense* or *T.b. rhodesiense*. Only a few drugs are available to treat the disease, which are either toxic, scarce, or do not readily pass the blood brain barrier to achieve therapeutic levels in the brain. A new drug is not in sight. The organo-arsenical compound melarsoprol is the main drug in use for therapy of the late stage of the disease, with central nervous system involvement. During melarsoprol treatment patients often suffer from serious adverse effects such as fever, diarrhea, pruritus and in 2-10% of the cases from reactive encephalopathy with fatal outcome in 50%. Although melarsoprol was introduced in 1949 there is a lack of data on its metabolites, pharmacologic or chemical properties.

The present PhD project had two objectives: Firstly to analyze, characterize and determine the pharmacokinetics of the metabolites of the trypanocidal drug melarsoprol (the pharmacokinetics of relapse patients were additionally investigated). Secondly to evaluate further possible drug candidates for treatment of trypanosomiasis by structure activity relationship: In this study a group of antibacterial agents, the fluoroquinolones, was examined.

A HPLC-UV method was developed to separate the parent drug melarsoprol from its potential metabolites. One metabolite could be identified after incubation of the drug with microsomes and also in serum of patients treated with melarsoprol: Melarsen oxide. Interestingly, this trypanocidal drug was in use before the development of melarsoprol, which is the condensation product of melarsen oxide and dimercaprol (an antidote for arsenic poisoning). After administration of 2.2 mg/kg of melarsoprol C_{max} of melarsen oxide was reached after 5-15 minutes, the clearance was found to be 21.5 ml/min/kg and a half life of 3.8 hours was calculated. The therapeutic value of melarsen oxide was investigated in *in vivo* mice experiments: It could be demonstrated that the drug passes the blood brain barrier, hence might be an alternative to melarsoprol.

Investigations on additional metabolites revealed that melarsen oxide or its arsenical derivatives are irreversibly bound to proteins. Covalent protein bound drugs are often associated with serious immunological adverse effects.

The bacteriocidal class of fluoroquinolones act via the inhibition of topoisomerases. These enzymes mediating topological changes in the DNA are essential for cell survival. Trypanosomes possess a condensed, topologically interlocked circular type of mitochondrial DNA. The high demand for topoisomerase activity in combination with the rapid proliferation of trypanosomes makes the inhibition of topoisomerases an interesting drug target. 160 fluoroquinolones were tested against *T.b. rhodesiense in vitro*, with several compounds found to be active in the nanomolar range. The R7 substitution of the quinolone core was of importance for activity, whereas structural modification of the quinolone structure at position R1, R2, R3, and R8 did not influence try-

panocidal activity. *In vitro* cytotoxicity was determined for active compounds and subsequently six compounds were selected for further *in vivo* evaluation. However, even after the administration of high doses (100 mg/kg) no parasitological cure of the mice could be achieved.

Zusammenfassung

Etwa 55-60 Millionen Menschen, in Gebieten südlich der Sahara, sind bedroht von Trypanosomen, den Erregern der afrikanischen Schlafkrankheit, infiziert zu werden. Für die Behandlung stehen nur wenige Medikamente zur Verfügung und zur Zeit werden keine neuen Medikamente entwickelt. Die organo-arsen Verbindung Melarsoprol ist die meist verwendete Substanz zur Therapie des Spätstadiums der Krankheit, nachdem die Erreger das Zentralnervensystems befallen haben. Die Therapie wird begleitet von schweren Nebenwirkungen wie Fieber, Durchfall, Juckreiz und in 2-10% der Fälle von reaktiven Encephalopathien, die in 50% tödlich enden. Melarsoprol wurde schon 1949 zur medikamentösen Krankheitskontrolle eingeführt, dennoch sind pharmakologische, pharmakokinetische oder chemische Eigenschaften bis heute weitgehend unerforscht.

Zwei Möglichkeiten, für die Entwicklung von Leitsubstanzen für die Therapie von Trypanosomiasis werden in der vorliegenden Doktorarbeit präsentiert: Erstens die Aufklärung, Charakterisierung und Pharmakokinetik der Metaboliten von Melarsoprol. mit zusätzlicher Untersuchung der Pharmakokinetik von Therapieversagern. Zweitens die Durchführung einer Struktur-Aktivitätsbeziehungsstudie gegenüber Trypanosomen am Beispiel der antibiotischen Substanzgruppe der Fluoroquinolone.

Für die Trennung von Melarsoprol von seinen Stoffwechselprodukten und deren Identifikation in Blutproben und Leberzellinkubationen wurde eine HPLC-UV Methode entwickelt. Mittels dieser konnte ein aktiver Metabolit bestimmt werden: Melarsenoxid, welches interessanterweise der Vorgänger von Melarsoprol, dem Kondensationsprodukt aus Melarsenoxid und Dimercaprol (ein Antidot gegen Arsenvergiftungen) war. Nach Applikation einer Dosis von 2.2 mg/kg Melarsoprol werden Spitzenplasmakonzentrationen von Melarsenoxid nach 5-15 Minuten erreicht, die durchschnittliche Halbwertszeit von Melarsenoxid ist 3.8 h und die Clearance beträgt 21.5 ml/min/kg. In einem *in vivo* Mausexperiment konnte die Blut-Hirngängigkeit von Melarsenoxid demonstriert werden, somit weist der hydrophilere Metabolit Potential für einen Einsatz in der Therapie des Spätstadiums der Krankheit auf.

Weitere Untersuchungen ergaben, dass Melarsenoxid, oder arsenhaltige Metaboliten dieser Substanz, irreversibel proteingebunden sind. Da kovalent gebundene Protein-Arzneikomplexe eine Immunantwort hervorrufen können, könnte dies eine mögliche Ursache der schweren Nebenwirkungen von Melarsoprol sein.

Fluoroquinolone, Gyrasehemmer werden häufig für die Therapie von bakteriellen Infektionskrankheiten eingesetzt. Diese Antibiotika hemmen lebenswichtige Enzyme, die sogenannten Topoisomerasen, die verantwortlich für das Schneiden von DNA Strängen sind, und dadurch das Absterben der Bakterienzelle bewirken. Da

Trypanosomen eine hohe Teilungsrate und eine grosse DNA Dichte besitzen, lässt sich eine Sensitivität gegenüber Fluoroquinolone vermuten.

In der Studie wurden 160 Fluoroquinolonderivate *in vitro* getestet, und einige dieser Substanzen wiesen Aktivität gegenüber Trypanosomen im Konzentrationsbereich von 100–1000 ng/ml auf. Für die Aktivtät gegenüber Trypanosomen war besonders die R7 Substitution des Quinolon- Grundgerüstes wichtig, wogegen die R1, R2, R3 und R8 Substitution nicht von Bedeutung zu sein scheint. Von den aktiven Substanzen wurde die *in vitro* Toxizität ermittelt, und anschliessend sechs Derivate mit geringer Toxizität *in vivo* getestet. Diese zeigten jedoch im Mausmodell auch in hohen Dosen keine Wirkung auf Trypanosomen.

Introduction

Biological and historical background of human African trypanosomiasis

The parasite

Human African trypanosomiasis (HAT), sleeping sickness, is caused by the protozoan parasites *Trypanosoma brucei (T.b.) gambiense* and *T.b. rhodesiense. T.b. brucei* is not infective to humans*. Trypanosoma brucei* belong to the genus *Trypanosoma* and are classified in the phylum *Sarcomastigophora*, the order *Kinetoplastida* and the family *Trypanosomatidae*. The parasites are transmitted to the mammalian host by the bite of tsetse flies (several *Glossina* species) during the probing and feeding process of both sexes. *T.b. gambiense* is endemic throughout West and Central Africa. The course of the disease is slow and chronic- sometimes years elapse before specific symptoms become obvious. Nevertheless, the outcome is fatal due to extensive involvement of the central nervous system. Transmission of *T.b. gambiense* infection is anthroponotic, humans are the preferred host, however pigs, dogs and cattle can act as reservoirs (Molyneux, Pentreath *et al.*, 1996).

T.b. rhodesiense, occurring in East Africa, is the causative agent of the acute form of the disease. Severe clinical signs are developed within days following the infection. A number of livestock and game animals can act as reservoirs for *T.b. rhodesiense*, *Glossina* spp. are preferentially bovid feeders and not attracted to humans, therefore only feed on humans when other hosts are not available (Molyneux, Pentreath *et al.*, 1996).

The economic and social impact of animal trypanosomiasis caused by *T. congolense*, *T. vivax*, *T. evansi* and *T. equiperdum* is tremendous: About seven million cattle die annually in the “tsetse-belt”, thus diminishing important protein sources (Hajduk, Adler *et al.*, 1992).

The life cycle of trypanosomes involves two hosts: A mammalian and an intermediate insect host or vector, which transmits the infection to a new mammalian host. Trypanosomes are characterized by the position of the nucleus relative to the kinetoplast, an organelle associated with the mitochondrion and containing a condensed network of circular DNA. Trypanosomes range in length from 15-35 μm (Bales, 1988). In the epimastigote stage, in the insect vector, the kinetoplast is anterior to the nucleus. Trypomastigotes, the slender forms found in the mammalian blood possess a subterminal kinetoplast.

The trypomastigote bloodstream forms of mammalian trypanosomes are basically lanceolate in shape, their body having the form of an elongated flattened blade, which is elliptic or oval in transverse section, while it ends taper to a point (Hoare, 1972).

Two distinct morphological forms are seen in the bloodstream infection: Early in the infection rapidly dividing long, slender forms predominate, which are even able to cross

the blood brain barrier and invade the cerebrospinal fluid in the advanced stage of the infection. Non-dividing short stumpy forms are seen at peak levels and during declining of parasitemia (Vickerman, 1985).

In 1909, the German scientist Kleine could prove that trypanosomes had to undergo a cycle of development in the tsetse fly. This cycle is complex with many morphological, physiological, biochemical changes and migration of the parasite: Briefly, trypomastigotes are ingested from an infected mammalian host by the tsetse fly during its blood meal. Only the stumpy forms of *T. brucei* are capable of initiating the cycle in the vector: In the midgut they transform to procyclic forms. The procyclics proliferate in the ectoperitrophic space between the midgut and the peritrophic membrane. After 10-12 days they move to the proventriculus. Transformed into the slender and non-infective epimastigotes, they migrate via the hypopharynx to the salivary glands. Epimastigotes further differentiate during 15-35 days, depending on temperature and humidity of the environment, into stumpy trypomastigote forms and mammal infective metacyclics. With the bite of the tsetse fly the metacyclics are injected into the mammalian skin where they transform into long slender trypomastigote forms and evade into the blood and lymphatic system (Vickerman, 1985).

The disease

Trypanosomiasis is an ancient disease and written records go back as far as the 14th century: An Arab writer described the death of Mansa Djata, the ruler of the Malli Kingdom (Niger):

"*His end was to be overtaken by the sleeping sickness which is a disease that frequently befalls the inhabitants of these countries especially their chieftains. Sleep overtakes one of them in such a manner that it is hardly possible to awake him. He (the king) remained in this condition during two years, until he died*".

In 1843 Gruby, a doctor from Vienna, isolated trypanosomes from the blood of a frog and created the generic name *Trypanosoma*. During the 35 years that followed little advance was made: In 1880 Griffith Evans, a veterinary officer, could demonstrate the pathologic connection of this organism to animal diseases. Sir D. Bruce made the most important contribution of subsequent investigations leading to the understanding of Nagana in cattle as a trypanosome infection (*T. brucei*). He also demonstrated that the disease was transmitted by tsetse flies and that game animals acted as reservoirs of the infection. In 1902 human trypanosomes were detected by Dutton in a European patient returning from Gambia and his irregular fever attacks related to the parasite (*T.b. gambiense*). The pathogenicity of trypanosomes was first demonstrated in 1903 by Castellani, who associated trypanosomes with sleeping sickness by finding them in the cerebrospinal fluid of an Ugandan patient ("*when examining a specimen of cerebrospinal fluid taken by lumbar puncture …I was surprised to observe a living trypanosoma…my eye had been attracted by a little fish like parasite darting about*") (Bentivoglio, Grassi-Zucconi *et al.*, 1994). Seven years later another trypanosomal or-

ganism was isolated from the blood of a European returning from Rhodesia *(T.b. rhodesiense)*. During the same period some of the most important trypanosomes affecting livestock and other domestic animals were described and their life cycles determined e.g. *T. equiperdum* in 1901 or *T. congolense* in 1904 (Hoare, 1972; Bales, 1988).

Several devastating epidemics occurred due to an increased movement of people after the arrival of European explorers and because of lacking knowledge of the epidemiology and transmission of the disease. The disease expanded from restricted foci into large endemic regions. Between 1896 and 1906 half a million people had died along the river Congo, and the outbreak on the northern shore of Lake Victoria killed about 250,000 people (Bales, 1988).

By the late 1950's the incidence of sleeping sickness has been greatly reduced by mass campaigns of active case finding, chemotherapeutic treatment and vector control. The independence from colonial power and subsequent civil disturbances, declining economies and reduced health financing led to a dramatic resurgence of HAT (Smith, Pepin *et al.*, 1998).

Actual situation

It is estimated that 60 million people living in around 200 discrete transmission zones in 36 sub-Saharan countries are at risk of acquiring sleeping sickness. The disease is only endemic in areas where tsetse flies are found, i. e. the tsetse belt: from approximately 14°N from Senegal in the West to 10°N in Southern Somalia in the East and 20°S corresponding to the northern fringes of the Kalahari and Namibian deserts (Molyneux, Pentreath *et al.*, 1996).

The incidence of annual cases is currently estimated at 300,000, in certain villages of the Democratic Republic of Congo and in Angola the reported prevalence is up to 80%. In some provinces the mortality rate of sleeping sickness is of the same order of magnitude as the one caused by AIDS (UNDP, 1997).

Although trypanosomiasis is considered less important than malaria or AIDS, it remains a continuous threat to humans. The symptoms are dramatic, the mortality rate is nearly 100%, if untreated, and epidemic outbreaks drive people away from their villages, thus reducing productivity and threatening domestic livestock.

While sleeping sickness has declined in a number of West African countries, vast areas of Central Africa from Southern Sudan and Uganda, through the Democratic Republic of Congo to Angola are experiencing progressive epidemic spread (Smith, Pepin *et al.*, 1998).

The situation in Angola

Gambian HAT was first recognized in northern Angola in the late 19th century. In response the Portuguese Colonial Government put emphasis on control and created a national surveillance program (*Missão de Combate as Tripanosomiase*). Each village was visited at least once every year and access to diagnosis and treatment to the whole population was guaranteed. The incidence of HAT was reduced from 5,000 cases in 1950 to only 3 cases 1974. In 1976, Angola became independent and the country was thrown into a cruel civil war until today. One of the consequences was the complete collapse of the health system and the discontinuation of the control program. Since 1994 governmental activities focus on the establisment of treatment centers, active case finding, health eduction and vector control (Smith, Pepin *et al.*, 1998).

Today, Angola is one of the most affected countries by HAT, especially the northern parts of the country, where sleeping sickness is the major cause of adult mortality (Smith, Pepin *et al.*, 1998). In the last six years, 22,560 cases have been diagnosed, almost exclusively through passive case finding. In 1997 alone, 8,275 new cases were detected, although active case finding was covering only 2% of the 4 million population at risk (Van Nieuwenhove, 1998).

Clinical manifestation and pathogenesis

Signs and symptoms are classified according to the clinical progression of the disease: the initial trypanosomal chancre, followed by the haematolymphatic or first stage, and the meningoencephalitic or second stage (when parasites have invaded the central nervous system (CNS). The clinical classification of the second stage of the disease is mainly limited to crude measurements of changes in the cerebrospinal fluid (CSF). The clinical signs vary between individuals and disease foci and are not always specific (WHO, 1998).

A few days after the bite of an infected tsetse fly a chancre, a tender, painful and local swelling, is frequently observed in *T.b. rhodesiense* infections. This primary lesion can last up to several weeks. In its fluid trypanosomes are detectable. The chancre is less common with *T.b. gambiense* infections.

Fever, resulting from an evasion of the parasites into the blood and their production of pharmacologically active substances is accompanied by other unspecific symptoms. Frequent complaints are headache, weight loss, joint pains, skin rashes, pruritus, anemia or endocrine disorders. Sometimes the symptoms are minor and may not alert the patients to seek medical advice. An enlargement of the lymph glands is often seen: In *T.b. gambiense* infections the posterior cervical lymph nodes (Winterbottom's sign) are firm, mobile, painless and vary in size (WHO, 1998). Lymphadenopathy is usually generalized in patients suffering from *T.b. rhodesiense* infections (WHO, 1998). In both forms of the disease an enlargement of the spleen and the liver can be observed.

The brain, heart and lung are the most severely affected organs: Dysrhythmia, heart murmurs and low blood pressure may be revealed. Pericardial effusion, myocarditis and cerebral and meningeal edema are frequent symptoms in the second stage of disease.

The involvement of the CNS may occur within a few weeks in *T.b. rhodesiense* infections or may not occur until years later (*T.b. gambiense* infections). The organisms penetrate into the brain tissue and produce inflammatory lesions. The onset of this stage is insidious with progressive involvement. Early changes and symptoms involve abnormal body movements, extrapyramidal signs, tremors, irritability, and alterations in mood, indifference or the classical reversal of the sleep rhythm with daytime somnolence and night insomnia. As the disease progresses the neurologic abnormalities become more marked: Epileptiform seizures may occur as well as local paralyses, maniacal chances, total indifference and somnolence leading to coma and death. Due to the long duration of *T.b. gambiense* infections extensive neurological changes might be observed, which lead next to acute infections or malnutrition to death. In acute *T.b. rhodesiense* disease death often occurs before CNS involvement because of cardiac failures, pneumonia or superimposed infectious diseases (Molyneux, Pentreath *et al.*, 1996).

The mechanisms of pathogenicity are only poorly understood; most theories suggest immunopathologic and biochemical processes. High parasitaemia results in an exposure of the host to high levels of toxic metabolites, lytic enzymes and immunosuppressive membrane components, which induce unregulated lymphocyte proliferation and destructive inflammatory responses (WHO, 1998). The pathogenesis is ultimately linked to the inability of the untreated patient to remove the parasite, since trypanosomes escape the immune system by varying their surface glycoproteine coat (Bales, 1988). These variant surface glycoproteins (VSG's) are strongly immunogenic and elicit high levels of IgM antibodies.

It has been suggested that high IgM levels increase osmotic pressure, erythrocyte sedimentation rate and blood viscosity. Circulating immune complexes activate the kallikrein, kinin, complement and blood coagulation systems, which lead to increased vascular permeability, edema, inflammation and tissue damage (WHO, 1998). In addition the production of autoantibodies directed against antigenic components of e.g. brain and heart, is often discussed (Bales, 1988).

Treatment

In 1902 the first *in vivo* model became available and experimental treatment of trypanosome infections were carried out. Out of a large number of preparations tested by P. Ehrlich only trypan red and arsenic compounds were found to be highly effective. Therefore chemotherapeutic research progressed mainly in two lines: the synthetic

dyes and related compounds (e. g. suramin) and the organic arsenicals (e.g. melarsoprol) (Drews, 1998).

Currently only four drugs are available for the treatment of HAT: Suramin (1922) and pentamidine (1940) are used for the treatment of the first stage, melarsoprol (1949) and eflornithine (1990) for the late stage of disease. These drugs have many adverse effects, are expensive and are not readily available (WHO, 1998).

The treatment with arsenical drugs

More than 12,000 arsenical compounds were synthesized in the first half of this century and screened for their activity against sleeping sickness (Friedheim, 1973). One of them, melarsoprol (Mel B) is still the drug of choice for treatment of the late stage of disease.

The treatment of trypanosomiasis with arsenicals has a long history:

Potassium arsenite was the first drug described to affect trypanosomes. The prime consideration appears to have been the tonic effect of the drug, it was commonly prescribed (in small doses!) against conditions of weakness, rather than the anti-protozoan action. Potassium arsenite, also known as Fowler's solution marks the beginning of chemotherapy of sleeping sickness in 1887: Trekking from the east African coast towards west and passing tsetse infested habitats, Dr. Livingstone's horse fell sick and was cured with a solution of 1% potassium arsenite (AsO_2). Unfortunately the improvement was followed by a relapse. After offering the horse another dose the animal turned its head and said: "dear Dr. Livingstone, I don't want your medicine, let me die in peace" (Friedheim and Distefano, 1989).

In 1905 the first organic arsenical **Atoxyl**â (sodium-p-amino-phenyl arsonate), which had already been synthesized in 1863 was brought to the market against febrile conditions and syphilis. The aliphatic arsenic compounds were prepared accidentally. Although Atoxyl®, which originated as a by-product of parafuchsin "did not exercise any destructive action on the parasites in the test tubes" it was found to be highly active in *T.b. gambiense* infections *in vivo* and 30 times less toxic than Fowler's solution (Williamson, 1970). A major drawback, however, was its toxicity affecting the optic nerve; moreover relapses were the rule and therefore it did not justify its early hopes. It cured only early cases of the disease and patients with *T.b. rhodesiense* infections remained incurable (Williamson, 1970, Friedheim, 1989).

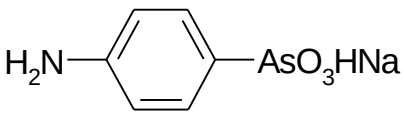


Fig.1 Atoxyl

In the following years many derivatives of Atoxyl® were synthesized and tested for their trypanocidal activity. It was demonstrated that trivalent arsenic compounds were more active, but also more toxic than the pentavalent arsenicals (Williamson, 1970). Among the derivatives of Atoxyl® were **salvarsan** (1910), a drug highly active against syphilis, and **tryparsamide** the first late stage drug of trypanosomiasis. Heidelberger and Jacobs developed tryparsamide in 1918, in order to improve the bad solubility and chemical instability of the trivalent salvarsan. Trials of tryparsamide were carried out in 1920 in the then Belgian Congo. The drug was significantly more active in second stage *T.b. gambiense* infections than Atoxyl® but not active in *T.b. rhodesiense* infections. Its therapeutic index was found to be six times higher than the one of Atoxyl®. Nevertheless, tryparsamide still exhibited a dose-dependent toxicity against the optic nerve leading to a disastrous incidence in 1930: A young lieutenant doubled the prescribed dose of the drug on a Monday morning resulting two days later in 800 blind patients. Furthermore, following mass treatment, tryparsamide resistant cases appeared in increasing numbers (Friedheim, 1959, Williamson, 1970).

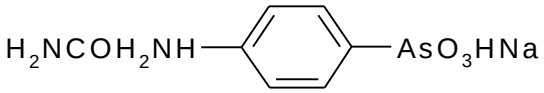


Fig.2 Tryparsamide

Enormous efforts were made to improve atoxyl and tryparsamide. In British, French and German laboratories 12,500 arsenic compounds had been synthesized by 1932. Also Dr. Friedheim was prompted by the fatal incidence on the optic nerve to develop alternative arsenical drugs not endangering vision.

He even converted his own kitchen into a laboratory. In his first line of investigations he linked sulphonic acid radicals to Atoxyl® (**compound 2654**) (Friedheim, 1959) to eliminate the toxic effects on the optic nerve. Sulphonic acids are largely dissociated under physiological conditions and the resulting ions have no negative effect on the brain and the optic nerve. The effectiveness of the drug could be demonstrated in a study in Nigeria but compound 2654 was later abandoned. One of the reasons for withdrawal might have been the colored urine due to elimination of the purple compound by the kidneys (Friedheim, 1959). In addition, the slow effect of the sulphonic acids on the trypanosomes and thus necessary long treatment motivated Friedheim to do further investigations. He noticed that all arsenicals possessing a significant trypanocidal activity contained nitrogen in one form or another. The melamine chemistry, famous in the manufacture of plastic, offered a favorable start for the synthesis of new compounds.

Friedheim started with 2,4-Dichloro-s-triazinyl-6 aminophenyl-arsonic acid, a toxic substance of no therapeutic value and replaced the two halogens by unsubstituted amino groups. This replacement resulted in a maximum trypanocidal effect combined with minimum toxicity in mice (Friedheim, 1944): This first melamine compound was **melarsen** (1938). The drug was used in therapy the following years but its therapeutic effect was controversial: Duggan and Hutchison (1951) reported the cure of 200 cases in Nigeria, Friedheim himself noticed severe adverse effects such as epileptic convulsions, especially in malnourished people (Duggan and Hutchinson, 1951, Friedheim, 1959). Melarsen was not totally abandoned and its efficacy was investigated in further trials. The drug was found to be less toxic than earlier drugs, water soluble, thus easy to administer but more expensive. This might have been the main reason for its withdrawal (Williamson, 1962).

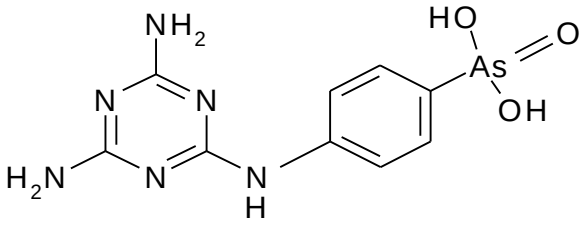


Fig.3 Melarsen

Bearing in mind Ehrlich's demonstration of the higher activity (up to 250,000 times) of trivalent arsenicals compared to the pentavalent forms, Friedheim synthesized in 1939 **melarsen oxide**. It was obtained from melarsen by simple reduction with sulfur dioxide in the presence of HCl (Friedheim, 1948). Good cures were achieved with melarsen oxide in several trials but the drug was later abandoned in favor of **melarsoprol**.

Melarsoprol is the condensation product of melarsen oxide with **dimercaprol**, also known as British Anti Lewisite (BAL), an arsenic antidote. The condensation product embedded the arsenic in a five-membered ring. The development of BAL and its ability to detoxicate trivalent arsenicals led to the combination of a number of BAL condensation products like BAL-atoxyl, BAL-tryparsamide or BAL-stovarsol. Several of them were tested but only one had come into general usage: Melarsoprol or Mel B.

Friedheim found melarsoprol to be 100 times less toxic than melarsen oxide and reported that its trypanocidal activity was by a factor of 2.5 lower (Friedheim, 1949).

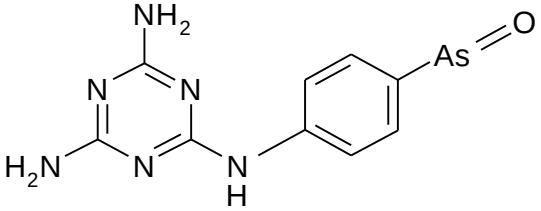


Fig.4 Melarsen Oxide

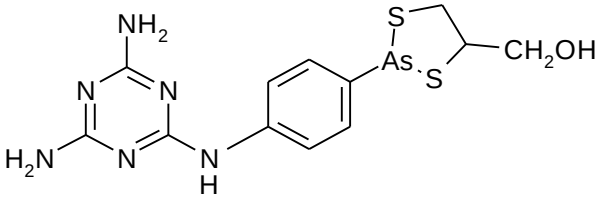


Fig.5 Melarsoprol

Melarsoprol

From 1950 onwards melarsoprol was widely used and its effectiveness against late stage *T.b. rhodesiense* infections was a major improvement (Apted, 1953). Other advantages over tryparsamide were its activity in tryparsamide resistant cases and that it showed no toxic effect on the optic nerve (Friedheim, 1959).

Melarsoprol is effective in both, the first and the second stage of the disease, but is exclusively used in the late stage because of the risk of serious adverse effects: Thrombophlebitis at the site of injection due to the propylene glycol solvent is frequently reported. Adverse effects such as cardiotoxicity, cutaneous reactions, polyneuropathy, diarrhea, tremors, fever, or albuminuria are quite common (Pepin, Milord *et al.*, 1994, Cook, 1995). A very serious complication, a drug induced encephalopathy occurs in 5-10% of the treated patients and is characterized by fever, headache, tremor and convulsions. The condition usually detoriates to deep coma within 1-2 days in 10-50% of these patients, followed by death (WHO, 1998). The cause for this severe reaction remains controversial, but generally an immunologic reaction is assumed to be involved (Pepin and Milord, 1991, Pepin and Milord, 1994).

Ceccaldi, Director of the Pasteur Institute in Brazzaville reported a 19% fatality rate with the remark: "*of all poisons circulating in Africa Friedheim's melarsoprol is certainly the most poisonous one*" (Friedheim and Distefano, 1989). Nevertheless, it remains the main drug in use for treatment of late stage trypanosomiasis.

An alarmingly high incidence of treatment failures of up to 20% was lately being reported from regions in Northern Angola, Northern Uganda or from the South of Sudan (Van Nieuwenhove, 1998).

Although the drug was introduced almost half a century ago, it is not well studied yet and its mode of action not well understood. The drug was initially thought to act by inhibiting the trypanosomal pyruvate kinase, which is a key enzyme in African trypanosomes for production of ATP (Flynn and Bowman, 1974). Recent investigations showed that trypanothion (N, N-bis-glutathionyl-spermidine), the equivalent to glutathione in African trypanosomes and a major cofactor involved in the thiol- disulfide redox balance, forms a stable adduct with melarsen oxide. This complex is an effective inhibitor of the

trypanothione reductase, leading to disturbance of the redox balance of the parasite and thus exposing the trypanosome to free radicals (Fairlamb, Henderson *et al.*, 1989). However, this theory was questioned and it was suggested that the phospho-fructokinase, an enzyme of the glycolytic pathway and interference with energy metabolism might be the main drug target (Wang, 1995).

Three related compounds are worth mentioning **melarsen oxide dithiomalate**, **melarsen oxide dimercaptosuccinate (Mel W)** and its diaethylamin analogue **Mel D** (Friedheim, 1959, Friedheim, 1970). Mel W and Mel D were used in therapy (Friedheim, 1959, Nodenot, 1960) but could never replace Mel B. The reason for their withdrawal remains unclear, since the drugs were described as water soluble, non toxic and as effective as Mel B (especially against *T.b. gambiense*) (Friedheim, 1959). One reason for their abandonment might have been the high incidence of relapses (Robertson, 1963). Melarsen oxide dithiomalate was found to be less active than melarsoprol, the advantages of cyclic over open chain arsenic dimercaptide structures were discussed (Friedheim, 1951).

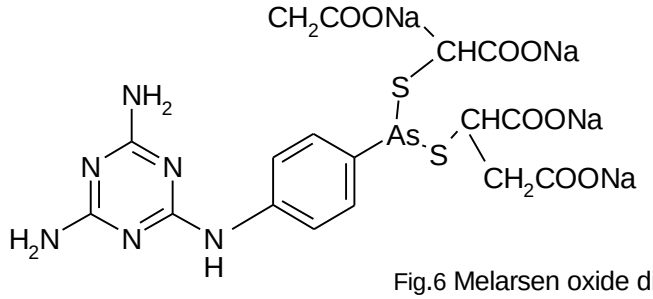


Fig.6 Melarsen oxide dithiomalate

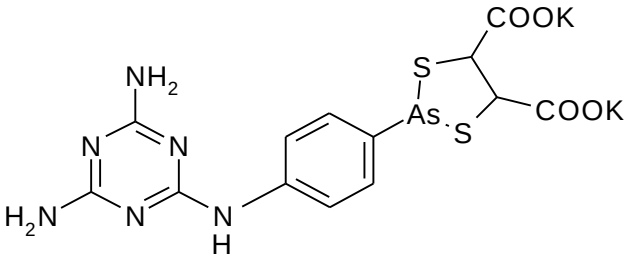


Fig.7 Mel W

Further open chain derivatives like a series of **dithioarsenites** or **p-biguanidophenylarsonic acids** (Banks, Controulis *et al.*, 1946) which were synthesized with the aim of producing better soluble compounds, failed in therapy (Tillitson, 1951).

No information is available on the biological activity of a number of **heterocyclic arsenic compounds** (Mann, 1950).

Parallel to the development of the melamin arsenicals, organic arsenicals related to phenylarsenoxide were synthesized. Gaillot and Baget prepared salts of tetracycline antibiotics with **phenylarsonate derivatives** (Gaillot and Baget, 1955) and studies with

oxophenarsine were conducted (Peters and Wright, 1943). Doak and Eagle tested a number of **phenylarsenoxides** with a variety of substituents, but without structure activity relationship of prognostic value. Most drugs were rejected because of their toxicity. Only one compound, **butarsen**, revealed sufficient activity to be tested in man, but it was only effective against the first stage of HAT, thus never came into general use (Doak and Eagle, 1951).

None of the further synthesized arsenicals and also none of the antimony analogues of melarsen oxide and Mel B replaced Mel B, although demonstrating to have similar activity (Friedheim, 1953)

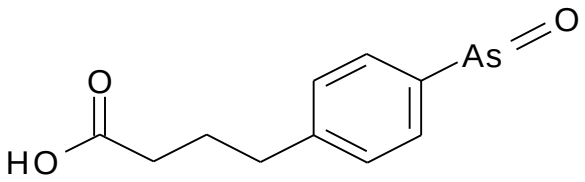


Fig.8 Butarsen

Recent achievements

Over the past 40 years only one compound was brought to the market and approved for use against human African trypanosomiasis: eflornithine (DFMO, ornidyl®) in 1990 (Nightingdale, 1991). It was developed originally by rational design as an antitumor drug. Eflornithine is a specific, enzyme activated, irreversible inhibitor of the ornithine decarboxylase (ODC). ODC catalyzes the formation of putrescine from ornithine, the rate-limiting step in the biosynthesis of the polyamines as putrescine, spermidine and spermine. The polyamines are essential for the growth and proliferation of prokaryotic and eucaryotic cells (Metcalf, Bey *et al.*, 1978). It was anticipated that an effective inhibition of the ODC would stop growth in tumor cells. However, its performance in antitumor trials was poor. Interestingly, it was found to have a good antitrypanosomal activity and field trials proved that DFMO was very effective against late stage *T.b. gambiense* infections (Pepin, Milord *et al.*, 1987). DFMO could not fulfill all hopes as the drug is not effective in *T.b. rhodesiense* infections, is considerably toxic, shows a moderate efficacy, a short half life and is almost unaffordable expensive.

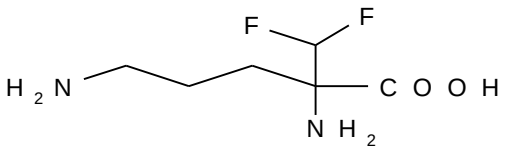


Fig.9 Eflornithine

Much of the present day antiparasite chemotherapy is reminiscent of practices from the 19th century: Melarsoprol, having its empirical origin from Fowler's solution, remains almost a century after the introduction of Atoxyl® the most powerful trypanocidal drug with a capacity to cure both stages of the disease. In accordance with the few other drugs in use, suramin, pentamidine and diminazene, it would not pass today's drug registration laws.

In view of the alarming increase of sleeping sickness cases and refractory cases in the treatment with melarsoprol there is an urgent need for alternative drugs.

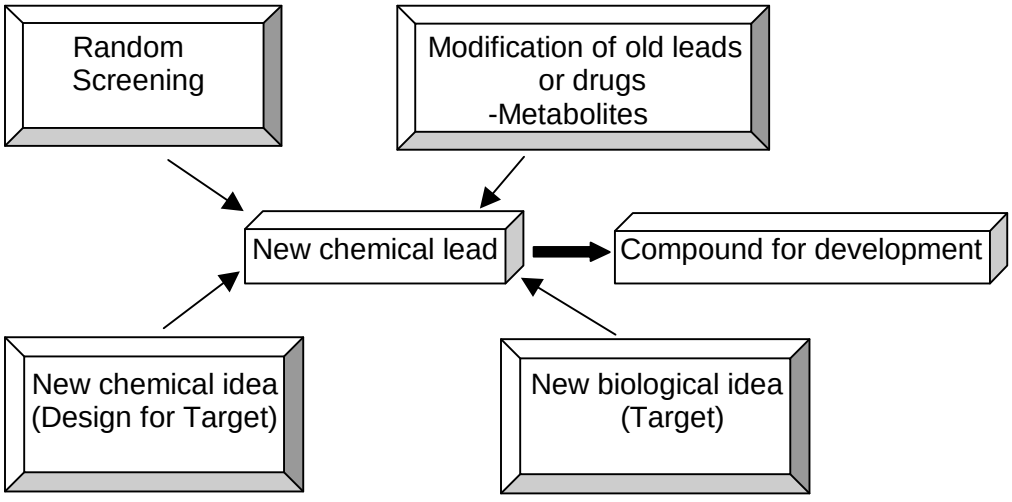
The ideal antitypanosomal drug must (1) be effective against both *T.b. rhodesiense* and *T.b. gambiense*, with a large therapeutic window; (2) pass the blood brain barrier; (3) be easy to administer, preferably without need for hospitalization of the patient; and (4) be affordable in African countries.

However, it is well documented that new drugs are only developed when sales expectations are likely to match the high costs for their development. In average, it takes six to twelve years from the identification of an active compound to the development of a new drug and out of 10,000 compounds synthesized only 1-2 drugs make it to the market. Between 1976 and 1996 the cost of bringing a new drug to the market has increased tenfold to more than 300 million US$ for a single drug.

Therefore, the industry has virtually abandoned large disease domains, including trypanosomiasis, that represent huge medical needs but have no financial return (Drews and Ryser, 1997). Two examples may illustrate this recent trend: (1) In Japan the annual drug expenditure per capita is 412 US$ compared to Mozambique's 2 US$; (2) 75% of the world's population living in developing countries consume only 21% of the global production of pharmaceuticals (UNDP, 1997).

Techniques used to discover a lead compound

Drug discovery is a process, which involves multiple disciplines and techniques:



Random Screening

The idea of the random screening approach is to evaluate the biological activity of a large number of compounds with a simple system in order to identify lead compounds. The odds of finding a usable compound lie only between one in 10,000 and one in 50,000. Therefore screening is invariably associated with assays that are able to manage high capacities of compounds and with libraries (containing several compounds as representatives of many structurally diverse chemicals) to facilitate the discovery process (Hudson, 1994).

In vitro assays cannot account for pharmacological and pharmacokinetic processes that take place in the body. The majority of current antiprotozoal drugs arose from leads selected by *in vivo* assays. Such assays pose the demanding requirements of good intrinsic activity against the target parasite coupled with appropriate pharmacokinetics (Hudson, 1994). *In vivo* assays are expensive and ethically restricted. Therefore *in vitro* assays coupled with the use of human hepatocytes or microsomal incubations, which partially mimic the metabolism in the body could serve as an alternative.

Search for drug targets

A rational approach in drug design is to undertake fundamental research on the biochemical, physiological or molecular features of the parasite. A drug target has to be unique or distinctively different from the mammalian host to allow selective inhibition, and it must be essential for the survival of parasites. African trypanosomes are amongst the most studied parasitic protozoa and many of their biological features have been documented. The molecular biology of trypanosomes is unique and numerous potential drug targets have been identified (Wang, 1995). The polyamine synthetic enzymes s-adenosylmethionine decarboxylase (SAMDC), ornithine decarboxylase (ODC), which are thought to play an important role in cell division and differentiation of trypanosomes could be targeted. Furthermore, glycolytic and purine salvage enzymes, the trypanothione synthase and reductase, essential for the cellular defense against oxidative stress or protein import can be viewed as attractive targets for antitrypanosomal chemotherapy (Wang, 1995). Topoisomerases could also serve as an interesting drug target: Kinetoplastid protozoa have a unique and complex type of DNA structure in the mitochondrion creating a high demand for topoisomerase activity (Englund, Hajduk *et al.*, 1982).

A major step towards a novel inhibitor of a target enzyme or receptor is to obtain its three-dimensional structure, which may be investigated by X-ray or NMR spectroscopy or computational biology based on homologous proteins of known structure. Computer designed drugs based on the structure and function of the enzyme are selectively synthesized or already known inhibitors and their binding tested (Douglas, 1994). No single drug, however, has ever been found using a purely rational method (Spilker, 1994). It is naïve to think that a computer designed molecule will already turn out to be the best drug, but rational drug discovery may inspire and provide an entry point for the

search. Interestingly dimercaprol comes closest to this ideal. Its synthesis was based on a rational concept, but before the antidote was discovered 100 different compounds were synthesized which failed in therapy (Spilker, 1994, Stocken, 1946).

Metabolic studies

Drug metabolism may also serve as a source of ideas for discovery of new compounds. After application most drugs are chemically changed in the body, mainly in the liver but also in the kidneys, lungs and other organs:

Although drugs are usually converted to less effective compounds, in some instances the metabolites are more active than the parent compound (some metabolites may even reveal an unexpected toxicity). A metabolite may have more desirable properties than the parent drug and it could serve as an alternative drug or even replace the parent drug (Spilker, 1994). In addition, knowledge of the metabolic pathway and the enzymes involved helps to avoid severe and toxic adverse effects.

Therefore pharmacological and metabolic studies play a key role in the multidisciplinary approach of drug research and development.

The entire process from identification to marketing of an active metabolite is less complex than the development of new active derivatives. Synthesis and screening activities can be omitted and the registration of a metabolite as an additional or alternative drug is often easier since documentation already exists (Spilker, 1994) In praxis numerous examples are known in which drugs have been replaced by one of their metabolites or have been improved based on the structure of their metabolites: Desmethylimipramine is a metabolite of imipramine and both are marketed for the treatment of depression; acetaminophen is a metabolite of phenacetin and has replaced the parent drug (Spilker, 1994, Lambe, 1995, Park, 1986). A recent example is fexofenadin, a metabolite of terfenadine, which was brought to the market in 1996. Fexofenadine has a longer duration of action compared to terfenadine and is therefore more patient-friendly. Moreover the metabolite lacks some of terfenadine’s adverse effects, including a fatal tachycardia. The withdrawal of terfenadine from the U.S. market has been announced recently (Gonzales and Estes, 1998).

Metabolic reactions of xenobiotics are generally classified into two types: Phase I reactions include oxidations, reductions and hydrolysis. Oxidative alterations of molecules are carried out by hemeproteins termed cytochromes P_{450}. The majority of P_{450}'s involved in drug metabolism appear to belong to four distinct gene families; each family is further divided into subfamilies. Many drugs may be largely dependent on single forms of P450 for their metabolism in the liver, because each P450 has a unique binding site. There are large differences among patients in the liver content and catalytic activity of P_{450}'s, reflecting genetic and probably non-genetic factors. This heterogeneity results in an individual response to the drug (Watkins, 1992).

Phase II reactions are enzyme-catalysed conjugations of the original compound or Phase I metabolite (provided now with a different functional group), which require the involvement of a high energy donor substrate (Watkins, 1992).

For the study of metabolic phase I reactions subcellular fractions (microsomes) and purified enzymes (cytochrome P450) are generally used. Other systems such as intact cells, intact organs, primary cultures, established cell lines and tissue slices are used to study both phase I and phase II reactions (Chiu, 1993).

Justification and aim

There is an urgent need for novel drugs for human African trypanosomiasis, which are safe, effective and affordable. In the present study two promising strategies to discover a lead compound for the treatment of sleeping sickness were investigated: First, research on targets for intervention with inhibitors of the topoisomerase selected. Second investigations on the metabolism of existing drugs. Melarsoprol is the current drug of choice for late stage trypanosomiasis but its pharmacological profile has never been studied in detail. The recently observed discrepancy between the results of two assays, HPLC and bioassay, in determining melarsoprol concentration emphasizes the need to carry out a thorough metabolic study for melarsoprol.

References

Apted, F. I. C. (1953). The treatment of advanced cases of Rhodesian sleeping sickness by Mel B and Arsobal. *Trans. R. Soc. Trop. Med. Hyg.* 47(5): 387-398.

Bales, D. J. (1988). African Trypanosomiasis. Hunter's Tropical Medicine. S. G. T. Philadelphia, Saunders Company: 617-628.

Banks, C. K., J. Controulis, *et al.* (1946). N-Derivatives of p-arsanilic acid. *J. Amer. Chem. Soc.* 68: 2102.

Bentivoglio, M., G. Grassi-Zucconi, *et al.* (1994). From trypanosomes to the nervous system, from molecules to behavior: a survey, on the occasion of the 90th anniversary of Castellani's discovery of the parasites in sleeping sickness. *Ital. J. Neurol. Sci.* 15(2): 75-87.

Bronner, U., R. Brun, *et al.* (1998). Discrepancy in plasma melarsoprol concentrations between HPLC and bioassay methods in patients with *T. gambiense* sleeping sickness indicates that melarsoprol is metabolized. *Trop. Med. Int. Health* 3(11): 913-917.

Burri, C., J. Blum, *et al.* (1995). Alternative application of melarsoprol for treatment of *T. b. gambiense* sleeping sickness. Preliminary results. *Ann. Soc. Belg. Med. Trop.* 75(1): 65-71.

Chiu, S. (1993). The use of *in vitro* metabolism studies in the understanding of new drugs. *JPM* 29(2): 77-83.

Doak, G. O. and H. Eagle (1951). Correlations between the chemical structure and biological activity of arsenosobenzenes. First Symposium on Chemical-Biological Correlation., Washington, D.C., National Research Council.

Douglas, K. T. (1994). Rational drug design in parasitology. *Parasitol. Today* 10(10): 389-392.

Drews, J. (1998). Die verspielte Zukunft: wohin geht die Arzneimittelforschung? Basel, Birkhäuser Verlag.

Drews, J. and S. Ryser (1997). Pharmaceutical innovation between scientific opportunities and economic constrains. *DDT* 2(9): 365-372.

Duggan, A., J. and M. P. Hutchinson (1951). The efficacy of certain trypanocidal compounds against *Trypanosoma gambiense* infection in man. *Trans. Roy. Soc. Trop. Med. Hyg.* 44(5): 535-544.

Englund, P. T., S. L. Hajduk, *et al.* (1982). The molecular biology of trypanosomes. *Annu. Rev. Biochem.* 51: 695-726.

Fairlamb, A. H., G. B. Henderson, *et al.* (1989). Trypanothion is the primary target for arsenical drugs against Arican trypanosomes. *Proc. Natl. Acad. Sci. USA* 86: 2607-2611.

Flieger, K. (1993). FDA finds new ways to speed treatment to patients. *FDA Consum.* 27(8): 14-18.

Flynn, I. W. and I. B. R. Bowman (1974). The action of trypanocidal arsenical drugs on *Trypanosoma brucei* and *Trypanosoma rhodesiense*. *Comp. Biochem. Physiol.* 48B: 261-271.

Friedheim, E. A. H. (1944). Trypanocidal and spirochetocidal arsenicals derived from s-triazine. *J. Amer. Chem. Soc.* 66: 1775-1778.

Friedheim, E. A. H. (1948). Melarsen oxide in the treatment of human African trypanosomiasis. *Ann. Trop. Med. Parasitol.* 42: 357-363.

Friedheim, E. A. H. (1949). Mel B in the treatment of human trypanosomiasis. *Am. J. Trop. Med. Hyg.* 29: 173-180.

Friedheim, E. A. H. (1951). Mel B in the treatment of tryparsamide resistant *T. gambiense* sleeping sickness: Observations on drug resistance in the trypanosomes of the french Cameroun. *Am. J. Trop. Med. Hyg.* 31: 218-235.

Friedheim, E. A. H. (1953). MSb and MSbB in the treatment of sleeping sickness due to infection with *Trypanosoma gambiense*. *Ann. Trop. Med. Parasitol.* 47: 350-360.

Friedheim, E. A. H. (1959). Some approaches to the development of chemotherapeutic compounds. *Ann. Trop. Med. Parasit.* 53: 1-9.

Friedheim, E. A. H. (1973). The chemotherapy and chemoprophylaxis of African trypanosomiasis. *XIIème Congrès International de thérapeutique, Genève, Suisse*: 277-282.

Friedheim, E. A. H. and D. Distefano (1989). Melarsoprol in the treatment of African sleeping sickness. International Scientific Council for Trypanosomiasis Research and Control. OAU/STRC. Mombasa.

Gaillot, P. and J. Baget (1955). Salts of sym-ethylenediamino-4,4-diphenylarsonic acid. French Patent 1096712.

Ganellin, C. R. (1993). Chapter 7: General approaches to discovering new drugs: an historical perspective. Medicinal Chemistry. The role of organic chemistry in drug research. C. R. Ganellin and S. M. Roberts. London, Academic press limited.

Gonzales, M. A. and K. S. Estes (1998). Pharmacokinetic overview of oral second generation H1 antihistamines. *Int. J. Clin. Pharmacol. Ther.* 36(5): 292-300.

Gutteridge, W. E. (1993). Chemotherapy. Modern Parasitology. F. E. G. Cox. Oxford, Blackwell Scientific Publications: 219-237.

Hajduk, S., B. Adler*, et al.* (1992). Molecular Biology of African Trypanosomes - Development of New Strategies to Combat an Old Disease. *Am. J. Med. Sci.* 303(4): 258-270.

Hoare, C. A. (1972). The trypanosomes of mammals (A zoological monograph). Oxford and Edinburgh, Blackwell Scientific Publications.

Hudson, A. T. (1994). The contribution of empiricism to antiparasite drug discovery. *Parasitol. Today* 10(10): 387-389.

Lambe J. (1995). Psychotropic drugs. *Aust. N. Z. J. Psychiatry* 29(3): 522-3

Mann, F. G. (1950). The heterocyclic derivatives of phosphorus, arsenic, antimony, bismuth, and silicon. New York, Interscience Publishers, Inc.

Metcalf, B. W., P. Bey, *et al.* (1978). Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogues. *Am. J. Med. Sci.* 100: 2551-2553.

Molyneux, D. H., V. Pentreath, *et al.* (1996). African trypanosomiasis in man. Manson's Tropical Diseases. G. C. Cook. London, W.B. Saunders Company Ltd.: 1171-1196.

Mutschler, E. (1991). Arzneimittelwirkungen. Stuttgart, Wissenschaftliche Verlagsgesellschaft mbH.

Nightingale, S. (1991). Drug for sleeping sickness approved*. JAMA. (From the Food and Drug Administration)*. 265: 1229.

Park, B. K (1986). Metabolic basis of adverse drug reactions. *J. R. Coll. Physicians Lond.* 20(3): 195-200

Pepin, J. and F. Milord (1991). African trypanosomiasis and drug-induced encephalopathy; risk factors and pathogenesis. *Trans. R. Soc. Trop. Med. Hyg.* 85: 222-224.

Pepin, J., F. Milord, *et al.* (1987). Difluoromethylornithine for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness. *Lancet* 81: 1431-1433.

Pepin, J., F. Milord, *et al.* (1994). Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy. *Trans. R. Soc. Trop. Med. Hyg.* 88(4): 447-52.

Pepin, J. and F. Milord (1994). The treatment of human African trypanosomiasis. *Adv. Parasitol.* 33: 1-46.

Peters, L. and H. N. Wright (1943). Trypanocidal activity and arsenic content of rat blood following intravenous administration of oxophenarsine hydrochloride. *Proc. Soc. Exper. Biol. and Med.* 52: 3.

Räz, B., M. Iten, *et al.* (1997). The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Trop.* 68(2): 139-47.

Robertson, D. H. H. (1963). The treatment of sleeping sickness (mainly due to *Trypanosoma rhodesiense*) with melarsoprol. *Trans. R. Soc. Trop. Med. Hyg.* 57(2): 122-133.

Smith, D. H., J. Pepin, *et al.* (1998). Human African trypanosomiasis: an emerging public health crisis. *Br. Med. Bull.* 54(2): 341-355.

Spilker, B. (1994). Multinational pharmaceutical companies. Principles and practices. New York, Raven Press.

Stocken L. A. H., Thompson R. S. (1946). Whittaker V. P. British Anti- lewisite. *Biochem. J.* 41: 47- 51.

Tillitson, E. W. (1951). Arsenical compounds. U.S.patent: 383.

UNDP, W. B., WHO (1997). African Trypanosomiasis. Geneva, WHO.

Van Nieuwenhove, S. (1998). Current sleeping sickness situation and control strategies. *International Colloquium Sleeping Sickness Rediscovered*. Antwerp.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *Br. Med. Bull.* 41(2): 105-114.

Wang, C. C. (1995). Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. *Annu. Rev. Pharmacol. Toxicol.* 35: 93-127.

Watkins, P.B. (1992) Drug metabolism by the cytochromes P450 in the liver and small bowel. *Gastrointestinal Pharmacol*. 3,511-522.

WHO (1998). Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. *World Health Organ. Tech. Rep. Ser.* 881: I-VI, 1-114.

Williamson, J. (1962). Chemotherapy and chemoprophylaxis of African trypanosomiasis. *Exp. Parasitol.* 12: 274.

Williamson, J. (1970). Chapt. 7: Review of chemotherapeutic and chemoprophylactic agents. The African Trypanosomiasis. H. W. Mulligan: 125-221.

Discrepancy in plasma melarsoprol concentrations between HPLC and bioassay methods in patients with T. gambiense sleeping sickness- an indication that melarsoprol is metabolised

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Keywords plasma, urine and CSF concentrations of melarsoprol, HPLC, bioassay, metabolism, *T. gambiense* sleeping sickness

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Summary

With the use of a recently developed specific high-performance liquid chromatography (HPLC) method and a bioassay which determines trypanocidal activity, concentrations of melarsoprol were studied in plasma, urine and cerebrospinal fluid (CSF) from 8 patients with late stage *Trypanosoma gambiense* sleeping sickness. The subjects were given their first series of melarsoprol injections- one dose i.v. per day for 4 days (1.2, 2.4, 3.0-3.6, 3.0-3.6 mg per kg b.w., respectively). Plasma samples were obtained before the first melarsoprol injection, immediately after and at 1h, 24 h and 5 days after the 4th injection. Urine was collected before start of melarsoprol therapy and at 0-24 h after the 4th injection. CSF samples were taken once before treatment and at 24 h after the 4th injection. The HPLC analyses showed that the plasma concentration immediately after the 4th injection varied from 2,200 to 15,900 nmol/l, at 1 h they were considerably lower, 0-1,800 nmol/l, and at 24 h no melarsoprol could be detected. In urine small amounts of melarsoprol were recovered. Melarsoprol could not be detected in CSF by HPLC. Immediately after injection, bioassay analyses showed plasma concentrations of the same magnitude as assayed by HPLC but at 1 h they were 4 to 65-fold higher than the levels assessed by HPLC. Still at 24 h and 5 days after the 4th injection, significant but decreasing activity, could be detected. Urine levels were 40 to 260-fold higher than the measured HPLC concentrations. In CSF low, but detectable, activity was found. The results indicate that melarsoprol is rapidly eliminated from plasma. The significant trypanocidal activity determined by bioassay and simultaneuos low or not detectable levels of melarsoprol assayed by HPLC, indicate that the compound is transformed into metabolites with parasiticidal activity.

Introduction

The arsenical melarsoprol was introduced already in 1949 for the therapy of human African sleeping sickness and is still the main drug for treatment of late stage disease caused by *Trypanosoma rhodesiense* and *Trypanosoma gambiense* (Pepin & Milord 1994). The efficacy of melarsoprol is high but its use is hampered both by serious adverse effects, e.g. reactive encephalopathy in 4-8 % of treated patients with *T. gambiense* (Pepin & Milord 1994) and complicated dosage regimens for repeated intravenous administration. The pharmacokinetics of the drug is just preliminary characterized due to lack of a specific analytical assay. In the only published pharmacokinetic study, a mean elimination half life of 35 h and a volume of distribution of 100 l were found in sleeping sickness patients (Burri *et al.* 1993). These calculations were based on a biological assay (Burri & Brun 1992) and atomic absorption spectrometry (Burri *et al.* 1993). Both methods are unspecific and cannot distinguish melarsoprol from other substances (e.g. melarsoprol metabolites) with trypanocidal properties.

In order to be able to assess the pharmacokinetics of melarsoprol, we have recently developed a sensitive, specific high-performance liquid chromatography (HPLC) method (Ericsson *et al.* 1997) for analyses of the melarsoprol concentration in biological fluids. We have now compared the concentrations of melarsoprol in plasma, urine and cerebrospinal fluid (CSF) during therapy of *T. gambiense* sleeping sickness using both the HPLC method and the bioassay, with the aim to unravel to what extent the bioassay codetermines biologically active metabolites of melarsoprol.

Patients and Methods

Patients

Eight consecutive patients with *T. gambiense* disease at the national research centre for trypanosomiasis (P.R.C.T.) in Daloa, Côte d'Ivoire, were included. Examination of peripheral blood using the mini-anion exchange centrifugation technique (Lumsden *et al.* 1979) demonstrated trypanosomes in all subjects. In four patients double centrifugation of CSF showed parasites and the remaining four had an increased CSF white cell count (median 253, range 36-954 x 10^6/l). Thus, the eight subjects were considered to suffer from late stage disease according to the WHO criteria (WHO 1986).

The median age was 26 (range 10-36) years and the median weight was 56 (range 24-61) kg. Four patients were females.

Study design

The subjects were given one daily intravenous dose of melarsoprol (Arsobal®, Specia, Paris France) for 4 days (1.2, 2.4, 3.0-3.6, 3.0-3.6 mg per kg b.w. respectively), which constituted the first of three series of melarsoprol injections. The daily dose never exceeded 180 mg melarsoprol as this dosage was considered to be the maximum dose at the centre. Between two and five days before the first melarsoprol injection, the patients received one dose of 4 mg pentamidine isethionate (Pentacarinat®, May & Baker, Rhône-Poulenc Rorer, Dagenham, England) per kg b.w. intramuscularly as well as a single dose of 0.5 mg betamethasone intramuscularly (Diprostene®, Schering-Plough, Levallois-Perret, France). Pentamidine was given in order to kill parasites in peripheral blood and betamethasone as prophylaxis against encephalopathy, according to the routine at centre.

Plasma samples were obtained before the 1st melarsoprol injection, immediately after and at 1 h, 24 h and 5 days after the 4th injection by separate venepunctures. Urine was collected during 24 h before the 1st melarsoprol injection and during 0-24 h after the 4th melarsoprol injection. CSF samples were obtained by lumbar puncture before the 1st melarsoprol injection and at 24 h after the 4th injection.

The study design was approved by the Ministry of Health, Abidjan, Côte d´Ivoire. All subjects or accompanying relatives gave verbal consent to the study after explanation of the protocol.

Handling of samples

Blood was collected in 4.5 ml Vacutainer tubes containing ethylene diaminetetraacetic acid (EDTA) and centrifuged within 15 min in a Beckman refrigerated centrifuge

(Beckman Instruments, Palo Alto, USA) at 1000 *g* for 10 min. The plasma was transferred to a NUNC® tube (InterMED, Roskilde, Denmark) and frozen at -196° C within a few minutes. Urine was collected in 2.5 L plastic (high-density polyethylene) containers (LAGAN-plast, Ljungby, Sweden), the volume was measured and 5 ml aliquots were transferred to two NUNC® tubes and frozen as above.

CSF was collected directly in NUNC® tubes and frozen as previously described.

The plasma, urine and CSF samples were kept frozen at -196° C in Africa, in dry ice during 24 h of transportation to Europe, and at -70° C in Europe pending assay.

Drug analyses

HPLC

The HPLC assay consists of a reversed-phase liquid chromatography system with UV detection recently described by us (Ericsson *et al.* 1997). The lower limit of determination is 9 nmol/L, 45 nmol/L and 10 nmol/L, in plasma, urine and CSF respectively, with a coeffecient of variation of 3-6%. All samples were analyzed in duplicates. 100-1,000 µl of plasma, urine or CSF were used per assay.

Bioassay

The bioassay determines parasiticidal activity in different body fluids using a *T. rhodesiense in vitro* model. The lower limit of determination is 22.6 nmol/l with a coeffecient of variation of 20% (Burri & Brun 1992). All determinations were made in triplicates with a sampling volume of 100 µl per assay.

Results

HPLC

Immediately after the 4th injection, the plasma melarsoprol concentrations varied between 2,230 and 15,900 nmol/l, at 1 h after dose they were considerably lower (0-1,780 nmol/l) and at 24 h and 5 days no melarsoprol could be detected (Table 1). In urine low concentrations (0-3,680 nmol/l) were recovered. The compound could not be detected in CSF (Table 2).

Bioassay

The plasma concentrations immediately after the 4th injection were similar to levels assessed by HPLC but at 1 h they were 4 to 65-fold (median 25-fold) higher. Still at 24 h and 5 days after the 4th melarsoprol injection, significant but decreasing activity could be detected (Table 1).

Urine levels were 40 to 260-fold (median 180-fold) higher than the measured HPLC concentrations. In CSF low, but detectable, activity was found (Table 2).

Before the 1st melarsoprol injection, low levels of trypanocidal activity could be determined by the bioassay in plasma in all subjetcs (Table 1). In urine and CSF, low levels were also recorded before the first injection in 6 and 3 patients, respectively (Table 2).

The marked difference between HPLC and bioassay determinations in plasma is clearly visualised in Fig. 1 (patient no. 5).

Discussion

The results of the present study demonstrate that melarsoprol is rapidly eliminated from plasma, i.e. within hours. As the number of sampling points were few, we were not able to calculate pharmacokinetic parameters but it is obvious that the elimination half life is much shorter than the 35 h previously reported.

Substantially lower levels of melarsoprol were determined in urine by HPLC compared to the bioassay. However, melarsoprol is not stable in urine at room temperature (Ericsson *et al.* 1997) and it is therefore not possible to determine the true concentration of melarsoprol in urine by HPLC since the urine was collected at the ward during 24 h.

The low trypanocidal activity detected by bioassay in plasma and CSF before the first melarsoprol injection is probably due to pentamidine still present in these body fluids, an observation explained by its pronounced tissue affinity and very slow elimination from the body (Bronner *et al.* 1995).

The trypanocidal activity measured in the pretreatment urine samples of patients no. 1, 7 and 8, was considerable and probably also a result of the pentamidine injected 2 to 5 days before melarsoprol.

In addition, patient no. 8 received a few doses of metronidazol 4 days before the 1st melarsoprol injection for therapy of an amoeba-infection. Metronidazol is reported to have clinical effect in the therapy of *T. rhodesiense* sleeping sickness (Foulkes 1996) and may therefore induce trypanocidal activity in the samples from this patient.

The significant trypanocidal activity determined by bioassay in plasma, urine and CSF and simultaneous low or not detectable levels of melarsoprol assayed by HPLC, indicate that melarsoprol is eliminated from plasma by transformation into metabolites with parasiticidal activity.

No melarsoprol was detected in CSF 24 h after the 4th injection while the bioassay showed low trypanocidal activity. This finding may also be explained by the presence of active melarsoprol metabolites.

The metabolic fate of melarsoprol is unknown but there are several ways by which melarsoprol can be transformed. A possible metabolite is melarsen oxide, which also can be formed from melarsoprol by non-enzymatic hydrolysis (unpublished data from our laboratories). It has trypanocidal activity and has been used in the therapy of African sleeping sickness (Friedheim 1948) but was abandoned because of its toxicity. Melarsoprol was originally synthesized by reacting melarsen oxide with BAL (Williamson 1970), and it was believed but not verified in comparative trials, that melarsoprol was less toxic than melarsen oxide.

Other possible metabolic pathways include N-oxidation, aromatic or aliphatic oxidation, and direct conjugation.

The ordinary treatment schedule for therapy with melarsoprol - 3-4 series of 3-4 injections with an interval of 7-10 days between the series- has been used for many years and is still advocated by the WHO (WHO 1986). A fear of drug accumulation has motivated the long drug-free intervals. However, investigations have not yielded any signs of drug accumulation (Cristau *et al.* 1975; Burri *et al.* 1994), and this complicated dosage schedule was recently questioned on the basis of new pharmacokinetic information mainly obtained by the bioassay method and computer simulations. An alternative schedule, consisting of ten daily doses of melarsoprol, was proposed (Burri *et al.* 1993) and has been successfully tested on 11 patients in Zaire (Burri *et al.* 1995).

The present finding of a rapid plasma elimination of melarsoprol and sustained trypanocidal activity in plasma and CSF probably caused by metabolites, clearly shows the need to use the bioassay when designing new treatment schedules. It is also obvious that the use of chromatographic techniques is crucial when trying to reveal active metabolites of melarsoprol.

Despite the introduction of eflornithine (DFMO) in the treatment of sleeping sickness (Bacchi *et al.* 1983; Van Nieuwenhove *et al.* 1985; Doua *et al.* 1987), melarsoprol is the only drug available for the majority of patients suffering from *T. gambiense* and *T. rhodesiense* sleeping sickness with CNS involvement. Thus, the improvement of its use is still of clinical relevance. The encephalopathy induced by this drug is of major clinical concern and the most important complication of melarsoprol treatment. Its etiology has not been elucidated and the role of metabolism in the disposition of melarsoprol has just been discovered. It can be hypothezised that melarsoprol is transformed into parasiticidal metabolites with more or less toxic properties. Further studies on the disposition and metabolism of melarsoprol are needed as they may shed light on the issue of melarsoprol toxicity and be very valuable when reevaluating the dosage regimen of this drug.

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References

Bacchi CJ, Garofalo J, Mockenhaupt D *et al* (1983) In vivo effects of a-DL-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **7**, 209-225.

Bronner U, Gustafsson LL, Doua F *et al* (1995) Pharmacokinetics and adverse reactions after a single dose of pentamidine in patients with *Trypanosoma gambiense* sleeping sickness. *British Journal of Clinical Pharmacology* **39**, 289-295.

Burri C & Brun R (1992) An in vitro bioassay for quantification of melarsoprol in serum and cerebrospinal fluid. *Tropical Medicine and Parasitology* **43**, 223-225.

Burri C, Baltz T, Giroud C, Doua F, Welker HA & Brun R (1993) Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* **39**, 225-234.

Burri C, Onyango JD, Auma JE, Burudi EME & Brun R (1994) Pharmacokinetics of melarsoprol in uninfected vervet monkeys. *Acta Tropica* **58**, 35-49.

Burri C, Blum J & Brun R (1995) Alternative application of melarsoprol for treatment of *T.b. gambiense* sleeping sickness. *Annales de la Societé Belge Médecine Tropicale* **75**, 65-71.

Cristau B, Placidi M & Legait J-P (1975) Étude de l'excrétion de l'arsenic chez le trypanosome traité au mélarsorpol (Arsobal®). *Medecine Tropicale (Marseilles)* **35**, 389-401.

Doua F, Boa FY, Schechter PJ *et al* (1987) Treatment of human late stage gambiense trypanosomiasis with alpha-difluoromethylornithine (eflornithine). Efficacy and tolerance in 14 cases in Côte d'Ivoire. *American Journal of Tropical Medicine and Hygiene* **37**, 525-533.

Ericsson Ö, Schweda E, Bronner U, Rombo L, Fridén M & Gustafsson LL (1997) Determination of melarsoprol in biological fluids by high-performance liquid chromatography and characterisation of two stereoisomers by NMR. *Journal of Chromatography B*, **690**, 243-251.

Foulkes JR (1996) Metronidazole and suramin combination in the treatment of arsenical refractory rhodesian sleeping sickness- a case study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **90**, 422.

Friedheim EAH (1948) Melarsen oxide in the treatment of human African trypanosomiasis. *Annals of Tropical Medicine and Parasitology* **42**, 357-363.

Lumsden WHR, Kimber CD, Evans DA & Doig J (1979) *Trypanosoma brucei*: miniature anion exchange centrifugation technique for detection of low parasitemias: adaptation for field use. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**, 312-317.

Pépin J & Milord F (1994) The treatment of human African trypanosomiasis. *Advances in Parasitology* **33**, 1-47.

Van Nieuwenhove S, Schechter PJ, Declercq J, Boné G, Burke J & Sjoerdsma A (1985) Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-alpha-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 692-698.

WHO (1986) *Epidemiology and control of African trypanosomiasis.* Geneva: World Health Organization, *Technical report series* no. 739.

Williamson J (1970) Review of chemotherapeutic and chemoprophylaxis agents: In *The African Trypanosomiases.* (ed. HW Mulligan & WH Potts), George Allen and Unwin Ltd, London, pp. 125-221.

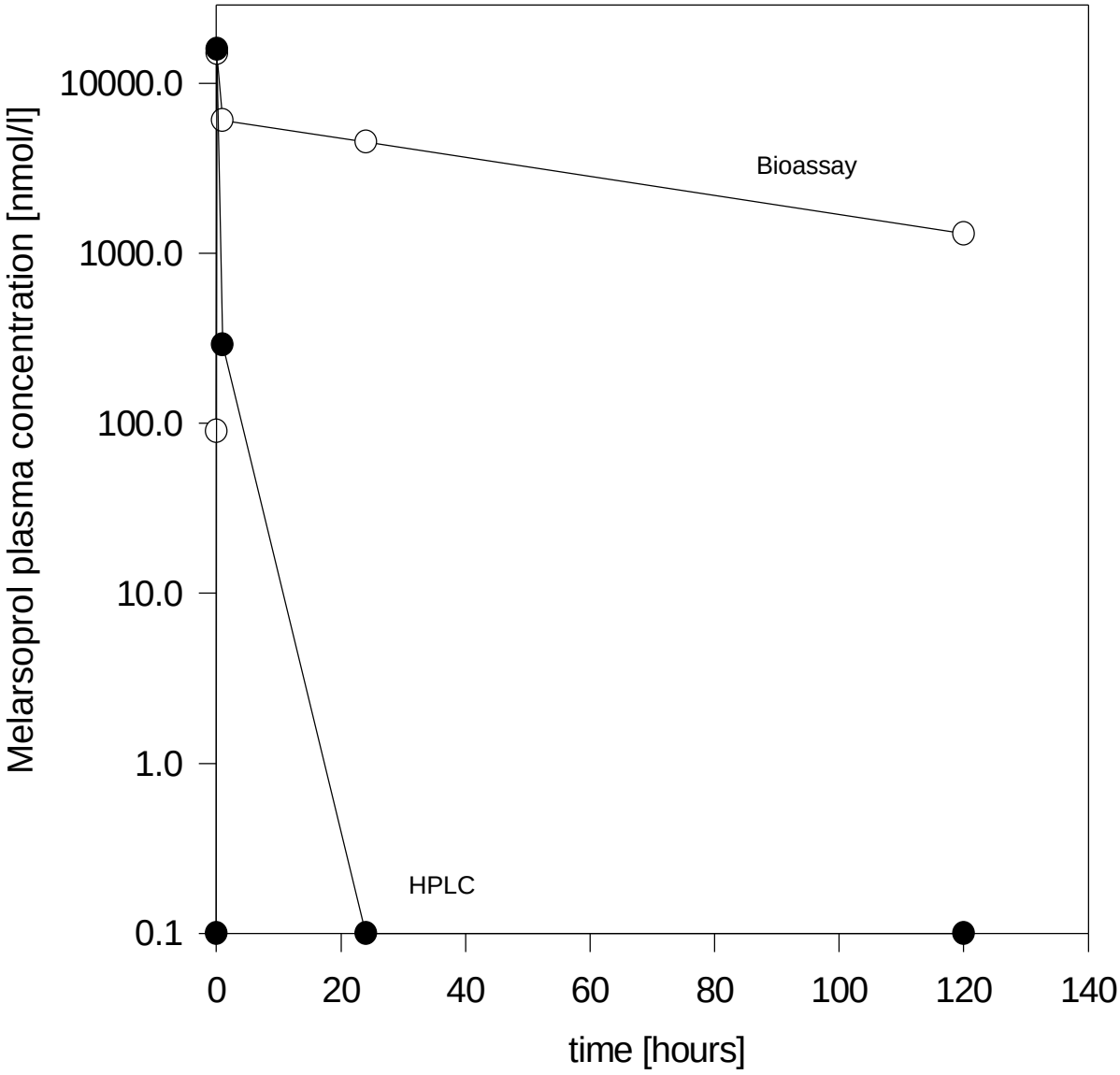

Figure 1

Plasma concentrations of melarsoprol 0-120 h after the 4th i.v. injection of 1.2-3.2 mg melarsoprol per kg b.w. in patient no. 5. Analyses by HPLC and bioassay of the same samples are shown.

arsoprol concentrations in plasma determined by HPLC and bioassay in 8 patients with *T. gambiense* sleeping sickness administered i.v. injections of 1.2- 3.6 mg melarsoprol per kg b.w.

Before 1st injection		**Immediately after 4th injection**		**1h after 4th injection**		**24h after 4th injection**		**5d after 4th injection**	
HPLC	bioassay	HPLC	bioassay	HPLC	bioassay	HPLC	bioassay	HPLC	bioassay
0	590	5500	11300	200	13160	0	9040	0	980
0	45	2230	9040	1780	6570	0	1790	0	280
0	45	8250	8380	310	9040	0	4550	0	720
0	90	ND	ND	ND	ND	0	3240	0	480
0	90	15900	15010	290	6040	0	4520	0	1300
0	1080	4400	9040	290	6390	0	4520	0	510
0	90	8900	10300	220	7530	0	3940	0	530
0	70	6180	8280	0	7750	0	2260	0	580

arsoprol concentrations in urine and CSF determined by HPLC and bioassay in 8 patients with *T. biense* sleeping sickness administered 4 i.v. injections of 1.2- 3.6 mg melarsoprol per kg b.w.

Urine				**CSF**			
0-24h before 1st injection		**0-24h after 4th injection**		**Before 1st injection**		**24h after 4th injection**	
HPLC	bioassay	HPLC	bioassay	HPLC	bioassay	HPLC	bioassay
0	12340	1280	139800	0	0	0	90
0	720	0	7230	0	0	ND	80
0	180	3680	139800	0	0	0	100
0	0	ND	ND	ND	0	0	140
0	100	1020	101230	0	10	ND	140
0	0	380	23470	0	50	0	80
0	7670	990	130150	0	0	0	45
0	17470	450	115690	0	20	0	180

mined

ere also given a single i.m. injection of 4 mg pentamidine isethionate 2-5 days before the first dose of melarsoprol.

Physico-chemical properties of the trypanocidal drug mela rsprol

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Keywords: Melarsoprol, protein binding, Trypanosomiasis, Sleeping sickness

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Introduction

Trypanosomiasis is a communicable disease, caused by the protozoan parasites *Trypanosoma brucei gambiense* and *T.b. rhodesiense.* 55 million people in 36 African countries are at risk of the disease. The recent epidemic outbreaks affecting several regions with about 300 000 new cases annually (Smith *et al.*, 1998) reinforces the need for efficient, safe and affordable drugs, or as a minimal temporary requirement the improved application of the existing ones.

Melarsoprol (Arsobal®) remains the drug of choice for the treatment of the late stage of sleeping sickness, in which the parasite has invaded the central nervous system. The only new compound, eflornithine, which has been introduced during the last 50 years, is hampered by its almost unaffordable costs, its ineffectiveness against *T.b. rhodesiense,* and the short duration of action and low efficacy which cause logistic problems.

Though melarsoprol was introduced in 1949 (Friedheim, 1949) remarkably little is known about the mechanism of action (Fairlamb *et al.*, 1989, Wang, 1995), and only limited data on its chemical, pharmacological and pharmacokinetic properties are available (Cristau *et al.*, 1975, Pepin and Milord, 1994, Burri *et al.*, 1993, Burri *et al.*, 1994). The drug is administered by intravenous injection as a 3.6% solution in propylene glycol. Adverse drug reactions like cutaneous reactions, polyneuropathy, diarrhea, fever or thrombophlebitis at the site of injection (Pepin and Milord, 1994, Molyneux *et al.*, 1996) are quite common. Encephalopathic syndromes, which are reported to occur in 2-10% and being fatal in 50-70%, are the most important complication of melarsoprol treatment. Its trigger remains controversial, but immune reactions are suggested (Hunter *et al.*, 1992, Haller *et al.*, 1986).

Plasma protein binding has a significant impact on the clinical pharmacology and pharmacokinetics of a drug, since normally only the unbound moiety is available for distribution, elimination and drug action (Wright *et al.*, 1996). Displacement of the drug from its protein binding is often responsible for drug interaction related adverse effects (Tillement *et al.*, 1984). The lipid solubility and dissociation constant are two other important physical parameters. As lipoid membranes, like the blood brain barrier, can only be passed by lipid soluble and unionized molecules these parameters essentially influence the pharmacokinetics of the drug (Brodie *et al.*, 1960). Since late stage of trypanosomiasis is characterized by the invasion of the parasites into the CSF, penetration of the blood brain barrier is a fundamental requirement for successful treatment. The melarsoprol levels in the CSF are about 50 times lower than those in serum (Burri *et al.*, 1993) which may be due to ionization or a strong protein binding of drug.

To elucidate essential basic pharmacological properties of the drug we studied the protein binding, the water / octanol coefficient of partition (P) and the dissociation constant (pK_b) of melarsoprol, as well as of one of its suggested metabolites melarsen oxide (Bronner *et al.*, 1998).

Methods and results

Drugs and Chemicals

Melarsen oxide was obtained from Rhône-Mérieux, Laboratoire de Toulouse, France; melarsoprol was purchased (Arsobal®, batch 701) from Specia, France. Solvents and chemicals were purchased from Merck.

Chromatographic system

Peak areas were determined with a chromatographic system consisting of Waters pump 510 (flow 1 ml/min), an ultraviolet detector Spectroflow 773 Kratos and a Spectra Physics SP 4270 integrator equipped with a Beckman RP-C_{18} ODS ultrasphere (5 µm, 4.4 mm I.D.∗ 15 cm) column and a C_{18} guard column (5 µm, 4.6 mm I.D.∗ 4.5 cm). The detection wavelength were for melarsen oxide 274 nm and for melarsoprol 283 nm. The mobile phase consisted of 4 ml of acetonitrile and 96ml of distilled water (HPLC quality) for melarsen oxide and 25 ml of acetonitrile and 75 ml of water for melarsoprol.

Determination of the coefficient of partition *P*

The partition of melarsoprol and melarsen oxide between buffer and n-octanol was analyzed with the shake flask method at physiological pH(Leo *et al.*, 1971, Pöyhiä and Seppälä, 1994): For melarsenoxide 5 ml n-octanol was added to 5 ml of a 6 µM solution of melarsen oxide in 0.1 M ammonium acetate buffer adjusted with 1 M NaOH to pH 7.4 in five test tubes each. A more lipophilic characteristic was predicted for melarsoprol, which made necessary an increased assay concentration to prevent levels in the water phase below the limit of detection. A maximum of 180 µM was achievable, limited by the poor water solubility of the compound.

The tubes were incubated for 1 hour in a rotation mixer at 22°C.For determination of *P* (C_{pre}-C_{post}/C_{post}) 20 µl of the aqueous phase was injected into the HPLC and the concentration calculated according to the areas.

The average value of *P* for melarsen oxide was determined as 8.4.

For melarsoprol the coefficient of partition between buffer and n- octanol was calculated of approximately 160. The exact value could not be determined due to the poor water solubility of the compound. The *P* value was previously determined spectrometrically to be 40 at pH 7 for melarsoprol (Cristau *et al.*, 1972), which corresponds to the value of 160 determined at pH 7.4.

Analysis of protein binding

The binding of melarsoprol and melarsen oxide to serum proteins, e.g. albumin (35 g/l), and α-1-acid glycoprotein (0.55 g/l) was studied based on a physical separation by ultrafiltration (Pöyhiä and Seppälä, 1994, Wright *et al.*, 1996). Prior to the experiment the occurrence of binding of the drug to the filter Centrex UF-0.5 (10000 kDa Schleicher & Schuell filters) was tested and could be excluded.

The protein solutions were spiked with 18 µM melarsoprol, which corresponds to therapeutic concentrations found in samples of human patients (Ericsson *et al.*, 1997) and 3 µM melarsen oxide. The samples were incubated for 30 minutes at 37°C in eppendorf tubes. The unbound drug was then separated by centrifugation at 12000g. The concentration of the free drug in the filtrate was determined by high performance liquid chromatography as described and the binding was calculated according to $c_{bound} = c_{prae} - c_{post}$. For each binding experiment the procedure was repeated five times.

For melarsoprol a total serum protein binding of 79%, an albumin binding of 79% and a glycoprotein binding of 70% was calculated. The total serum protein binding of melarsen oxide solution was 72%, the binding to albumin 46% and to α-1-acid glycoprotein 37%.

Ionization constant

The protonation constant was determined potentiometrically: 25 ml of a 0.9 mM melarsoprol solution and of a 1.1 mM melarsen oxide solution in water were pre-acidified to pH 2.2 - 2.9 with 1 M HCl and were then titrated alkalimetrically with 1 mM NaOH to a high pH. Three titrations were carried out for each drug at room temperature, the pK_a values of the corresponding acids were calculated with the Henderson-Hasselbalch equation (Latscha and Klein, 1990).

The pK_a was calculated as 4.8, the corresponding pK_b 9.2 for melarsoprol and the pK_a 4.5 and pK_b 9.5 for melarsen oxide, respectively.

Discussion

The lipid solubility of the unionized drug molecule is the physical property largely governing the rate of entry into the CSF (Brodie *et al.*, 1960). The *P* values calculated for melarsenoxide and especially melarsoprol indicate a high lipophilicity. However, for highly ionized drugs, a relationship exists between the degree of ionization and the CSF permeability constant; completely ionized compounds like sulfosalicylic acid hardly penetrate into the CSF, partially unionized molecules as for example salicylic acid have a permeability constant, roughly relating to the proportion of unionized drug (Brodie *et al.*, 1960). At a physiologic pH of 7.4 only a small quantity of both compounds is unionized (0.2% of melarsoprol and 0.1% of melarsenoxide), and thus only a small amount of drug is available to cross lipoid membranes.

The results obtained are supported by the earlier finding that the CSF concentrations are very low compared to the plasma levels in samples of patients treated with melarsoprol. The levels found in the serum were in the range of 1.5 - 2 µg/ml after the fourth injection of each therapy course, whereas the levels measured in the CSF at the same time were about 50 times lower (mean in the range of 30 ng/ml determined by bioassay) and in one third of the patients the concentrations were below the limit of detection of 9 ng/ml (Burri *et al.*, 1993).

Drugs with a protein binding of 50 to 85% are considered medium binding drugs (Klotz, 1984., Wright *et al.*, 1996). The extent of the protein binding of both compounds may be responsible, together with the ionization constant, for the slow CSF concentration adaptation of melarsoprol and its potential metabolites (Burri *et al.*, 1993, Burri *et al.*, 1994, Bronner *et al.*, 1998). Only a small fraction of the drug is available to add to the concentration gradient which drives the distribution into CSF.

The *in vivo* situation might be more complicated: Large interindividual variability of drug levels is a common feature (Rowland and Tozer, 1989). Sleeping sickness patients often have elevated IgG or IgM levels (Molyneux *et al.*, 1996), which may result in an increased total protein binding. Concomitant diseases like malaria, hepatitis and malnourishment (Wright *et al.*, 1996) may cause a modification of the plasma concentrations of albumin and α-1-acid glycoprotein. For melarsoprol up to 10-fold concentration differences were determined by a biological assay at equal timepoints in serum and in CSF (Burri *et al.*, 1993). These interindividual differences combined with the low therapeutic index may provide a partial explanation of the distribution of adverse effects frequently seen in the therapy with the arsenical drugs. A decreased level of bound drug may result in an increase of toxic effects, because of the resulting elevated levels in compartments and tissues otherwise poorly reached. The metabolic pathway of melarsoprol is largely unknown and differences in the individual metabolic capacity of patients may further complicate the situation. In order to better understand the adverse events compromising the use of melarsoprol it would be interesting to elucidate the metabolic pathway and to conduct more detailed pharmacokinetic studies.

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References

Brodie, B. B., Kurz, H. and Schanker, L. S., 1960. The importance of dissociation constant and lipid-solubility in influencing the passage of drugs into the cerebrospinal fluid. J. Pharmacol. Exp. Therap. 130, 20-25.

Bronner, U., Brun, R., Doua, F., Ericsson, O., Burri, C., Keiser, J., Miezan, T. W., Boa, Y. F., Rombo, L. and Gustafsson, L. L., 1998. Discrepancy in plasma melarsoprol concentrations between HPLC and bioassay methods in patients with *T. gambiense* sleeping sickness indicates that melarsoprol is metabolized. Trop. Med. Int. Health. 3, 913-917.

Burri, C., Onyango, J. D., Auma, J. E., Burudi, E. M. and Brun, R., 1994. Pharmacokinetics of melarsoprol in uninfected vervet monkeys. Acta Trop. 58, 35-49.

Burri, C., T., B., Giroud, C., Doua, F., Welker, H. A. and Brun, R., 1993. Pharmacokinetic properties of the trypanocidal drug melarsoprol. Chemotherapy. 39, 225-234.

Cristau, B., Chabas, E. and Placidi, M., 1975. Routes and kinetics of arsenic excretion in the guinea pig after injection of some organoarsenic drugs. Ann. Pharm. Fr. 33, 577-589.

Cristau, B., Soyfer, J. C. and Oddo-de-Garidel-Thoron, M. F., 1972. Ultraviolet spectrometry of some organoarsenic drugs. Ann. Pharm. Fr. 30, 65-76.

Ericsson, O., Schweda, E., Bronner, U., Rombo, L., Friden, M. and Gustafsson, L. L., 1997. Determination of melarsoprol in biological fluids by high-performance liquid chromatography and characterisation of two stereoisomers by nuclear magnetic resoncance spectroscopy. J Chromatogr. B. 690, 243-251.

Fairlamb, A. H., Henderson, G. B. and Cerami, A., 1989. Trypanothion is the primary target for arsenical drugs against African trypanosomes. Proc. Natl. Acad. Sci. USA. 86, 2607-2611.

Friedheim, E. A. H., 1949. Mel B in the treatment of human trypanosomiasis. Am. J. Trop. Med. 29, 173-180.

Haller, L., Adams, H., Merouze, F. and Dago, A., 1986. Clinical and pathological aspects of human African trypanosomiasis (*T.b. gambiense*) with particular reference to reactive arsenical encephalopathy. Am. J. Trop. Med. Hyg. 35, 94-99.

Hunter, C. A., Jennings, F. W., Adams, J. H., Murray, M. and Kennedy, P. G. E., 1992. Subcurative chemotherapy and fatal post-treatment reactive encephalopathies in African trypanosomiasis. Lancet. 339, 956-958.

Klotz, U., 1984. Klinische Pharmakokinetik. Gustav Fischer, New York.

Latscha, H. P. and Klein, H. A., 1990. Analytische Chemie. Springer, Berlin.

Leo, A., Hansch, C. and Elkins, D., 1971. Partition coefficients and their uses. Chem. Rev. 6, 527-554.

Molyneux, D. H., Pentreath, V. and Doua, F., 1996. In Manson's Tropical Diseases (Ed, Cook, G. C.) W.B. Saunders Company Ltd., London, pp. 1171-1196.

Pepin, J. and Milord, F., 1994. The treatment of human African trypanosomiasis. Adv. Parasitol. 33, 1-47.

Pöyhiä, R. and Seppälä, T., 1994. Liposolubility and protein binding of oxycodone *in vitro*. Pharmacol. Toxicol. 74, 23-27.

Rowland, M. and Tozer, T. N., 1989. Clinical pharmacokinetics. Lea & Febiger, Philadelphia.

Smith, D. H., Pepin, J. and Stich, A. H., 1998. Human African trypanosomiasis: an emerging public health crisis. Br. Med. Bull. 54, 341-55.

Tillement, J. P., Houin, G., Zini, R., Urien, S., Albengres, E., Barre, J., Lecomte, M., D'Athis, P. and Sebille, B., 1984. The binding of drugs to blood plasma macromolecules: recent advances and therapeutic significance. Adv. Drug Res. 13, 59-94.

Wang, C. C., 1995. Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. Annu. Rev. Pharmacol. Toxicol. 35, 93-127.

Wright, J. D., Boudinot, F. D. and Ujhelyi, M. R., 1996. Measurement and analysis of unbound drug concentrations. Clin. Pharmacokinet. 30, 445-462.

Investigations on the metabolites of the trypanocidal drug melarsoprol

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Clinical Pharmacology and Therapeutics in press

Abstract

Background Melarsoprol (Arsobal®) remains the first choice drug for trypanosomiasis (Human African sleeping sickness). To contribute to the sparse pharmacological data and to better understand the etiology of the frequent serious adverse reactions we have investigated the metabolism of this 50 years old organoarsenic compound.

Findings The half life of melarsoprol was < 1 h (HPLC), compared to 35 hours by bioassay and atomic absorption spectroscopy (AAS) indicating the existence of active metabolites. One metabolite, melarsen oxide, was identified by HPLC-UV after incubation of melarsoprol with microsomes. The metabolite is mainly formed by hydrolysis and not enzymatically. In humans c_{max} of melarsenoxide was reached 15 min after application, the clearance was 21.5 ml/min/kg and the half life of free melarsen oxide was 3.9 h. Either melarsen oxide, or a yet undiscovered further active metabolite, is irreversibly bound to proteins as shown by ultrafiltration, precipitation experiments and AAS. Due to the poor pharmaceutical properties of melarsoprol the therapeutic potential of melarsen oxide was investigated. In an acute infection rodent model 20/20 mice were cured (0.1-1 mg/kg i.v. or 2.2 mg/kg i.p.). In a CNS infection rodent model 5/6 mice survived for more than 180 days (5 mg/kg i.v), indicating a sufficient melarsen oxide penetration across the blood brain barrier.

Interpretation The perspectives for the future of trypanosomiasis treatment are deplorable. Therefore pharmacological investigations to improve the use of the first line drug melarsoprol are required. The presented results may build a basis for further research on the etiology of severe adverse reactions.

Keywords: Melarsoprol, Melarsen oxide, metabolites, Trypanosomiasis, Sleeping sickness, HPLC, bioassay, AAS, covalent binding, drug metabolism

Introduction

Sleeping sickness (Human African Trypanosomiasis) is caused by *Trypanosoma brucei* sp., kinetoplast parasites, transmitted by the bite of the tsetse fly (Glossina sp.). The disease is found over vast areas of sub-Saharan Africa and exists in two clinical forms: The West and Central African form (*T. brucei gambiense*) is a chronic disease, which continuously aggravates over several years, before death occurs, while the East African form (*T. brucei rhodesiense*) develops acutely. The onset of the disease is uncharacteristic with fever and general malaise. In the second stage, after invasion of the central nervous system by the parasite, severe mental and endocrinic disturbances like delirium, mania, paranoia, schizoid attacks, aggressive behaviour and severe motor problems are the main signs. Without appropriate treatment both forms of the disease are fatal [1].

An estimated 55-60 million of people are at risk of becoming infected with trypanosomes, but only four million of them are under active surveillance or have access to health centres where reliable diagnosis and treatment is available [2]. While the disease had been brought under control at the end of the 1960's, the situation has deteriorated since 1970 and major outbreaks are reported from countries like the Democratic Republic of Congo, Angola and Sudan [3]. The number of infected persons is currently estimated at over 300'000 [2].

Chemotherapy has made very little progress over many decades: the main drugs for the treatment of the first stage of disease are suramin and pentamidine, introduced 1922 and 1941. Due to a lack of valid alternatives the treatment of the second stage is mainly realised by the organoarsenic drug melarsoprol [p-(4,6-diamino-S-triazinyl-2-yl)-aminophenylarsenoxide]-2,3-dimercaptopropanol (Arsobal®) introduced in 1949 [4]. The drug is administered by intravenous injection as a 3.6% solution in propylene glycol. Adverse effects like cutaneous reactions, polyneuropathy, diarrhoea, fever or thrombophlebitis at the site of injection are quite common [5,6]. Encephalopathic syndromes are the most important complication of melarsoprol treatment, and occur at a rate of 5-10% and are fatal in 10-50% [2]. The trigger of the encephalopathies remains controversial, but immune reactions are suggested [7,8].

Only one drug, eflornithine (α-difluoromethylornithine), was brought to the market in the last decade [9]. However, the compound is only active against *T.b. gambiense* and is almost unaffordably expensive [2].

Despite the long utilisation, remarkably little is known on the chemical, pharmacological and pharmacokinetic properties of melarsoprol [5,10-12]. Recent results indicate that melarsoprol is metabolised to one or several active products. A terminal half life of 35 hours was determined by a biological assay, while the corresponding parameter determined by a HPLC method was 1 hour. The bioassay measures overall trypanocidal activity, whereas melarsoprol is specifically detected by HPLC [13].

Metabolites often have pharmacological properties different from their parent drugs and may be responsible for the activity of a drug as well as for adverse effects[14]. Hence, the metabolic pathway of melarsoprol was studied to get a better knowledge of the drug and to better understand the nature of the serious adverse effects related to its use.

Material and Methods:

Chemicals

Melarsen oxide was obtained from Rhone-Mérieux, Laboratoire de Toulouse; melarsoprol (Arsobal®, batch 701) was purchased from Specia, France. All solvents and chemicals were purchased from Merck (analytical quality).

Biological samples

Blood and cerebrospinal fluid (CSF) samples were obtained from five *T.b. gambiense* infected patients treated with melarsoprol at the Trypanosomiasis Reference Centre of Viana, Angola. The baseline characteristics of the patients are given in table I. The patients received 10 x 2.2 mg/kg of melarsoprol spaced by 24 h according to the protocol of Burri [11]: To investigate the nature of the metabolites and to determine their pharmacokinetic properties, serum was collected by venepuncture before, and 15 min, 1, 3, 6, 8 and 24 h after the first drug application. CSF was obtained by spinal tap from two patients at 1 and 6 h respectively, and from one patient at 24 h after the first drug application. In addition, to determine the level of metabolites at the end of treatment, samples collected 24, 48 and 72 h after the third course of melarsoprol injection were used. Those samples were collected for pharmacokinetic investigations at the Projet de Recherches Cliniques sur la Trypanosomiase, PRCT, Daloa, Ivory Coast in 1990 [11]. All samples were frozen and shipped on dry ice or in liquid nitrogen to the Swiss Tropical Institute, where they were stored at – 80°C. The chosen storage conditions of the samples were evaluated by accelerated stability tests.

Analytical procedure for the incubation of microsomes

Microsomes were prepared from pooled human liver samples. The protein content was measured as described by Lowry [15]. The microsomes were incubated at 37 °C with a 50 µM or 100 µM solution of melarsoprol in the presence of 2 mM NaDPH, 2.5 mM $MgCl_2$ and 50 mM TrisHCl for 30 min. The reaction was terminated with 0.05 M zinc sulphate in 50% v/v acetonitrile or with 100% methanol. All reactions were performed in triplicates, and samples without NaDPH or drug served as controls. After centrifugation for 10 min at 3000g the supernatant was evaporated under a stream of nitrogen and the pellet reconstituted in acetonitrile/distilled water (1:1 v/v) and analysed by HPLC-UV. Isocratic runs were performed using a mobile phase of 0, 2, 4, 8, 12, 20 and 30 ml of acetonitrile with distilled HPLC water (to a total of 100 ml) in order to detect all products at satisfactory retention times according to their polarity.

High performance liquid chromatography (HPLC) for the determination of melarsen oxide

The HPLC system consisted of a Waters pump 510 (flow 1 ml/min), a Beckman RP-C_{18} Ultrasphere ODS column with 5 µm particle size (4.4 mm I.D.∗ 15 cm length) with a C_{18} guard precolumn (4.4 mm I.D.∗ 4.5 cm length), an ultraviolet detector Spectroflow 773 (Kratos) and a Spectra Physics 4270 integrator. The isocratic mobile phase consisted of 4 ml acetonitrile and 96 ml distilled water of HPLC quality. The detection wavelenght for melarsen oxide was 274 nm and the peak areas were measured. The limit of determination was 16 ng/ml.

Determination of melarsen oxide in serum and CSF samples

100 - 1000 µl of serum or CSF were pipetted into 12 ml polypropylene tubes and 200 - 2000 µl 0.05 M zinc sulphate in 50% v/v water/acetonitrile were added for precipitation of the serum proteins. After mixing for 10 sec using a vortex mixer the samples were allowed to stand for 10 min. The tubes were then centrifuged for 10 min at 3500g. The supernatant was transferred to a new test tube, evaporated gently to 20 - 50 µl under a stream of nitrogen and directly injected into the chromatographic system. All samples were assayed in duplicates. Standard curves were prepared by adding known amounts of melarsen oxide to drug free serum and the samples treated as described above. The resulting peak areas were plotted versus concentration. The limit of quantification was 50 ng/ml (R.S.D: 7.9%). No peak interference with quinine, pentamidine, phenobarbital or chloroquine was observed.

In human samples melarsen oxide was identified by retention time and *in vitro* confirmation of the activity of the fraction containing the respective peak. The fraction was evaporated under nitrogen, dissolved in sterile distilled water and the trypanocidal activity tested following the method described by Räz[16].

Determination of melarsoprol with HPLC

Melarsoprol concentrations in serum and CSF were determined using a previously described method[17].

Determination of the binding of active products to serum proteins

1 ml of human serum collected at least 24 h after the last injection of melarsoprol was pipetted into test tubes and an equal amount of precipitating solvents added. The following solvents were tested: 0.05 M zinc sulphate in 50% v/v water/acetonitrile, β-mercaptoethanol, DMSO, 7 M NaOH, tri-chlor-acetic acid (TCA) and proteinase K. After mixing for 10 sec on a vortex, the samples were allowed to stand for 10 min at room temperature. Then they were centrifuged at 3000g and the supernatant and the protein

pellet were separated. Both phases were analysed by atomic absorption spectroscopy (AAS). In one additional experiment the protein pellet was boiled with 1 M KOH at 80°C for 1 h and the supernatant was examined by AAS.

For the analysis of active components bound to proteins in the CSF a total of 7 ml was pooled from 3 patients in a test tube and 0.05 M zinc sulphate in 50% v/v water/acetonitrile were added. After centrifugation at 3000g for ten min the supernatant was discarded, and the pellet was analysed by AAS.

For ultrafiltration 1 ml of human serum sampled either 24, 48 or 72 h after the last injection of melarsoprol was centrifuged at 10'000g for 15 min in tubes equipped with Centrisart I (Sartorius) filters of either 5 kD, 10 kD and 20 kD or 100 kD cut off. The activity of the filtrate and of the residue was analysed by bioassay.

Determination of the irreversible binding of melarsen oxide to macromolecules

6 µM of melarsen oxide were incubated in blank human serum for 48 h at 37 °C. The serum proteins were precipitated with equal amounts of 0.05 M zinc sulphate in 50% water/acetonitrile v/v. After vortexing the sample and centrifugation at 3000g the arsenic content of the protein pellet was determined by AAS. The same experiment was conducted in presence of 5 mM of the thiol blocking agent p-hydroxymercuribenzoate, to test whether melarsen oxide is bound to a sulphur group of plasma proteins.

Determination of arsenic in serum, urine and CSF by atomic absorption spectroscopy

Electro thermal atomic absorption spectroscopy (AAS) for determination of the arsenic content of the samples was performed on a Varian AA-400 Zeemann equipped with a graphite furnace operated at 193.7 nm, by Amt für Umwelt und Energie, Basel. The material was mineralised in a microwave oven with 2 ml 65% HNO_3 and 2 ml hydrogen peroxide 30% at 150 W for 10 min. All samples were assayed in duplicates. Three spiked control samples for AAS determination were prepared at concentrations of 1440, 3600 and 7200 ng/ml of melarsoprol. In the first spike 1510 ng/ml or 111% of the arsenic in the other two samples 103% and 85% of the arsenic was recovered. The limit of determination was 50 ng/ml of arsenic using a 250 µl specimen and the RSD was 1.6%.

In vitro activity testing and bioassay determination of drug levels

The trypanosome clone STIB 900, a cloned derivative of *T.b. rhodesiense* STIB 704, isolated 1982 in Tanzania, was used for *in vitro* growth inhibition studies. Bloodstream forms were cultured in Minimum Essential Medium (MEM) with Earle's salts (powder, GIBCO/BRL No.072-01100 P) supplemented with 25 mM HEPES, 1 g/l additional glu-

cose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM nonessential amino acids (100x). The medium was further supplemented according to Baltz[18].

To test the activity of melarsoprol metabolites the Alamar Blue® assay was used as previously described [16]. The tested maximum concentration for all substances was 100 µg/ml. Stock solutions in dimethyloxide (DMSO) were prepared and diluted with culture medium. Bloodstream forms of *T.b. rhodesiense* were inoculated into 96-well microtiter plates (Costar, USA). The trypanosomes were incubated with serial drug dilutions for 70 h at 37 °C in 5% CO_2. Then 10 µl Alamar Blue® were added and after 2 h of incubation the fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 590 nm determined. The IC_{50} values were calculated by linear interpolation [19].

For determination of the total trypanocidal activity of biological fluids, calculated as melarsoprol equivalents, a modification of the Alamar Blue® assays with a limit of determination of 4 ng/ml was used [20]. For determination by bioassay 100 µl of the patient sample were required.

In vivo testing of activity

Acute rodent model

Animals: Female CD1 mice (20 - 25g) were purchased from Charles River, Germany.

Groups of five animals were used; animals were infected intraperitoneally with $2*10^5$ trypanosomes of STIB 704. Treatment was started 24 h after infection. On four consecutive days, several doses of the drug were administered intraperitoneally and intravenously. One control group without treatment was included in the experiment. Animals were checked twice a week for parasites in tail vein blood. Animals surviving >30 days were considered as cured.

Relapse infection rodent model

The ability of melarsen oxide to pass the blood brain barrier and its curative effectiveness in the central nervous system were also examined in the TREU 667 second stage CNS model infection [21]. Mice were infected with 10^6 parasites. After 21 days of development of the infection mice were treated with 5 mg/kg of intravenous melarsen oxide for seven days. After the end of treatment mice were checked weekly for parasites, and they were considered to be cured if they survived for at least 180 days. A group of mice obtaining once 40 mg/kg Berenil served as control. Berenil does not pass the blood brain barrier. The drug clears the trypanosomes from the circulation and organs other than the brain.

Results

Microsomal study

Melarsen oxide was the only product that could be detected by HPLC analysis after microsomal incubation followed by precipitation. The peak was identified by comparison of the retention time with a melarsen oxide solution. Melarsen oxide could also be detected at the same concentration range in the control run without NaDPH. This indicates that melarsen oxide is not formed in a microsomal reaction but by hydrolysis. Whether melarsen oxide is also formed enzymatically to a limited extent can not be concluded from this experiment.

Concentration- time profile of melarsen oxide in serum

The pharmacokinetic profile of melarsen oxide was investigated by HPLC in five *T.b. gambiense* patients after application the first of ten doses of 2.2 mg/kg of melarsoprol (table II).

The maximum levels (C_{max}) of melarsen oxide ranged from 461 to 848 ng/ml (average 636 ± 157*)* and were detected 15 min after drug administration (Table III) Within 8 h they fell to 7-11% of C_{max} and thereafter a half-life of 3.9 h (s = 0.7) accounted for the area under the concentration time curve. The average clearance was calculated as 21.5 (s = 3.3) ml/min*kg. (The calculation of $t_{1/2}$ and C_L was based on the value of patients one to four due to outlier results). In samples taken 24 h after the first application the melarsen oxide concentrations were below the limit of quantification of 50 ng/ml (Table III).

To confirm the melarsen oxide levels, serum samples previously collected from eight patients [13] receiving the fourth injection (first series) of melarsoprol were analyzed. C_{max} were reached five min after application and ranged from 758 to 2371 (average 1147) ng/ml. After 1 h 247-889 (average 640) ng/ml could be detected, in samples taken 24 h after injection the levels of melarsen oxide were below the limit of quantification (data not shown).

Determination of arsenic in serum samples by AAS

The average serum concentration- versus time curve is presented in Fig. 2.

No arsenic was detected in samples taken before treatment. Immediately after the injection (t= 15 min) of melarsoprol 527-770 ng/ml of arsenic was found (average 657± 90). 124-171 ng/ml of arsenic (average 147± 20 ng/ml) was measured 24 h after application (Table IV).

Determination of melarsoprol by HPLC and bioassay

Before the first injection of melarsoprol the activity in the serum determined by biological assay was insignificant (8 - 19 ng/ml), and no melarsoprol was detected by HPLC. The average C_{max} concentration of melarsoprol after 15 min determined by HPLC was found to be 720 ng/ml compared to 4925 ng/ml calculated by bioassay. With HPLC the determined half life of melarsoprol was calculated to be less than an hour. In samples taken 3 h after application no melarsoprol was found by HPLC (Table V and VI).

Drug levels in CSF

In CSF samples taken at various time points no arsenic, melarsoprol or melarsen oxide levels above the limit of quantification could be detected by AAS (50 ng/ml) and HPLC (16 ng/ml, 50 ng/ml). In contrast, low concentrations were determined by bioassay: 1 h after application 28 and 32 ng/ml (patient 1 and 2), 6 h after the application 78 and 139 ng/ml (patient 5 and 4) and 24 h after application 34 ng/ml (patient 3) were found. No arsenic could be detected by AAS at any time point in the CSF samples of the five patients (see Tables III; IV; V; VI).

Evidence for further metabolites

The molar melarsoprol concentrations determined by bioassay corresponded to the molar concentrations of arsenic of the samples. However, the HPLC concentrations of melarsen oxide and melarsoprol were not equal to the sum of the concentrations determined by bioassay. In samples taken 24 h after the injection, no melarsoprol and melarsen oxide could be determined by HPLC, but activity and arsenic could still be detected.

In conclusion there is evidence that melarsoprol is metabolised into melarsen oxide, which rapidly undergoes further transformation into one or more active compounds still containing the arsenic moiety.

Characterisation of further metabolites

Several derivatives and fragments of melarsoprol were tested *in vitro* for their trypanocidal activity. It could be demonstrated that the activity is associated with the arsenical part of the compound: The trivalent arsenicals, melarsen oxide, melarsoprol and phenylarsen were highly active (Minimum Inhibitory Concentration MIC = 1 - 6.5 ng/ml). The pentavalent form melarsen and the inorganic arsenic oxide (MIC = 50 µg/ml and 111 ng/ml) were considerably less active. Melamine and dimercaprol (BAL, British Anti Lewisite), two non-arsenic chemical constituents of the drug melarsoprol, were inactive (see Table VII).

The discrepancy between the short half life of melarsen oxide and the long terminal half life of activity as determined by bioassay indicates that further active products of melarsoprol are formed. The *in vitro* testing of fragments and derivatives of melarsoprol suggests that an arsenic metabolite is formed.

However, no further metabolites could be detected either by HPLC-UV using 0, 5, 10, 15 and 30 ml acetonitrile up to 100 ml HPLC water in the supernatant of microsomal incubations or in the supernatant of precipitated samples of melarsoprol treated patients. Determination of the activity by *in vitro* assay of the chromatographic fractions of the supernatant of precipitated human samples did not reveal any activity. The results are supported by AAS determination of the arsenic concentration in precipitated human serum and CSF samples with several solvents as described. In all the samples only traces of arsenic were found in the supernatant, but most of the arsenic was detected in the protein pellet. Also the precipitation of a pooled liquor sample and analysis of the pellet for arsenic revealed a high concentration of 400 µg/kg of arsenic bound to proteins (Table VIII). These results indicate a strong binding of the metabolite to serum and also to CSF proteins. An irreversibly bound drug is defined as one only liberated from the isolated protein by saponification[22].

To test further whether the drug is irreversibly bound to plasma proteins according to the definition, the protein pellet was boiled at 80°C for one hour in KOH. Still only 30% of the total arsenic could be detected in the supernatant. The result was further confirmed by ultrafiltration: A 10 fold higher activity in the filter residue was determined compared to the filtrate using filters exclusion sizes of 5 kD, 10 kD and 20 kD. Only filters with large pores (100 kD) yielded an equal activity of the residue and the filtrate, indicating that the metabolite is bound to macromolecules larger than 20 kD.

A confirming *in vitro* experiment gave further evidence for the irreversible nature of the binding of melarsen oxide to serum proteins: 900 µg/kg of the arsenic was recovered in the protein pellet from a melarsen oxide spiked serum sample. In presence of p-hydroxymercuribenzoate 1300 µg/kg of the arsenic could be detected in the protein pellet, which rules out an exclusive As-S binding (Table VIII).

In vivo activity studies of melarsen oxide

In vivo acute rodent model infections

100% cure rates were achieved by administering the drug intraperitoneally (2.2 mg/kg) and intravenously (range 0.1 – 1 mg/kg) (Table VII).

In vivo relapse rodent model infections

Three mice receiving one dose of 40 mg/kg berenil, a drug which is not capable of passing the blood brain barrier died on day 13, 52, and 55 after treatment. Of six mice treated with 5 mg/kg of melarsen oxide for seven subsequent days 5 survived for 180 days; while one died on day 83.

Discussion

Drug metabolites may contribute or be solely responsible for drug action or adverse effects [14]. Occasionally their pharmacological properties are superior to those of the parent compound [23]. Thus, the metabolic pathway must mandatorily be sufficiently documented for each new drug. Surprisingly, the pharmacology of the first choice drug for treatment of 2nd stage sleeping sickness, melarsoprol is still poorly elucidated half a century after its development. Adverse effects with an essentially unexplained background hamper the application of the drug [2]. Therefore, we have attempted to study the metabolism of this organoarsenical drug.

The active drug dominating in the body is melarsen oxide and not melarsoprol, which has a half life of less than 30 minutes in plasma. We could demonstrate that melarsen oxide is the first metabolite, confirming a recently published hypothesis [13]. Melarsen oxide is rapidly formed in the body and maximum levels (C_{max}) are reached after 15 minutes. It then disappears fast from the plasma with an apparent half-life of 3.8 hours. Previous experiments showed that melarsen oxide is formed from melarsoprol by hydrolysis [13]. However, the breakdown of melarsoprol measured *in vitro* after incubation with serum at room temperature was found to be a slow process with a half-life of 3 days [17]. Hence, it cannot be excluded that melarsen oxide may also be formed to some extent by enzymatic reactions. Since melarsoprol is the BAL derivative of melarsen oxide, another possible explanation for the discrepancy might be the known instability of British Anti Lewisite (dimercaprol, BAL) complexes *in vivo* [24].

Melarsoprol's first metabolite, melarsen oxide was synthesized by E. Friedheim in 1939 and in use for several years [25]. However, after the discovery of BAL the drug was abandoned in favor of melarsoprol.

The very short half-life of melarsen oxide is not solely due to a fast elimination. It was demonstrated that one of the metabolic products formed is bound to plasma proteins of a size of more than 20 kD. Both proteins which are commonly responsible for drug binding, albumin (66 kD) and α1-acid-glycoprotein (44 kD), fall into this category. The drug might also become bound to antibodies, tissue proteins or red blood cells. Due to the irreversible nature of the binding the protein-drug product cannot longer be determined by HPLC, but activity remains detectable by bioassay. The present data do not allow to conclude whether melarsen oxide alone, or also melarsoprol or further unrecognised metabolic products are bound to proteins. The mechanism of the covalent binding and the reactive groups of the proteins are unknown. NH_2 groups of the proteins could serve as reactive nucleophiles for the arsen oxide of melarsen oxide. Hence the formation of a Schiff base and following spontaneous Amadori rearrangements may be proposed [3].

The bound active compound acts as a reservoir that is depleted very slowly. This is in line with the recently published serum and CSF multiple-dose kinetics. A long half life in serum ($t_{1/2}$ = 35 hours) and a very slow elimination from the CSF ($t_{1/2}$= ca. 120 hours) were calculated, and a delayed increase of the CSF levels was proposed[11, 12]. In contrast, the drug can be detected in the CSF already after 1 hour by both, the bioassay and HPLC.

Melarsoprol is only soluble in propylene glycol, an irritating solvent. Administration is very painful and thrombophlebitis at the injection side is a common feature. Little attention has been paid in the past years to improve the way of administering the drug, though earlier small scale studies indicated even oral activity [26]. Melarsen oxide is more polar, a 36 mg/ml stock solution in propylene glycol could be diluted 1:2 in water compared to the necessity of a 1:500 dilution of melarsoprol.

The use of melarsen oxide as a trypanocidal compound was abandoned due to its toxicity [4]. However, other reports state equivalence of toxicity when melarsen oxide and melarsoprol were compared [27]. It could be demonstrated that dimercaprol (BAL) does not contribute to the trypanocidal activity. In contrast, its contribution to drug toxicity cannot be excluded. BAL is a toxic compound with a LD_{50} of 1.48 mmol/kg if applied intraperitoneally to rats [28]. Further on BAL complexes of trypanocidal drugs may even be more toxic than the arsenical drug itself. For example, the complex of dimercaprol and oxophenarsine has a substantially higher toxicity compared to oxophenarsine[29]. To confirm the activity we tested melarsen oxide in a relapse infection mouse model. Mice were treated for 7 days with 5 mg/kg melarsen oxide i.v. The results indicate that melarsen oxide passes the blood brain barrier in sufficient concentration.

Adverse effects are common in melarsoprol treatment and the underlying reasons may be manifold. Covalent binding of drugs or drug metabolites have been reported to be cause of severe adverse effects due to immune reaction of type I to IV, as the altered macromolecules may turn into an immunogen. The extent of such reactions is influenced by genetic and host factors and the disposition of the conjugate[30]. The interaction of penicillines with the immune system is a classical example of such a hapten formation: The carbonyl moiety of the opened β- lactam ring forms an amide linkage to the amino groups of proteins. The resulting penicilloyl- protein conjugate is the trigger for anaphylaxis [31].

The trigger for the encephalopathic syndromes is still unknown. Generally, an involvement of the immune system is proposed today, but the underlying causes are still controversial. Hunter and colleagues suggested that insufficient drug levels leading to complete elimination of trypanosomes from the bloodstream but not the CSF, may provoke a violent inflammatory response of activated astrocytes after restoration of the immune competence [8]. Other researchers proposed that high initial doses result in

massive killing of trypanosomes leading to the release of immuno-complexes attracting subsequently an array of antibodies [32]. On the other hand it was suggested that the immune response was directed against the arsenical or its derivatives bound to parasite proteins, because the reactions were observed in both stages of disease unrelated to dose and schedule [7]. Considering the diversity of hypothesis, at least a part of the reactions may be due to a immune reaction against the protein bound metabolite.

It is clear that all organoarsenic compounds are far from optimal. However, there will not be any satisfactory alternatives available for clinical use in sleeping sickness therapy for a long time to come. In this light the elucidation of the cause of severe adverse reactions is of great importance and the presented results may build a basis for a more rational approach. The elimination of the severe adverse effects may not be possible, but it must be a primary goal to provide appropriate information on adequate responses to these life threatening situations. In addition, the findings on melarsen oxide indicate that an appropriate pre-clinical evaluation of this compound versus melarsoprol might be of interest.

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References

1. Molyneux DH, Pentreath V, Doua F. African trypanosomiasis in man. In: G.C. Cook, editor. Manson's Tropical Diseases London: W.B. Saunders Company Ltd.; 1996. p. 1171-96.

2. WHO. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser 1998;881:I-VI,1-114.

3. Smith DH, Pepin J, Stich AH. Human African trypanosomiasis: an emerging public health crisis. Br Med Bull 1998;54:341-55.

4. Friedheim EAH. Mel B in the treatment of human trypanosomiasis. Am J Trop Med 1949;29:173-80.

5. Pepin J, Milord F, Khonde A, Niyonsenga T, Loko L, Mpia B. Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy Trans R Soc Trop Med Hyg 1994;88:447-52.

6. Cook GC. Adverse effects of chemotherapeutic agents used in tropical medcine. Drug Saf 1995;13:31-45.

7. Haller L, Adams H, Merouze F, Dago A. Clinical and pathological aspects of human African trypanosomiasis (*T. b. gambiense*) with particular reference to reactive arsenical encephalopathy. Am J Trop Med Hyg 1986;35:94-9.

8. Hunter CA, Jennings FW, Adams JH, Murray M, Kennedy PGE. Subcurative chemotherapy and fatal post-treatment reactive encephalopathies in African trypanosomiasis. Lancet 1992;339:956-58.

9. Nightingale S. Drug for sleeping sickness approved JAMA. (From the Food and Drug Administration) 1991;265:1229.

10. Cristeau B, Placidi M, Legait JP. Etude de l'excretion de l'arsenic chez le trypanosomé traité au melarsoprol. Méd Trop 1975;35:389-401.

11. Burri C, Baltz T, Giroud C, Doua F, Welker HA, Brun R. Pharmacokinetic properties of the trypanocidal drug melarsoprol Chemotherapy 1993;39:225-34.

12. Burri C, Pharmacological aspects of the trypanocidal drug melarsoprol, PhD thesis, Swiss Tropical Institute. 1994, University of Basel: Basel, Switzerland.

13. Bronner U *et al.* Discrepancy in plasma melarsoprol concentrations between HPLC and bioassay methods in patients with *T. gambiense* sleeping sickness indicates that melarsoprol is metabolized. Trop Med Int Health 1998;3:913-7.

14. Rowland M, Tozer TN. Clinical pharmacokinetics. 2nd ed. Philadelphia: Lea & Febiger; 1989. p. 546.

15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265- 75.

16. Räz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense and T.b. gambiense*) *in vitro.* Acta Trop 1997;68:139-47.

17. Ericsson O, Schweda E, Bronner U, Rombo L, Friden M, Gustafsson LL. Determination of melarsoprol in biological fluids by high-performance liquid chromatography and characterisation of two stereoisomers by nuclear magnetic resoncance spectroscopy. J Chromatogr B 1997;690:243-51.

18. Baltz T, Baltz D, Giroud C, Crockett J. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense.* EMBO J 1985;4:1273-77.

19. Huber W, Koella JC. A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. Acta Trop 1993;55:257-61.

20. Onyango JD, Burri C, Brun R. An automated biological assay to determine levels of the trypanocidal drug melarsoprol in biological fluids submitted to Acta Trop 1999.

21. Jennings FW, Gray GD. Relapsed parasitaemia following chemotherapy of chronic *T. brucei* infections in mice and its relation to cerebral trypanosomes. Contrib Microbiol Immunol 1983;7:147-54.

22. Smith PC, McDonagh AF, Benet LZ. Irreversible binding of zomepirac to plasma protein *in vitro* and *in vivo.* J Clin Invest 1986;77:934- 939.

23. Spilker B. Multinational pharmaceutical companies. Principles and practices. 1st ed. New York: Raven Press; 1994.

24. Mutschler E. Arzneimittelwirkungen. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH; 1991.

25. Friedheim EAH. Melarsen oxide in the treatment of human African trypanosomiasis. Ann Trop Med 1948;42:357-63.

26. Onyango RJ, Ogada T, Mbwabi D. The use of oral Mel B in the treatment of *T. rhodesiense* sleeping sickness. EATRO Report 1968;82-3.

27. Jonchère H, Gomer J, Reynaud R. Contribution a l'étude de produits a radical melaminyl dans le traitement de la trypanosomiase humaine. Bull Soc Path Exot 1953;46:386-96.

28. Mückter H, Liebl B, Reichl F-X, Hunder G, Walther U, Fichtl B. Are we ready to replace dimercaprol (BAL) as an arsenic antidote? Human & Experimental Toxicology 1997;16:460-65.

29. Weatherhall M. The antidotal activity of some dithiols and acetydithiols in mice poisened with oxophenarsine. J Pharmacol 1949;1:576-92.

30. Park BK, Kitteringham NR. Drug-protein conjugation and its immunological consequences. Drug Metabol Rev 1990;22:87-144.

31. Hess DA, Rieder MJ. The role of reactive drug metabolites in immune-mediated adverse drug reactions. Ann Pharmacother 1997;31:1378-86.

32. Pepin J, Milord F. African trypanosomiasis and drug-induced encephalopathy; risk factors and pathogenesis Trans R Soc Trop Med Hyg 1991;85:222-4.

Figure 1: Serum drug levels determined by bioassay, HPLC and AAS

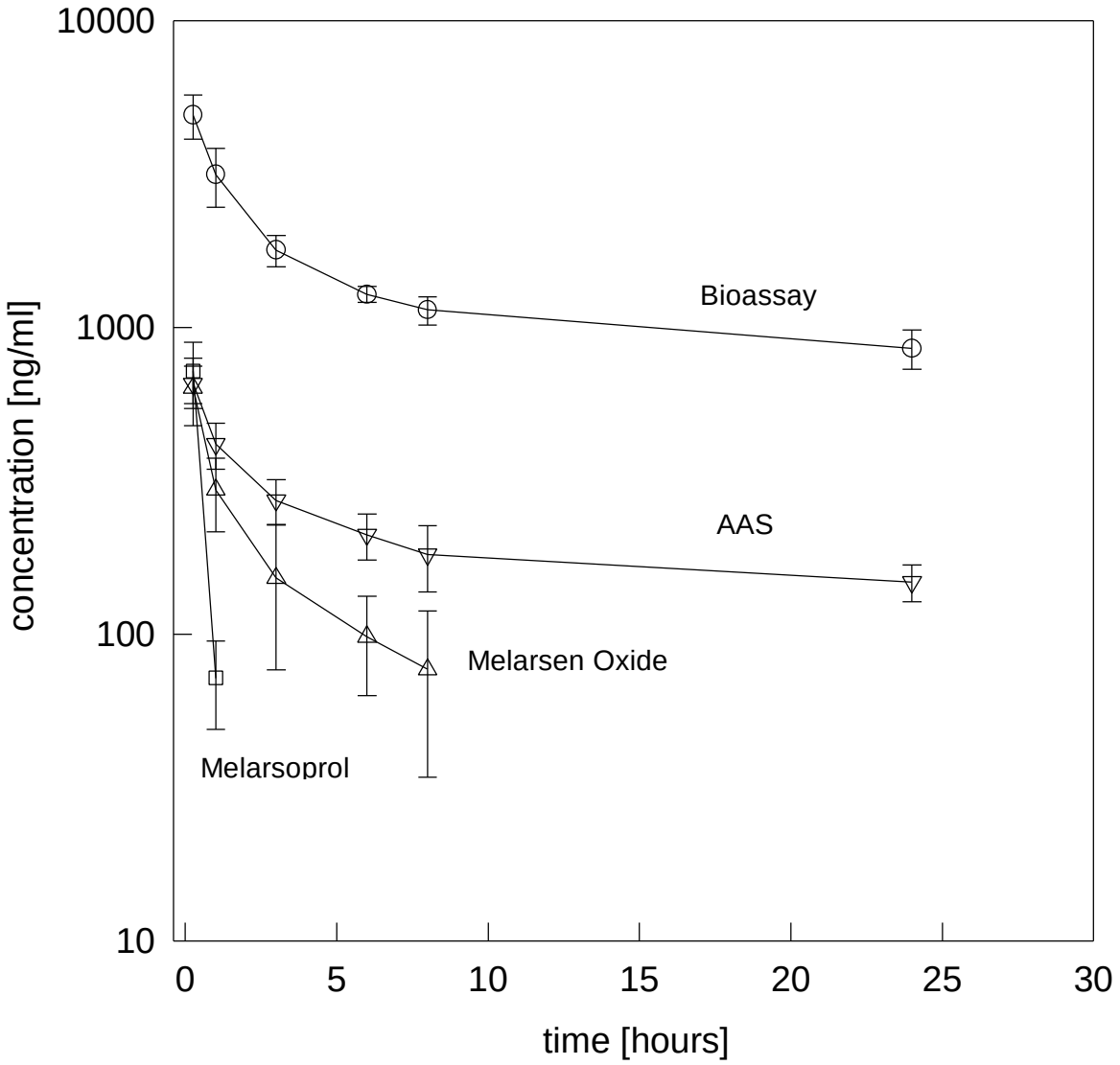


Table I: Baseline characteristics of patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex	male	male	female	female	female
Age (years)	36	20	20	21	33
Weight (kg)	48	52	37	37	38
Height (cm)	170	166	151	155	152
WBC (No/mm^3 in CSF)	400	160	88	64	64
Hb (mmol/l/dl)	5.9	7.7	5.5	5.2	6.0
Glucose (mmol/l)	4.0	1.2	3.2	3.5	1.9
Urea (mmol/l)	5.2	<3.5	<3.5	<3.5	4.0
Creatinine (µmol/l)	49.5	49.2	56.6	<45	58.3
Bilirubine (µmol/l)	9.9	8.6	8.7	<8.5	18.8
GPT (U/l)	7.7	5.8	4.5	7.2	22.7

Table II: Pharmacokinetic properties of melarsen oxide as determined in human patients

Pharmacokinetic properties	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**	**(Patient 5)**	**Average**	**SD**
Clearance C_L [ml/min*kg]	17.6	20.7	22.6	25.4	(5.3)	21.5	3.3
Area under the curve AUC [ng/ml*h*kg]	2090	1770	1630	1440	(6820)	1733	274
Volume Vss [l/kg]	4.7	5.5	6.1	7.7	(15.6)	6.0	1.2
Half life $t_{½}$ [hours]	4.7	3.2	3.5	4.0	(34.7)	3.9	0.7

Table III: Melarsen oxide concentrations in ng/ml determined by HPLC in serum and CSF

Time	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Average	SD
Serum							
Before application	<50	<50	<50	<50	<50	<50	n.a.[1]
After application:							
15 minutes	848	508	715	648	461	636	157
1 hour	426	302	288	225	238	296	80
3 hours	129	256		150	(75)[2]	178	68
6 hours		77	90	76	(150)[2]	81	8
8 hours	62	56		49	(140)[2]	56	7
24 hours	<50	<50	<50	<50	<50	<50	n.a.[1]
CSF							
Various timepoints	<50	<50	<50	<50	<50	<50	n.a.[1]

[1]not applicable; Empty cells represent missing samples

[2]data not used for calculation of pharmacokinetic parameters due to assaying difficulties

Table IV: Arsenic concentrations in ng/ml determined by AAS in serum and CSF

Time	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Average	SD
Serum							
Before application	<50	<50	<50	<50	<50	<50	n.a.[1]
After application:							
15 minutes	691	770	676	527	619	657	90
1 hour	351	530	378	381	443	417	72
3 hours	228	282	241	346	271	274	46
6 hours		176	209	261	198	211	36
8 hours	139	163	184	256	167	182	44
24 hours	124	147	165	171	132	147	20
CSF							
Various timepoints	<50	<50	<50	<50	<50	<50	n.a.[1]

[1]not applicable; Empty cells represent missing samples

Table V: Melarsoprol concentrations in ng/ml determined by bioassay in serum and CSF

Time	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Average	SD
Serum							
Before application	8	7	19	12	17	12	n.a.[1]
After application:							
15 minutes	4550	4675	6300	4200	4900	4925	809
1 hour	2930	4090	2900	2290	3550	3152	688
3 hours	1478	1920	1900	1241	1860	1789	209
6 hours		1300	1200		1350	1283	76
8 hours	998	1100	1100	1320	1185	1140	120
24 hours	796	1070	747	816	846	855	125
CSF							
Various timepoints	28 (1 hour)	32 (1 hour)	34 (24 hours)	139 (6 hours)	78 (6 hours)	n.a.[1]	n.a.[1]

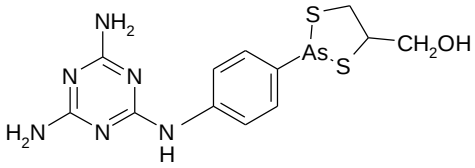
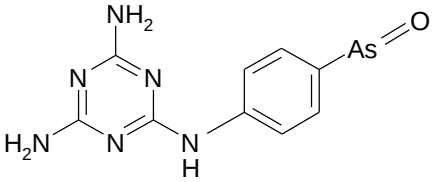
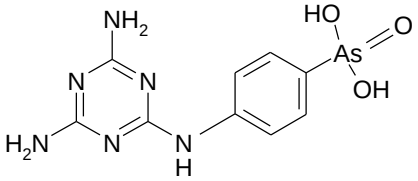
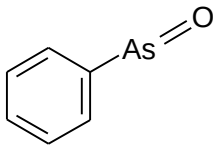
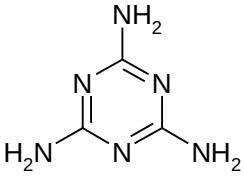
[1]not applicable; Empty cells represent missing samples

Table VI: Melarsoprol concentrations in ng/ml determined by HPLC in serum and CSF

Time	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Average	SD
Serum							
Before application	0	0	0	0	0	0	n.a.[1]
15 minutes	602	1026	668	601	706	720	176
1 hour	71	103	75	38	72	72	23
3 hours	0	0	0	0	0	0	n.a.
6 hours		0	0		0	0	n.a.
8 hours	0	0	0	0	0	0	n.a.
24 hours	0	0	0	0	0	0	n.a.
CSF							
Various timepoints	<50	<50	<50	<50	<50	n.a.[1]	n.a.[1]

[1]not applicable; Empty cells represent missing samples

Table VII: In vitro activity of melarsoprol and related compounds

Compound tested	Formula	MIC[1]
Melarsoprol		6.5 ng/ml
Melarsen oxide		6.5 ng/ml
Melarsen		50 µg/ml
Phenylarsen		1 ng/ml
Arsenic oxide	As_2O_3	111 ng/ml
Melamine		>100 µg/ml
Dimercaprol	$C_3H_5OH(SH)_2$	11 µg/ml

[1]Minimal Inhibitory Concentration

Table VIII: Arsenic content of precipitated samples

Solvents used for precipitation of serum samples	**Total melarsoprol concentration (bioassay) in µg/L**	**Arsenic concentration (AAS) of supernatant in µg/L**
0.05 M zinc sulphate in 50% water/ acetonitrile	765	8
7 M NaOH	380	54
TCA	531	10
DMSO	380	12
Proteinase K	540	4
Boiling 1 hour with 1M KOH	1440	60
β-mercaptoethanol	500	2

Table IX: *In vivo* activity of melarsen oxide in an acute infection model

Dose of melarsen oxide (mg/kg/day)	No. mice cured/ No. mice total	% mice cured
Control	0/5	0
2.2 mg/kg i.p	5/5	100
Control	0/5	0
0.1 mg/kg i.v.	5/5	100
0.5 mg/kg i.v.	5/5	100
1 mg/kg i.v.	5/5	100

Pharmacokinetic investigations on patients refractory to melarsoprol treatment from Northern Angola

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Abstract

Melarsoprol, an organo-arsenical, drug has been the drug of choice for late stage trypanosomiasis since 50 years. Due to the lack of alternatives any abatement of this medication will have a dramatic negative impact on the perspectives for the treatment of the infected. A high number of patients refractory to melarsoprol treatment was recently reported from Northern Uganda and Northern Angola. A study was carried out in Northern Angola to investigate whether interpatient pharmacokinetic differences influence the outcome of melarsoprol treatment. Drug levels were determined by a biological assay in serum and cerebrospinal fluid of a total of 22 patients. 9 patients could be successfully treated, 8 were refractory and of 5 the outcome was unclear or no adequate follow up information was available. No differences in the pharmacokinetic parameters (maximum serum concentration C_{max}, half-life $t_{1/2}$, total clearance C_L and the volume of distribution V_{ss}) could be detected between the groups. The serum and CSF concentrations for all patients were in the expected range. This result indicates that other underlying factors are responsible for treatment failures.

Introduction

The estimated number of people infected with *Trypanosoma brucei gambiense* (sleeping sickness) dramatically increased during the last decade. The countries currently most affected are the Democratic Republic of Congo (DRC) and Angola (Smith *et al*. 1998). Treatment of trypanosomiasis is still mainly based on melarsoprol and any abatement of the value of this drug will have a dramatic negative impact on the perspectives for the treatment of the infected. Until today treatment failures were a known feature, but remained at rather low and constant levels of 1 - 10% (Pepin & Milord 1994; WHO 1986) during the last fifty years. However, this situation has radically changed after recent reports of much higher rates of treatment refractory patients from Northern Uganda and Northern Angola. The Ugandan National Sleeping Sickness Control Program (NSSCP) had started a control program in collaboration with Médecins sans Frontières (MSF) in the Arua District and reported a rate of treatment failures of 26.9% during an observation period of 24 months (Legros *et al*. 1999b). The Angolan National Institute for Combat and Control of Trypanosomiasis, ICCT reported 25% patients refractory to treatment from a center in Mbanza-Congo, Zaire Province run by Angotrip, Caritas Angola (Angotrip Caritas Angola, unpublished data). Treatment failures were defined as trypanosomes detectable in any sample of a patient within one month after treatment, or a complete lack of clinical response during the course of treatment, followed by rapid deterioration of the patients and death (unpublished data).

Treatment failures may have different causes: Possible reasons are the emergence of parasites with divergent biological features permitting either to advance to deeper compartments or to counteract the activity of drugs, pharmacological differences of certain individuals or populations leading to insufficient drug levels in critical body compartments, and reinfections. The approaches and strategies to bring this menacing situation under control are obviously different for either reason and thus it is of importance to rapidly elucidate which event underlies this novel situation.

Generally, the effects of a drug in terms of activity or toxicity may show considerable interpatient variation (Rowland & Tozer 1989), either resulting from different concentrations at the site of action (kinetic differences) or from different responses to a given drug concentration (dynamic differences). Differences in drug metabolism with a pharmacogenetic background are common (Rowland & Tozer 1989), and may be increased by external influences, like food or concomitant drug application.

The aim of the presented study was to investigate the influence of pharmacokinetic differences on the outcome of melarsoprol treatment of late stage trypanosomiasis. We have also conducted ancillary experiments on isolated trypanosomes from the patients enrolled in the study to further elucidate the background of the treatment failures.

Patients and methods

Study design

The study was carried out at the trypanosomiasis treatment center of M'banza Congo, Zaire Province, Angola, which is under the responsibility of the ICCT and maintained by Angotrip, Caritas Angola.

The inclusion criteria of the study were a confirmed second stage *T. b. gambiense* infection, which was either confirmed serological or parasitological (trypanosomes detected in the cerebrospinal fluid (CSF) and/or >5 white blood cells (WBC) /mm^3 detected in the CSF by microscopical investigation) and an age ≥14 years. Patients, or their responsible guardians in severe cases, were informed about the trial and asked for consent.

Melarsoprol treatment was performed according to the schedule of the ICCT (see Figure 1). All patients were pre-treated with chloroquine (1500 mg base) and mebendazole (100mg/bid/3d). Prednisolone was given at 1 mg/kg bw, 0.5 mg/kg, 0.25 mg/kg during the first, second, and third series of standard treatment, respectively. One tablet of a folic acid-iron combination was given daily.

For pharmacokinetic analysis the patients were categorized into three groups: (i) "treatment successful" i.e. no parasites detectable by microscopy in blood, lymph and/or CSF samples in any sample collected at treatment control performed within 48 hours after the last application of melarsoprol, and/or the first follow up examination attempted thirty days thereafter; (ii) "treatment failure" i.e. patient died during treatment with no clinical improvement, parasite positive in any sample examined at treatment control or upon the first follow up examination; (iii) "outcome unknown" i.e. patients without retrievable follow up information or unclear results (e.g. trypanosome positive with a very high WBC count, death for unconfirmed reasons within the first follow up period).

No regular follow up of the patients for 24 months according to the guidelines of the ICCT was possible due to the deteriorating political situation in the study area, which escalated in the evacuation and partial destruction of the center nine months after the study period and made access to the region impossible.

The study protocol was approved by the Ethics Committee of the University Hospital in Basel, Switzerland, and the Ethics Committee of the Ministry of Health in Luanda, Angola.

Patient population

Overall, 22 patients (13 female and 9 male) under treatment for late stage sleeping sickness were enrolled and subjected to sampling. 11 patients had been treated in the center before (19 – 45 days, median 43 days, after termination of last treatment), and 11 were cases reporting for the first time. Both groups were treated identically (see Figure 1). The age, body mass index (weight/$height^2$) and WBC count were determined at hospital admission.

Parasitological assessment

Patients reporting to the center were initially screened using the CATT-test (Magnus *et al.* 1978; WHO 1998). Positive cases or suspect patients with negative CATT results underwent lymph node puncture. The aspirate was microscopically checked for trypanosomes. If the result was negative, capillary blood was examined. Lumbar puncture was carried out for all positive cases detected by any method. The disease stage was determined by microscopical examination of the CSF for trypanosomes, and by counting of WBC.

Sampling and determination of drug levels

One full blood sample for the isolation of trypanosomes and KIVI kit was taken before treatment.

A total of 6 serum samples for drug level determination were collected from each patient: Before, 0.5, 6, 24, 48 and 72 hours after the last melarsoprol application of one of the series of treatment.

Samples were taken during the first treatment course from 3 patients, during the second treatment course from 8 patients, and during the third treatment course from 10 patients. One CSF sample for parasitological assessment and drug level determination was taken by spinal tap 24 or 48 hours after the last injection of melarsoprol, coinciding with the respective serum sample. The CSF collected upon diagnosis was used as blank control sample if available.

Serum samples were prepared from full blood and left for coagulation at ambient temperature for 30 minutes, centrifuged with a hand centrifuge (Hettich, Tuttlingen, Germany) for 10 minutes, frozen in a dry shipping liquid nitrogen container (Harsco CP 65, Taylor-Wharton, Camp Hill, PA, USA), transported to Luanda, transferred into an other dry shipping liquid nitrogen container (Harsco CP300, Camp Hill, PA, USA) and transferred to the Swiss Tropical Institute in Basel, Switzerland for analysis. CSF treatment samples were frozen in liquid nitrogen immediately after collection and shipped accordingly.

The drug levels in the samples collected were determined using a bioassay (Onyango *et al.* 2000).

Pharmacokinetic analysis

For categorization of the patients see study design. The data of one patient (patient 1, group “treatment failure”) were not used for analysis because of severe difficulties with preparation of his serum samples (only marginal serum amounts retrievable).

Samples of the three treatment courses were treated equally, as the levels and decay melarsoprol concentrations of each therapy course are comparable (Burri *et al.* 1993).

For calculation of the pharmacokinetic parameters a two compartment model was fitted to the individual concentration vs. time data of the patients with equation (1) using the Topfit software (Heinzel, 1993).

$$C_p = A * e^{-t} + B * e^{-t} \quad (1)$$

The differences among the three treatment groups were tested by ANOVA.

Genetic analysis

Trypanosome karyotyping of two strains isolated from refractory patients was done following the method of Van der Ploeg (Van der Ploeg *et al.* 1984), and described in detail by Degen (Degen *et al.* 1995). The method used was appropriate to separate chromosomes between 225 and 2200 kb. *T.b. gambiense* strains from Northwest Uganda and the Democratic Republic of Congo were used as a reference.

Results

Of the 22 patients enrolled 9 (41%) were considered successfully treated, remaining parasitologically negative at the first follow up examination planned after 30 and performed within 22 - 90 days (median 42 days) after the last application of melarsoprol. 5/9 (56%) of the successfully treated patients had a history of one previous unsuccessful melarsoprol treatment (median 43 days before the second treatment).

8 patients (36%) were considered treatment failures according to the definitions (see study design). 2 patients died during the ongoing treatment without any clinical improvement. 2 patients were trypanosome positive in blood and CSF at control examination within 48 hours after the last application of melarsoprol. 4 patients were found positive upon the first follow up examination performed 22 – 38 days (median 34 days). 4/8 (50%) patients of this group were previously treated once with melarsoprol without success. The data of one patient (patient 1, group “treatment failure”) were not used for analysis because of difficulties with serum sample preparation (see methods).

5 patients had an unknown or unclear treatment outcome. Of 2 of those no information was retrievable, two patients died within 2 months after treatment without any further parasitological examination, and one was trypanosome negative but had a WBC count of 367 mm^3 44 days after treatment. 2/5 (40%) patients were previously treated once with melarsoprol without success (overview see Tables 3, 4).

The continuous variables at baseline among the three groups were within a similar range (weight: $F_{2,18}$= 0.34 (p= 0.71); body mass index: $F_{2,13}$= 2.38 (p= 0.13); WBC: $F_{2,14}$= 0.93 (p= 0.42) (Table 1).

Results of the pharmacokinetic analysis

Maximum drug serum levels (determined 0.5 hours after the last application of melarsoprol ranged from 3635 ng/ml to 10838 ng/ml with an overall mean of 6209 ng/ml. After 24 hours drug concentrations between 874 ng/ml and 2190 (mean 1300 ng/ml) were determined. Half an hour after drug administration serum levels were considerably higher in the “treatment failure” and “unknown outcome” groups. At all subsequent timepoint no differences were found between any of the groups (see Figure 2).

The average drug concentration in the CSF 24 hours after melarsoprol application were 38.9 ng/ml in the “successful treatment” group (n=3) and 70.5 ng/ml for treatment refractory patients (n=3). A sample from one patient of the successfully treated group and the treatment failure group were collected after 48 hours (19.3 ng/ml collected after the second treatment series; 60.3 ng/ml after the third series) (Figure 3).

No differences for any of the calculated pharmacokinetic parameters was found between the three groups by ANOVA analysis: Clearance (C_L): $F_{2,18}$= 0.61 (p= 0.55); AUC: $F_{2,18}$= 0.54

(p= 0.59), volume of distribution (V_{SS}): $F_{2,18}$= 1.2 (p= 0.32); half life ($t_{1/2}$): $F_{2,18}$= 0.13; (p= 0.88). The detailed pharmacokinetic parameters are given in Table 2.

Results of the genetic analysis

The analysis of the intermediate-large sized chromosomes revealed for both isolates an identical intermediate pattern, which was similar to the reference strains (both isolates expressed one additional band). Also the results of the large size chromosome analysis showed a similarity of the isolates to the gambiense reference strains, only the chromosomes larger than 2.2 Mb were distinguishable from the reference strains. These results confirm the *T.b. gambiense* nature of the isolated trypanosomes.

Discussion

Recent activities in Northern Uganda and Angola revealed high frequencies of treatment failures with melarsoprol of 26.9% (Legros *et al*. 1999a; Legros *et al*. 1999b) and 25%, respectively (personal communication Dr. Mendoza Casillas, Caritas Angola). Interestingly, a single earlier report indicates a rate of up to 40% of treatment failures in the Lower-Zaire (Democratic Republic of Congo) (Ruppol & Burke 1977), including patients who remain parasite positive in the blood during the course of treatment. This report was reiterated by Pepin (Pepin & Milord 1994), who indicated the close proximity of the Lower-Zaire and the Northern Angola region. After 1975 there was a considerable migration in both directions due to the political situation in the area.

None of the enrolled patients were previously treated with melarsoprol more than once. The strategy of Caritas was to treat all refractory patients with DFMO. However, only very limited quantities were available at this point of time, which explains the attempt to retreat one of the studied patients with melarsoprol, after a first unsuccessful attempt. *T.b. gambiense* infected patients who are retreated with melarsoprol after failing to respond to treatment are less likely to be cured compared to those treated for the first time (Legros *et al*. 1999b; Pepin & Milord 1994). The therapeutic success in the investigated patient population is summarized in table 3, but our data set is too small to perform statistical significance tests for this finding.

Prior to study initiation the following sources of treatment failures were excluded: Inferior Arsobal® (melarsoprol) quality and inadequate performance of hospital staff. The quality of melarsoprol (Lot 705) was confirmed by HPLC analysis and the conduct of biological drug susceptibility assays. The handling and administration of the drugs were checked upon an auditing visit and found to be appropriate.

We could not find any evidence for differences between the pharmacokinetics of melarsoprol in patients successfully treated compared to those refractory to treatment. The average drug levels analyzed in all three groups are similar to those previously published:

The melarsoprol concentrations in plasma immediately after the last melarsoprol application of a treatment series (6273 ng/ml; SD 2108 ng/ml; n=19) were slightly higher but in the same range compared to those recently reported by Bronner (Bronner *et al*. 1998) (4045 ng/ml; SD 964 ng/ml; n=7). In samples taken 24 hours after the last melarsoprol application of a treatment series the drug concentrations were similar at 1342 ng/ml (SD 437 ng/ml; n=20) compared to 1686 ng/ml (SD 881 ng/ml; n=8) (Bronner *et al*. 1998). Also the CSF drug levels of 64 ng/ml (SD 38 ng/ml; n=9) 24 hours after the last drug application of a series were similar to those previously reported (42 ng/ml; SD 17 ng/ml; n=8) (Bronner *et al*. 1998). The calculated average half life ($t_{1/2}$) of 42 hours, and the average total clearance (C_L) of 1 ml/min*kg

are comparable to the respective values from preceding studies ($t_{1/2}$ 35 hours, C_L 1 ml/min*kg) obtained from late stage patients treated in the Ivory Coast (Burri *et al*. 1993).

The mechanisms leading to the increased rate of failures of melarsoprol treatment are presently under investigation.

Reinfections may account for a varying proportion of treatment failures, especially in areas with high transmission rates. However, the sharp increase of treatment failures and the observation of patients with trypanosomes detectable in body fluids within less than thirty days after treatment termination is not likely to be caused by this event. Due to the defined control period of only thirty days, the influence of reinfections on the outcome can almost be excluded.

Among several possible mechanisms of drug resistance in trypanosomes (Maser & Kaminsky 1997), a decrease in drug uptake is the most frequently reported. Cellular uptake of diamidines and melaminophenyl arsenicals is thought to occur via an adenosine transport system designated P2 (Carter & Fairlamb 1993). A gene, *TbAT1*, encoding a P2-type adenosine transporter has recently been cloned from *T. b. brucei* (Maser *et al*. 1999). Expression of *TbAT1* in yeast enabled adenosine uptake and conferred susceptibility to melaminophenyl arsenicals. A mutant, apparently non-functional variant of *TbAT1* has been found in drug-resistant laboratory and field strains of *T. brucei* spp. (Maser *et al*. 1999). The isolation from the enrolled patients of trypanosomes for drug susceptibility assessment was attempted by KIVI kit (Truc 1996) and by cryopreservation. However, of 15 KIVI samples and 22 collected by cryopreservation only two procyclic isolates could be cultivated from KIVI kits. Of the cryopreserved isolates none could be grown in rats. Transformation to bloodstream forms was unsuccessfully attempted by *in vitro* metacyclogenesis (M. Fasler, personal communication). The passage of the trypanosomes to tsetse flies was not attempted for the potential danger to laboratory staff. Procylic forms cannot be used for testing of drug susceptibility therefore no results are available. Due to persistent political unrest the M'banza Congo region remains completely inaccessible and additional collection of samples for further analysis was obstructed.

Alternative mechanisms of reduced drug susceptibility are discussed. A reduction in lipoic acid content has been observed in melarsen-resistant trypanosomes (Fairlamb *et al*. 1992), and selection for resistance to mycophenolic acid resulted in amplification of the inosine monophosphate dehydrogenase gene in *T. b. gambiense* (Wilson *et al*. 1994). The pathways involved in environmental sensing in trypanosomatids were recently reviewed (Parsons & Ruben 2000). It may be hypothesized that differences in the signal pathways may produce distinct affinities of such trypanosomes, permitting to advance to deeper compartments where only marginal drug levels can be reached.

No satisfactory clinical alternative to melarsoprol will be available in the near future. The occurrence of high treatment failure rates stresses the urgent need for research to develop new

drugs for human African trypanosomiasis. We provide evidence that pharmacokinetic differences among the patients can be excluded. This stresses the need for further studies of the causes of melarsoprol treatment failures.

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References:

Bronner U, Brun R, Doua F*, et al.* (1998) Discrepancy in plasma melarsoprol concentrations between HPLC and bioassay methods in patients with *T. gambiense* sleeping sickness indicates that melarsoprol is metabolized. *Tropical Medicine and International Health* **3**, 913-917.

Burri C, Baltz T, Giroud C*, et al.* (1993) Pharmacokinetic properties of the trypanocidal drug melarsoprol. *Chemotherapy* **39**, 225-234.

Carter NS and Fairlamb AH (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature* **361**, 173-176.

Degen R, Pospichal H, Enyaru J and Jenni L (1995) Sexual compatibility among *Trypanosoma brucei* isolates from an epidemic area in southeastern Uganda. *Parasitology Research* **81**, 253-257.

Fairlamb AH, Carter NS, Cunningham M and Smith K (1992) Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology* **53**, 213-222.

Legros D, Evans S, Maiso F, Enyaru JCK and Mbulamberi D (1999a) Risk factors for treatment failure after melarsoprol for *Trypanosoma brucei gambiense* trypanosomiasis in Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**, 439-442.

Legros D, Fournier C, Etchegorry MG, Maiso F and Szumilin E (1999b) Therapeutic failure of melarsoprol among patients treated for late stage of *T.b. gambiense* human African trypanosomiasis in Uganda. *Bulletin de la Société de Pathologie Exotique et ses Filiales* **92**, 171-172.

Magnus E, Vervoort T and Van Meirvenne N (1978) A card agglutination test with stained trypanosomes (CATT) for the serological diagnosis of *T.b. gambiense* trypanosomiasis. *Annales de la Société Belge de Médicine Tropicale* **58**, 169-176.

Maser P and Kaminsky R (1997) The mechanisms of drug resistance in *Trypanosoma brucei* spp. *Recent Research Developments in Antimicrobial Agents and Chemotherapy* **2**, 113-125.

Maser P, Sutterlin C, Kralli A and Kaminsky R (1999) A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **285**, 242-244.

Onyango JD, Burri C and Brun R (2000) An automated biological assay to determine levels of the trypanocidal drug melarsoprol in biological fluids. *Acta Tropica* **74**, 95-100.

Parsons M and Ruben L (2000) Pathways involved in environmental sensing in trypanosomatids. *Parasitology Today* **16**, 56-62.

Pepin J and Milord F (1994) The treatment of human African trypanosomiasis. *Advances in Parasitology* **33**, 1-47.

Rowland M and Tozer TN (1989) *Clinical pharmacokinetics*. Philadelphia: Lea & Febiger.

Ruppol JF and Burke J (1977) Follow-up des traitements contre la trypanosomiase expérimenté à Kimpangu (République du Zaire). *Annales de la Société Belge de Médicine Tropicale* **57**, 481-494.

Smith DH, Pepin J and Stich AH (1998) Human African trypanosomiasis: an emerging public health crisis. *British Medical Bulletin* **54**, 341-355.

Truc P (1996) A miniature kit for the *in vitro* isolation of *Trypanosoma brucei gambiense*: a preliminary field assessment on sleeping sickness patients in Cote d'Ivoire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 246-247.

Van der Ploeg LHT, Schwartz DC, Cantor CR and Borst P (1984) Antigenic variation in *Trypanosoma brucei* analyzed by electrophoretic separation of chomosome-sized DNA molecules. *Cell* **37**, 77-84.

WHO (1986) Epidemiology and control of African trypanosomiasis. *WHO Technical Report Series* **739**.

WHO (1998) Control and surveillance of African trypanosomiasis. *WHO Technical Report Series* **881**, 1-114.

Wilson K, Berens RL, Sifri CD and Ullman B (1994) Amplification of the inosinate dehydrogenase gene in *Trypanosoma brucei gambiense* due to an increase in chromosome copy number. *Journal of Biological Chemistry* **269**, 28979-28987.

Table 1: Comparison of continuous variables at hospital admission

	Group								
	Treatment successful			**Treatment failure**			**Outcome unknown**		
	N	Mean	c.v.	n	Mean	c.v.	n	mean	c.v.
Age (years)	9	39.0	46.8	7	34.3	32.4	5	27.6	45.4
Weight (kg)	9	51.1	10.1	7	49.1	10.3	5	48.8	16.1
Height (cm)	6	162.7	3.9	7	165.3	5.7	3	164.7	2.9
BMI (kg/m^2)	6	19.2	6.6	7	18.1	14.2	3	16.1	8.9
WBC	9	175[1]		3	78[1]		5	428[1]	
Total patients	9			7			5		

[1]Median

c.v. = coefficient of variation

mparison of pharmacokinetic parameters in serum

roup											
reatment successful					Treatment failure				Outcome unknown		
Mean	Min	Max	c.v.	n	Mean	Min	Max	c.v.	n	mean	Min
5303	3900	7235	23.21	7	7006	3635	10786	32.50	5	6544	3935
1267	986	1885	28.1	7	1344	874	2087	36.3	5	1296	1008
1.03	0.68	1.57	31.8	7	1.05	0.595	1.83	39.4	5	0.85	0.61
3.48	1.71	4.8	32.8	7	3.14	1.98	4.61	32.8	5	2.56	1.12
21’211	12’700	29’200	28.9	7	21’328	10’900	33’600	33.8	5	24’720	17’900
42.9	27.9	70.3	31.1	7	42.8	28.1	74.8	39.4	5	39.3	28.6

successful treatment (9), 1 ser 1, 4 ser 2, 4 ser 3

reatment failure (7) 1 ser 1, 1 ser 2, 5 ser 3;

unclear outcome (5) 1 ser 1), 3 ser 2, 1 ser 3

atient failing treatment were excluded due to sampling problems

ent of variation

Table 3: Categorization of patient based on treatment outcome and previous treatment

Actual treatment	**Previous treatment**		
	Yes	**No**	**Total**
Successful	5	4	9
Failure (within 30 days)	4[1]	4	8[1]
Not known	2	3	5
Total	11	11	22[1]

[1]The data of one additional patient were not used for evaluation due to problems at sample processing and consequently largely deviating results

atment outcome (previous and actual)

Treatment outcome	Interval since last treatment [days][1]	Follow up period [days][2]	Information available
failure)[3]	43	35	Trypanosomes in blood, CSF negative Retreatment with DFMO
ailure	n.a.[4]	died during treatment (parasitology unclear)	No clinical response to treatment
ailure	35	1[5]	Trypanosomes in blood, CSF positive Retreatment with DFMO
uccess	n.a.	60	Blood negative, CSF positive
uccess	n.a.	48	Blood negative, CSF positive
ailure	n.a.	1[5]	Trypanosomes in blood, CSF positive
uccess	45	90	Fair condition, no details
nknown	44	n.d.	none
uccess	42	55	Blood negative, CSF negative
ailure	45	died during treatment	No response; treatment switched to DFMO, no response, died under treatment
ailure	n.a.	38	Trypanosomes in lymph
ailure	n.a.	22	Remained critically ill under treatment

			Blood negative, CSF positive
			died 3 days after examination
ailure	40	33	Trypanosomes in lymph
			Blood negative, CSF negative
nknown	n.a.	44	Blood negative, CSF negative
			367 WBC in CSF
nknown	n.a.		Died for unknown reason max. 2 months after treatment (exact time-point unknown)
uccess	n.a.	30	Blood negative, CSF negative
nknown	19	n.d.	none
uccess	44	30	Blood negative, CSF negative
nknown	n.a.	28	Died for unknown reasons
			(CSF negative at discharge)
uccess	49	49 (360)[6]	Blood negative, CSF negative
uccess	40	n.d.	Oral information, patient in good condition
uccess	n.a.	70	Blood negative, CSF negative

rol application of previous treatment to hospital admission

rol application to first follow up examination

luded in pharmacokinetic evaluation because of sampling problems

rol treatment

1 24 hours after treatment termination

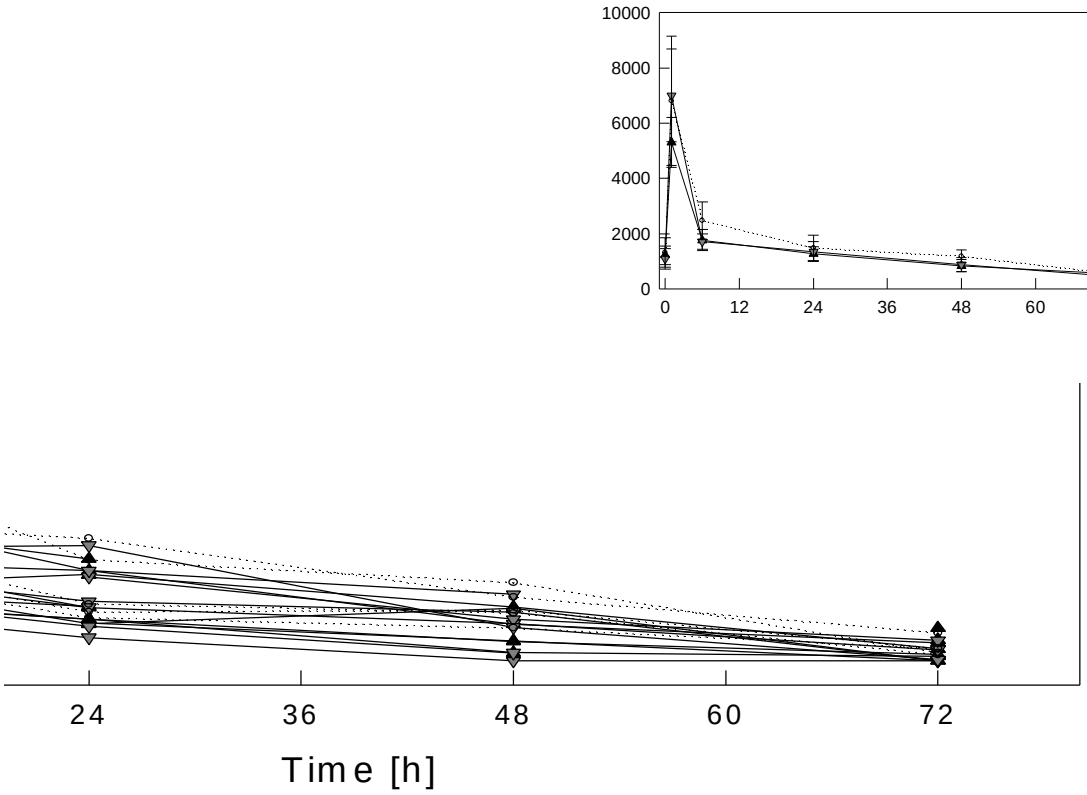
ollow up to Trypanosomiasis Reference Center, Viana Luanda

Figure 1: Schedule for late stage sleeping sickness treatment with melarsoprol (National sleeping sickness program Angola)

Day of drug application

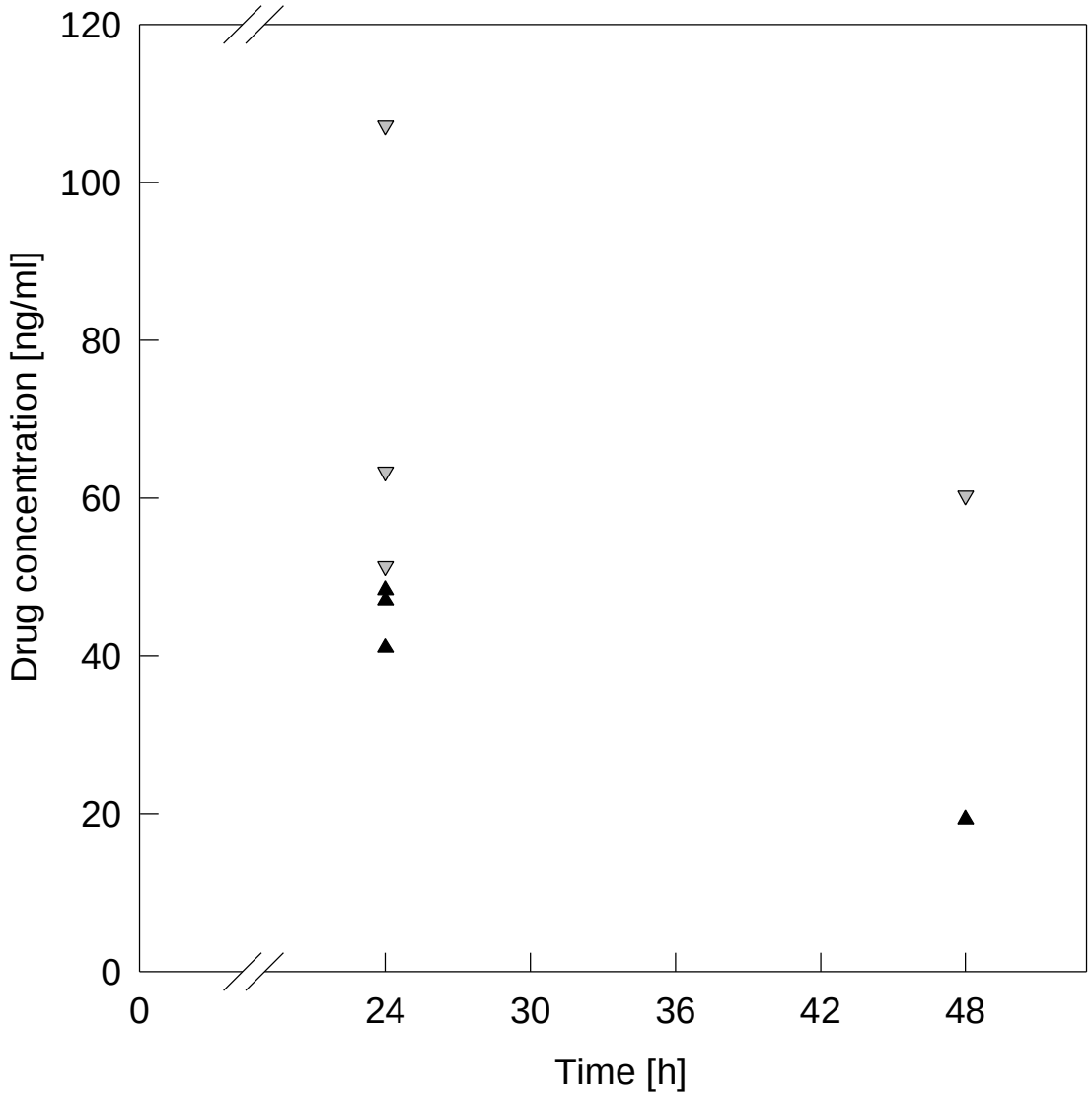
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
M^1	M^2	M^3	M^3								M^1	M^2	M^3	M^3								M^1	M^2	M^3	M^3

M^1= melarsoprol 1.2 mg/kg bw; M^2= 2.4 mg/kg bw; M^3= 3.6 mg/kg bw



erum drug levels determined by bioassay

Figure 3: Drug concentrations in the CSF



Evaluation of Quinolone Derivatives for Antitrypanosomal Activity

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Summary

160 fluoroquinolones and derivatives were tested for antitrypanosomal activity in a drug sensitivity assay followed by fluorometric evaluation. The most active quinolone compounds had IC_{50} values in the range from 100 ng/ml to 900 ng/ml, while several derivatives were not active at a concentration of 100 µg/ml. A structure activity relationship study was carried out: structural modification of the quinolones at position R1, R2, R3, and R8 did not influence trypanocidal activity. An exchange of the fluor at position 6 may contribute to an increase in activity but does not entirely control the activity. Pyrrolidine substituents at position R7 were in general more active than other substituents at this position. Tetracyclic quinolone derivatives were amongst the most active compounds with IC_{50} values in the range of 0.3-8.8 µg/ml.

The *in vitro* cytotoxicity on HT-29 cells was determined for active compounds with IC_{50} values below 1 µg/ml. In addition six drugs with an IC_{50} below 1 µg/ml and a selectivity index of more than 10 were chosen for *in vivo* experiments. Dose escalation experiments with a maximum dose of 100 mg/kg/bid were performed in a mouse model without central nervous system involvement. For unknown reasons the *in vitro* effect of the drugs could not be confirmed *in vivo*, but the class of compound remains of interest for their mode of action, the low toxicity, pharmacological properties and the availability of a large number of synthesized compounds.

Introduction

Human African trypanosomiasis is a fatal disease caused by the protozoan parasites T*rypanosoma brucei gambiense* in Central and West Africa, and *T.b. rhodesiense* in East Africa. About 55 million people in 36 countries are at risk of acquiring the disease (WHO, 1998) and the number of people infected is dramatically increasing (Smith *et al.*, 1998). There is no prospect of an antitrypanosomal vaccine, due to the ability of the parasite to readily change its surface antigens which results in an inadequate immunity response of the host (Wang, 1995). Therefore the clinical management of sleeping sickness relies entirely on chemotherapy. However, only a few drugs are available which all may cause severe adverse effects, and are generally expensive (WHO, 1998). Chemotherapy has made very little progress over many decades: the main drugs for the treatment of the first stage of disease are suramin and pentamidine were introduced in 1922 and 1941. The organo-arsenic drug melarsoprol (Arsobal®) (Friedheim, 1949) is still the drug of choice for treatment of the late stage, when the parasites have invaded the central nervous system. Only one drug, eflornithine (α-difluoromethylornithine) was brought to the market in the last decade. However, the compound, originally developed as an antitumor drug, is only active against *T.b. gambiense* and almost unaffordably expensive (WHO, 1998). Recent reports on treatment failures and drug resistant trypanosomes are source of new concerns (Pepin *et al.*, 1994, Van Nieuwenhove, 1998) In consequence new antitrypanosomal compounds are urgently needed.

Fluoroquinolones are a class of synthetic antibacterials, which offer a broad spectrum of activity, combined with excellent pharmacological and pharmacokinetic properties like high plasma levels, high clearance, oral and parenteral applicability, chemical stability and rare side effects. The penetration of the CNS has been demonstrated kinetically and clinically (Scheld, 1989). Early compounds of this class of drugs were active against Gram-negative bacteria, whereas newer ones have a broad spectrum affecting Gram-negative and -positive bacteria, including anaerobic pathogens (Wolfson and Hooper, 1989). Fluoroquinolones are specific inhibitors of topoisomerases, which are ubiquitous enzymes essential for cell survival. Topoisomerases mediate the mechanistic interactions like supercoiling, relaxation, knotting or catenating of DNA double helices (Maxwell and Gellert, 1986). Kinetoplastid protozoa have a unique and complex type of mitochondrial DNA, composed of thousands of topologically interlocked circular DNA molecules, creating a high demand for topoisomerase activity (Englund *et al.*, 1982). The required enzyme activity in combination with the rapid proliferation of trypanosomes makes the inhibition of topoisomerases an interesting potential drug target. *In vitro* activity against trypanosomes, *Plasmodium falciparum* and *Leishmania donovani* of quinolones has already been reported (Croft and Hogg, 1988, Raether *et al.*, 1989, Nenortas *et al.*, 1999).

We have determined the *in vitro* activity against human trypanosomes of 160 selected quinolones with chemical variations in order to evaluate structure-activity rela-

tionships and antiparasitic selectivity. Six compounds were furthermore evaluated *in vivo*.

Material and Methods

Drugs

Quinolones were kindly supplied by DuPont pharmaceuticals (DuPont 5a-5w, 15a-e, 9a-c, 16b, 16c, 17, 19, 22, 24-29); Kanebo Ltd. Pharmaceutical Division (Kanebo 8a-c, 8f-h); Johannes Gutenberg Universität Mainz, Fachbereich Chemie (UM 35, UM 36); University of Ljubljana, Department of Chemistry and Chemical Technology (Metal quinolones UL 1-5); University of Barcelona, Laboratory of Microbiology, Department of Sanitary Microbiology and Parasitology, Division of Health Sciences, Faculty of Pharmacy (E-4535, E-4534, E-4528, E-4527); Daiichi Pharmaceutical Co. Ltd. (DU 6859a); Hokuriku Seiyaku Co. Ltd. (HSR 903); Dong Wha Pharm Ind. Co. Ltd. (DW 116, DW 271); Pharmazeutisches Institut der Universität Bonn (H 1-H 10); Universita di Messina Istituto di Microbiologica Dipartimento di Scienze Farmaceutiche Universita Degli Studi Di Firenze (VG 6/1, MF 5137, MF 5168, MF 5101, MF 5142, MF 5124, WF 1); Laboratorios Dr. Esteve (E-4696, E-4629, E-4628, E-4591, E-4811, E-4555, E-4695, E-4630, E-4715, E-5091, E-4619, E-5112, E-4625, E-4626, E-4724, E-4685, E-4936, E-4868, E-4935, E-5100, E-4548, E-4487, E-4497, E-4654, E-4549, E-4550, E-4572, E-4627); Biotech Research Institute (LB 20304); Bayer AG (Gatifloxacin); Grünenthal GmbH (Moxifloxacin); Glaxo Wellcome, Verona (G 64070, G 67012, G 71258, G 59030, G 72800, G 71286); Universidad Nacional de Cordoba, Facultad de ciencias quimicas (Sulfanilyl Fluoroquinolones); Universidad Central de Venezuela (UV 1-17); Dipartimento di Scienze Farmaceutiche, Università di Trieste (V 1, V 2). Stock solutions of 10 mg/ml in 0.1 M NaOH or DMSO were prepared and stored at – 20°C.

In vitro testing of activity

The trypanosome clone STIB 900 a cloned derivative of *T.b. rhodesiense STIB 704,* isolated 1982 in Tanzania, was used for *in vitro* growth inhibition studies. Bloodstream forms were cultured in Minimum Essential Medium (MEM) with Earle's salts (powder, GIBCO/BRL No. 072-01100 P) supplemented with 25 mM HEPES, 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM nonessential amino acids (100x). The medium was further supplemented according to Baltz (Baltz *et al.*, 1985). The Alamar Blue sensitivity assay was carried out as previously described (Räz *et al.*, 1997): bloodstream forms of *T.b. rhodesiense* were inoculated into 96-well microtiter plates (Costar, USA). The trypanosomes were incubated with the serial drug dilutions for 70 hours at 37°C in 5% CO_2. Then 10 µl Alamar Blue were added and af-

ter two hours of incubation the fluorescence was determined at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

HT-29 cells (human adenocarcinoma cells) were cultivated in MEM supplemented with 10% heat inactivated foetal bovine serum and maintained at the same conditions as the trypanosomes. The highest drug concentration used for all substances was 100 µg/ml. The IC_{50} (concentration inhibiting the growth by 50%) values were calculated by linear interpolation (Huber and Koella, 1993). The cytotoxicity assay was performed according to the method of Pagé (Pagé *et al.*, 1993) with minor modifications. 5000 HT-29 cells/100 µl culture medium were incubated in the presence of the drugs in various concentrations. The assays were analysed as previously described.

In vivo testing of activity

Selection of compounds

Substances with an IC_{50} value for trypanosomes below 1 µg/ml and a selectivity index (defined as the ratio of the minimum inhibitory concentration of mammalian cells to that of trypanosomes) (Kaminsky *et al.*, 1996) of more than ten fold were selected for *in vivo* testing.

Acute infection model

Animals: female CD1 mice (20-25 g) were purchased from Charles River, Germany.

The animals were infected intraperitoneally with $2*10^5$ trypanosomes of STIB 704. Experimental treatment with the respective compounds was started 24 h after infection. The initial drug doses were calculated such that the plasma concentration would correspond to the IC_{50} levels as determined in the *in vitro* assays for the respective drugs. For calculation a volume of distribution of 3 l/kg was assumed. During the experiment the doses were increased to a maximum of 100 mg/kg. The drugs were administered intraperitoneally twice a day for two consequent days (see Table 2). Untreated control groups (n= 2-4) were included. Animals were checked for parasites twice weekly in tail vein blood. Animals surviving for more than 30 days were considered as cured.

Results

In vitro studies

160 quinolones derivatives were tested for activity against a clone of *T.b. rhodesiense* using serial dilutions with a maximum concentration of 100 µg/ml following the method described by Räz (Räz *et al.*, 1997). The IC_{50} values determined *in vitro* are given in Table 1 (see appendix). The metal quinolone UL 5 was the most potent compound with IC_{50} values of 100 ng/ml. DW 271, Kanebo 8g, Kanebo 8h, and the two sulfanyl fluoroquinolones $N(CH_3)_2SNx$ and OCH_3SNx were reasonably active, with IC_{50} concentrations in the range of 300 ng/ml up to 900 ng/ml. Some of the DuPont compounds, developed as antitumor drugs, were highly active but also showed high cytotoxicity on HT-29 cells.

In vivo studies

No parasitological cure of mice could be achieved with any of the compounds tested in dose escalation experiments up to100 mg/kg i.p. The detailed results are shown in Table 2. No signs of toxicity were observed during the experiments.

Discussion

In the past years the quinolones compounds progressed to the most important drugs in antibacterial therapy due to their broad spectrum against gram-positive and gram-negative bacteria and their minor adverse effects and toxicity. They possess many pharmacological advantages which have made them to excellent therapy for urinary, respiratory, gastrointestinal, skin or sexually transmitted diseases (Neu, 1990). Due to an increasing resistance problem new compounds are continuously developed, and an array of structural derivatives of the quinolone nucleus have been synthesized. Fluoroquinolones are known to inhibit topoisomerases, but GABA, amino acid, and adenosine receptor interactions have also been reported (Takayama *et al.*, 1995). For those reasons we have launched a project to investigate the curative potential of this class of drugs against sleeping sickness.

In vitro structure activity relationship

We tested 160 fluoroquinolones and their derivatives against cultured bloodstream forms of *T.b. rhodesiense*. Each structural modification of the quinolone core (see Figure 1) leads to characteristic *in vitro* activity and toxicity. By comparison of the trypanocidal *in vitro* activities structure relationships of the compounds tested were elaborated and are discussed.

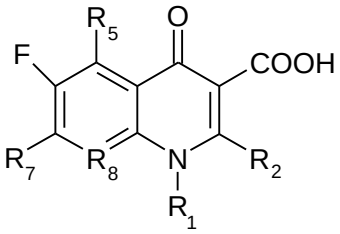

Figure 1

Position R1

Substitution at N_1 is not prerequisite for trypanocidal activity (e.g. various DuPont entities).

Position R2

Derivatization at this position is not directly associated to antitrypanosomal effect. Chemically different moieties like hydrogen (e.g. DW 271), phenyl (DuPont 5s) and styrylphenyl (e.g. DuPont 16b, 19, 24) exist in active drugs. However, modification with a melaminylgroup of the positions 1 and 2 yields generally moderately active compounds. The same groups are present in most drugs currently in use active

against human and animal trypanosomiasis (melarsoprol, pentamidine, berenil and samorin).

Position R5

This position is known to control bacterial potency and Gram positive activity, the optimal groups being NH_2 and OH followed by CH_3 (Domagala, 1994). However, we found no evidence for an impact on trypanosomal activity. Compounds with a Cl- or NH- or methyl-substitution were generally not trypanocidal. The activity of DW 271 can rather be explained by the substitution at position 7.

Position R6

Most marketed quinolones are F substituted at position 6 (fluoroquinolones). Replacement at this position of the F with Cl, Br or MeO yielded activity in the µM range in some instances (e.g. DuPont 5v, 9b, 9c or 24), but the effect was not consistent. These drugs were developed as anticancer drugs. Their mode of action is known to inhibit the tubuline function of cells leading to a high cytotoxicity against mammalian cells (Hamel *et al.*, 1996). The DuPont derivatives were therefore not followed *in vivo.* Drugs with modification at R6 only, yielded an increase of activity in the following order: anthra> methyl> AcO> F. In conclusion position 6 may contribute to, but does not exclusively control for activity.

Position R7

Pyrrolidine derivatives offer the highest therapeutic efficacy against Gram- positive bacteria, while the piperazines derivatives have better efficacy against Gram- negative strains (Domagala, 1994). Screening for trypanocidal activity yields higher activity for a pyrrolidine substituent (e.g. DW 271, LB 20304, HSR 903, DU 6859a,Moxifloxacin) over piperazin (e.g. Ciprofloxacin), azedinyl (e.g. E-4535) or NH substitution (e.g. DuPont 25). Further substitution of the pyrrolidine ring does not seem to lower the activity (e.g. DW 271). However, the substitute at positions 1 (large group) or 8 (electronegative) may completely abolish the activity of pyrrolidine derivatives. From the compounds screened it is not possible to deduct which of the positions has a larger influence (DU 6859a and HSR 903). The sulfaninyl-fluoroquinolones, see Table 1, were active though possessing a piperazine ring.

Phenyl- piperazinyl- butoxyl substitution at R7 yields inconsistent results, but the activity was generally low.

Position R8

In bacteria the optimal groups at this position for high *in vivo* and *in vitro* activity are N, CF, and CCl (Domagala, 1994). For trypanocidal activity substitution at R8 is not essential. However, it may modify the potency in conjunction with substitution at other positions (see R7).

Other structures

The thiazolopyrazines tested are quinolones with a unique tetracyclic structure and a modified morpholino group at position 7. Besides the quinolones modified at position 7 with a piperazinyl group, this set of compounds was most consistent in terms of *in vitro* activity. Particularly those drugs carrying a single side chain at the morpholino rest (Kanebo 8f – 8h) were active *in vitro* in the nanomolar range.

In vivo **activity**

The registered drugs ciprofloxacin and fleroxacin have a volume of distribution of 0.4 l/kg and 2.5 l/kg (Chukwuani *et al.*, 1998). For calculation of the initial doses of the compounds to be tested *in vivo* an average volume of distribution of 3 l/kg was assumed. The initial doses were defined such that the plasma concentrations achieved would correspond to the IC_{50} levels determined in the *in vitro* assays for the respective drug. Doses were applied twice a day since the drugs are known to have a short half-life, DW-271 having a half-life of 2.9 hours (Won Yong Lee, Dong Wha Pharm Ind. Co. Ltd., personal communication). The experiment was continued by dose escalation to a maximum of 100 mg/ml (UL 5 max. 10mg/kg due to limited solubility). In contrast to the promising *in vitro* results none of the compounds showed any curative effect at any dosage level and the blood of treated mice was not cleared from trypanosomes. No signs of toxicity were observed during the experiments.

The reason for the *in vivo* ineffectiveness cannot be explained by the results at hand, as no drug level determination in the plasma of the treated mice was performed. It may be hypothesized that the compounds have a very short half-life due to rapid elimination or metabolism, or have a very large volume of distribution all potentially leading to insufficient drug levels.

In conclusion several of the investigated quinolones revealed a remarkable *in vitro* activity. This, in combination with the complete lack of any toxic signs at all dosage levels tested *in vivo,* makes this class of compounds very attractive for further search for trypanocidal compounds despite the failure to confirm the promising *in vitro* activity in this set of experiments. Based on the results obtained special attention should be paid to the R7-substituted- and the tetracylic derivatives.

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References

Baltz, T., Baltz, D., Giroud, C. and Crockett, J., 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. EMBO J. 4, 1273-1277.

Chukwuani, C. M., Coker, H. A., Oduola, A. M., Ifudu, N. D. and Sowunmi, A., 1998. Single-dose pharmacokinetic study of ciprofloxacin and fleroxacin in healthy adult Nigerian volunteers. Chemotherapy. 44, 369-376.

Croft, S. L. and Hogg, J., 1988. Limited activity of bacterial DNA topoisomerase inhibitors against *Leishmania donovani* and *Trypanosoma cruzi* amastigotes *in vitro*. Trans.R.Soc.Trop.Med.Hyg. 82, 856.

Domagala, J. M., 1994. Structure-activity and structure-side-effect relationships for the quinolone antibacterials. J. Antimicrob. Chemother. 33, 685-706.

Englund, P. T., Hajduk, S. L. and Marini, J. C., 1982. The molecular biology of trypanosomes. Ann. Rev. Biochem. 51, 695-726.

Friedheim, E. A. H., 1949. Mel B in the treatment of human trypanosomiasis. American Journal of Tropical Medicine. 29, 173-180.

Hamel, E., Lin, C. M., Plowman, J., Wang, H. K., Lee, K. H. and Paull, K. D., 1996. Antitumor 2,3-dihydro-2(aryl)-4(1H)-quinazolinone derivatives. Biochem. Pharmacol. 51, 53-59.

Huber, W. and Koella, J. C., 1993. A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. Acta Trop.55, 257-261.

Kaminsky, R., Schmid, C. and Brun, R., 1996. An "*in vitro* selectivity index" for evaluation of cytotoxicity of antitrypanosomal compounds. In vitro toxicity. 9, 315-324.

Maxwell, A. and Gellert, M., 1986. Mechanistic aspects of DNA topoisomerases. Advances in Protein Chemistry. , 69-107.

Nenortas, E., Burri, C. and Shapiro, T. A., 1999. Antitrypanosomal activity of fluoroquinolones. Antimicrob. Ag. Chemother. 43, 2066 - 2068.

Neu, H. C., 1990. Quinolones in perspective. J. Antimicrob. Chemother. 26, 1-6.

Pagé, B., Pagé, M. and Noèl, C., 1993. A new fluorometric assays for cytotoxicity measurements *in vitro.* International Journal of Oncology. 3, 473-476.

Pepin, J., Milord, F., Khonde, A., Niyonsenga, T., Loko, L. and Mpia, B., 1994. Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy. Trans. R. Soc. Trop. Med. Hyg. 88, 447-52.

Raether, W., Seidenath, H. and Hofmann, J., 1989. Potent antibacterial fluoroquinolones with marked activity against Leishmania donovani *in vivo*. Parasitol. Res. 75, 412-3.

Räz, B., Iten, M., Grether-Buhler, Y., Kaminsky, R. and Brun, R., 1997. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. Acta Trop. 68, 139-47.

Scheld, W. M., 1989. Quinolone therapy for infections of the central nervous system. Revue of Infectious Diseases 11 Suppl 5, 1194-1202.

Smith, D. H., Pepin, J. and Stich, A. H., 1998. Human African trypanosomiasis: an emerging public health crisis. Brit. Med. Bull. 54, 341-55.

Takayama, S., Hirohashi, M., Kato, M. and Shimada, H., 1995. Toxicity of quinolone antimicrobial agents. Journal of Toxicology and Environmental Health. 45, 1-45.

Van Nieuwenhove, S. 1998. In *International Colloquium Sleeping Sickness Rediscovered* Antwerp.

Wang, C. C., 1995. Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. Annu. Rev. Pharmacol. Toxicol. 35, 93-127.

WHO, 1998. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organisation Technical Report Series. 881, I-VI, 1-114.

Wolfson, J. S. and Hooper, D. C., 1989. Fluoroquinolone antimicrobial agents. Clinical Microbiology Review. 2, 378-424.

Table 2: In vivo activity of selected derivatives

Compound	Dose	Number cured vs. number total	Survival time
Kanebo 8g	2.4 mg/kg	0/4	10/12 days
Kanebo 8g	9.6 mg/kg	0/2	10 days
Kanebo 8g	100 mg/kg	0/2	
Kanebo 8h	0.9 mg/kg	0/4	10/12 days
Kanebo 8h	3.6 mg/kg	0/2	10/11 days
Kanebo 8h	100 mg/kg	0/2	10/11 days
DW 271	2.1 mg/kg	0/4	10/11 days
DW 271	8.4 mg/kg	0/2	9 days
DW 271	100 mg/kg	0/2	
Metal quinolone 5	0.3 mg/kg	0/4	10 days
Metal quinolone 5	1.2 mg/kg	0/2	9/10 days
Metal quinolone 5	10 mg/kg	0/2	10/11 days
$N(CH_3)_2SNx$	2.7 mg/kg	0/4	12 days
$N(CH_3)_2SNx$	10.8 mg/kg	0/2	9 days
$N(CH_3)_2SNx$	100 mg/kg	0/2	
OCH_3SNx	2.7 mg/kg	0/4	13 days
OCH_3SNx	10.8 mg/kg	0/2	9 days
OCH_3SNx	100 mg/kg	0/2	
Control (included for each dose elevation)	-	0/4	9 days

Consequences of the biotransformation of melarsoprol, a discussion

The aim of this PhD thesis was to investigate the metabolism of melarsoprol, the main drug in use for the treatment of human African trypanosomiasis. The apparent discrepancy between the results of two assays (bioassay and HPLC) in determining melarsoprol concentrations in human body fluids encouraged to carry out a thorough study of melarsoprol's metabolism. Metabolic studies are, next to the strategies of screening and research on targets, a promising method for the discovery of a new drug for sleeping sickness: Metabolites often have more desirable properties than the parent drug and knowledge of the metabolic pathway and the enzymes involves helps to avoid severe adverse effects.

Generally, the process of drug metabolism can be regarded as a mechanism of detoxification: In order to facilitate the excretion of foreign, lipid soluble, nonpolar compounds (e.g. many drugs) from the body, they are converted into more hydrophilic compounds. Metabolic reactions of xenobiotics are classified into two types: Phase I reactions involve intramolecular rearrangements as oxidative, reductive or hydrolytic reactions. The metabolites may undergo further metabolism in phase II, enzyme catalysed conjugations. Less commonly the metabolite is formed spontaneously because of inherent chemical instability.

Under some circumstances, however, metabolism can lead to the formation of products, which possess a higher activity as the parent drug and augment its effect. On the other hand some metabolites have a different pharmacologic profile and may even be the cause of adverse effects or toxicity (Rowland and Tozer, 1989).

We have found that melarsoprol is hydrolyzed within minutes to the active drug melarsen oxide due to instability of the dimercaprol (BAL) complex. Melarsen oxide is the synthetic precursor of melarsoprol before the complexation with BAL, but is no longer commercially available. Melarsen oxide has a short half-life of about 3.8 hours; the reactive oxide is capable of binding covalently to macromolecules. It was demonstrated that melarsen oxide and potential metabolites of melarsoprol are irreversibly bound to serum proteins, resulting in a stable drug complex.

These interesting, but also problematic results of the study of melarsoprol's metabolism demonstrated difficulties researchers might face in the process of drug development: The developed drugs or metabolites lack activity, are toxic or as in our case reveal unexpected properties. The result that melarsoprol has inherently problematic pharmacologic properties encouraged to pursue in parallel a second strategy well established in our laboratory: To screen chemicals of a certain class in order to discover a lead compound acting on a biological target of trypanosomes.

The discovery of the rapid hydrolysis of the BAL complex raises the two main questions I would like to discuss: First, whether it is actually beneficial to complex melar-

sen oxide with BAL and second whether the numerous adverse effects that occur in the treatment with melarsoprol are due to the irreversible bound metabolite

Melarsen oxide versus melarsoprol

The development of melarsoprol

Melarsen oxide was synthesized by Friedheim in 1939 and subsequently used as an antitrypanosomal drug in a series of trials mainly in the 1940's and 1950's (see Table 1).

In view of these trials it can be summarized that in sufficient doses and applied intravenously, melarsen oxide seemes to be able to cure late stage trypanosomiasis. However, the reports on the concentration of the effective intravenous dose of melarsen oxide ranged from 1.5 mg/kg (Friedheim, 1948) to 3 mg/kg as the minimum effective dose (Jonchere, Gomer *et al.*, 1953). The reports on oral effectiveness are questionable and most publications do not provide any information on relapse cases and follow up studies.

In 1946, first results on a new detoxifier of arsenic, British Antilewisite (BAL or dimercaprol) were published. Applied to animal skin the substance revealed protection against the vesicant action of the chemical warfare agent lewisite (Danielli, Danielli *et al.*, 1946). However, animal trials with rats showed that BAL possessed a low LD_{50} of 50 mg/kg if applied intramuscular (Danielli, Danielli *et al.*, 1946) and 110 mg/kg if applied subcuteanously (Webb, 1946). It was supposed that the toxicity of BAL was due to its affinity to a large number of enzymes (Webb, 1946). The high toxicity and the necessity to administer the antidote soon after arsenic contamination restricted the systemic use of BAL (Danielli, Danielli *et al.*, 1946). Today BAL is hardly used as an arsenic antidote and most clinicians believe that the drug is useless (Mückter, Liebl *et al.*, 1997, Apted, 1980). Recently an increase of mortality after the administration of BAL for treatment of melarsoprol induced encephalopathic syndromes has been reported (Pepin, Milord *et al.*, 1995).

Nevertheless, Friedheim started to combine arsenicals with BAL, having in mind an increase of tolerance of the drug melarsen administered in combination with a "high protein diet in form of Swiss cheese" (Friedheim, 1959); patients in the Volta region received during treatment "large chunks of cheese" and Friedheim speculated that the decisive factor of the reduced incidences of adverse effects might be the sulfur content of the cheese. The trivalent arsenic was capped with the sulfur groups of the dimercaptan. Among the complexed drugs were atoxyl, stovarsol, oxophenarsine, osanine, tryparsamide and melarsen oxide (Friedheim, 1959). However, investigations on the complexed compounds revealed an increased toxicity, the LD_{50} of oxophenarsine was found to be 15 mg/kg compared to 4 mg/kg of the Marph-BAL complex when applied intramuscularly to rats (Peters and Stocken, 1946). In contrast, com-

plexes of oxophenarsine with the better tolerated and less toxic antidote dimercaprol glycoside did not show an enhancement of toxicity. It was discussed that an increase of toxicity of the complexed compounds could be due to the instability of the complex, the dissociation of dimercaprol (Peters and Stocken, 1946).

However, Friedheim did not abandon dimercaprol and based on promising results from trials with melarsen oxide (melarsen oxide was reported to be the first drug to cure late stage *T. b. gambiense* infections without toxic incidences in very high numbers) he focused on the melarsen oxide-BAL complex melarsoprol (Mel B) (Friedheim, 1959). Mel B received considerable attention terminating the use of melarsen oxide though the advantages of melarsoprol compared to melarsen oxide were questionable. Adverse effects were common during treatment with melarsoprol despite the arsenic theoretically being detoxified by the capping. Several clinicians assessed the advantages and disadvantages of melarsoprol over melarsen oxide: A direct comparison of both drugs demonstrated a similar toxicity of both compounds (Jonchere, Gomer *et al.*, 1953):

Table 2: A comparison of Melarsoprol and Melarsen oxide (Jonchere, Gomer *et al.*, 1953)**:**

	Melarsen oxide	**Melarsoprol**
Arsenic content	22.3 %	18.8 %
LD_{50} (mouse) expressed in means of arsenic content of drug	7.3 mg/kg	6.5 mg/kg
LD_{50} (mouse) expressed as total drug	32 mg/kg	30 mg/kg
Total dose used without complications	10.5 mg/kg (7 injections of 1.5 mg/kg)	10.8 mg/kg (3 injections of 3.6 mg)

Findings and conclusions

Today this controversy on the treatment of sleeping sickness has still not been resolved, but it has even been forgotten. Melarsoprol is still the drug of choice for treatment of the late stage of the disease but its future production is questionable.

Our single dose kinetic study indicated that the dissociation of melarsoprol, into melarsen oxide and BAL, in the body is rapid. One hour after application of the first of ten doses of 2.2 mg/kg only 38-103 ng/ml of melarsoprol and after three hours no drug could be detected by HPLC in serum of patients. C_{max} of melarsen oxide is reached already 5-15 minutes after the intravenous drug application (see Investigations of the trypanocidal drug melarsoprol).

We demonstrated that dimercaprol itself possessed no activity on trypanosomes. It is a rather toxic compound with a LD_{50} of 1.48 mmol/kg (mouse, i.p.) (Mückter, Liebl *et al.*, 1997). Therefore, contributions of BAL to melarsoprol's toxicity cannot be excluded.

Melarsoprol is only soluble in propylene glycol due to the lipophilicity of dimercaprol. Because of its viscosity it is difficult to administer the drug. The application is painful and a thrombophlebities at the injection site is frequently developed. In contrast melarsen oxide is more hydrophilic and would thus be better water soluble and easier to administer.

Interestingly BAL has been shown to redistribute arsenic to the brain and tissues (Mückter, Liebl *et al.*, 1997). Therefore this molecule may enhance the passage of melarsoprol through the blood brain barrier.

With the exception of a series of clinical trials conducted in the mid of the century, which revealed contradictory results, no data are available on the ability of melarsen oxide to pass lipophilic membranes as the blood brain barrier. Therefore we investigated in a late stage infection mouse model whether melarsen oxide is capable of passing the blood brain barrier in a sufficient amount for cure: The mice were followed up for 180 days and 5/6 mice could be cured receiving 7 doses of 5 mg/kg intravenous (see: Investigations on the trypanocidal drug melarsoprol).

Future possibilities

In view of our findings melarsen oxide appears to be a valid alternative to melarsoprol for clinical use. Therefore, in depth preclinical studies on several doses and routes of application in an appropriate animal model might be indicated to compare melarsen oxide with melarsoprol. The optimal dosage for melarsen oxide for cure of human African trypanosomiasis, with lowest rate of relapses and adverse effects should eventually be determined. If successful, the logical continuation would be a clinical trial in the setting of an "expanded access" protocol:

Under current US law, for example, all new drugs need proof that they are effective and safe and have to undergo extensive clinical testing before they are approved for marketing. This is a time-consuming and expensive process. In the case of metabolites this process might be facilitated and more rapid as baseline data already exist. The parent drug melarsoprol, however, is a drug, which is known to cause adverse

effects. Surprisingly to date hardly any appropriate information on its safety profile or chemical and kinetic properties exist. Therefore no data can be employed for its metabolite melarsen oxide. However, the Food and Drug Administration launched initiatives for urgently needed drugs for life-threatening diseases, that have no acceptable treatment so far: These innovative programs allow the compounds to be used in “expanded access” protocols, which are unrestricted studies in order to learn more about the drug while providing treatment. One prominent example is zidovudine: 4,000 AIDS patients were treated with zidovudine before it was approved and the drug was finally brought to the market only 107 days later(Flieger, 1993).

ırsen oxide in the treatment of Human African trypanosomiasis

ɔose	Treatment schedule	No. cured/ No. total	Observations	Reference
50 mg oral	Not known	Not known	No adverse effect	(Van Hoof, 1946)
5 mg ıtravenous	Not known	Not known	No adverse effect	(Van Hoof, 1946)
.1 mg/kg ıtravenous	7 days	5/5 first stage 1/3 late stage	-	(Weinmann and Franz, 1945)
mg/kg ral	5-8 days	4/4 first stage 11/12 late stage (followed up 4 month)	Arsenic recovered in brain, liver and kidneys after melox administration	(Weinmann and Franz, 1945)
.5 mg/kg intravenous	7 days, 2 series with 1 month interval	No results on follow up	-	(Nodenot, 1947)
mg/kg oral	8 days, 2 series	?/13 (results published soon after end of treatment)	-	(Nodenot, 1947)
Oral	Two series of seven days with 10 days break	1/6 late stage	-	(Rouzic, 1949)
.0015 mm/kg intravenous	Two series of seven days with one month break	5/7 late stage	-	(Rouzic, 1949)
cg oral	Two times 1cg	9/9 first stage 0/5 late stage	Doses of 3 cg tolerated. Single dose of melox effective	(Trinquier and Pellisier, 1948)
.5 mg/kg intravenous	2 series of 7 injections	54/54 late stage (immediate effect) 5/5 follow up 10 month	-	(Friedheim, 1948)
.5 mg/kg intravenous	Two series of seven days with one month break	10/10 late stage	Adverse effects observed	(Ceccaldi, Arnoult *et al.*, 1949)
.1 mg/kg intravenous	7 days	?/74 late stage (relapses after 2 years reported)	Adverse effects observed	(Duggan and Hutchinson, 1951)

.5 mg/kg ıtravenous	Three injections during 3 days	10/12 late stage	Dose insufficient	(Jonchere, Gomer *et al.*, 1953)
.5 mg/kg ıtravenous	Two series of three injections with three weeks break	4/5 late stage	Dose insufficient	(Jonchere, Gomer *et al.*, 1953)
mg/kg intrave- ous	Three injections during 3 days	9/9 late stage only 3 month follow up	Adverse effects observed	(Jonchere, Gomer *et al.*, 1953)
mg/kg intrave- ous	Two series of three injections with three weeks break	5/5 late stage only 3 month follow up	Adverse effects observed	(Jonchere, Gomer *et al.*, 1953)

Bound metabolites and associated adverse effects

General remarks

Adverse effects appear to be an inevitable complication of chemotherapy. They account for a large amount of fatal reactions and are responsible for a significant number of patient's deaths: Adverse reactions are reported to have an overall incidence of 10-20%, to cause 2-3% of consulations in general practice and up to 3% of admission to acute care hospital wards (Laurence and Bennett, 1993)

Various classifications of adverse drug reactions have been proposed(Park, Pirmohamed *et al.*, 1992; Hess and Rieder, 1997) They are often divided into four types: unpredictable (idiosyncratic) drug reactions, predictable reactions, reactions associated with long term drug therapy and delayed effects.

(1) Intolerance, allergy (they account for 10-20% of all adverse drug reactions and may even have a fatal outcome) or pseudoallergy are typical reactions of unpredictable adverse effects. So far, mechanisms of unpredictable adverse effects are not well understood, they are unpredictable from the knowledge of the basic pharmacology of the drug, unrelated to the intended pharmacologic effect of the medication, do not show any simple dose-response relationship and occur in only a small percentage of the population (hence the term idiosyncratic). Knowledge of the pharmacology of the drug does not assist to the identification of individual patients at risk (Hess and Rieder, 1997). To alleviate the adverse reaction a drug discontinuation rather than dose reduction is required (Park, Pirmohamed *et al.*, 1992). Aplastic anaemia occuring after chloramphenicol administration is a typical example for unpredictable adverse effects (Pirmohamed, Kitteringham *et al.*, 1994).

(2) In contrast, predictable adverse drug reactions are dose- dependent host independent and can be anticipated from the known pharmacology of the drug. Predictable adverse effects are common and include overdosing of drugs, drug-drug interactions or drug-disease interactions. Careful planning of treatment can reduce the incidence of predictable, dose dependent adverse effects (Hess and Rieder, 1997). Examples of this type of reaction include hypoglycaemia with oral hypoglycaemics and hypotension with anti- hypertensives (Park, Pirmohamed *et al.*, 1992).

(3) Examples of adverse effects associated with long term therapy, as benzodiazepine dependence or analgesic nephropathy are well described and can be anticipated (Park, Pirmohamed *et al.*, 1992).

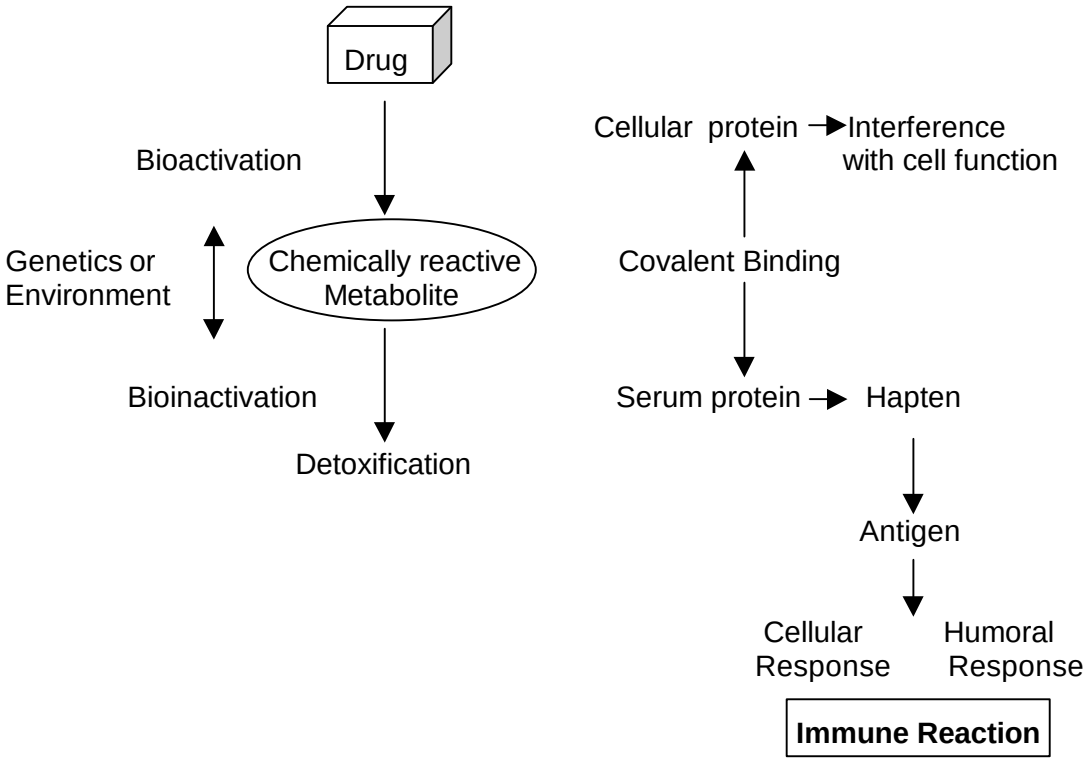
(4) Delayed effects such as carcinogenicity and teratogenicity are classified as type 4 reactions (Hess and Rieder, 1997). Today such toxicities should be precluded by the extensive programme of preclinical mutagenicity and carcinogenicity studies that a new

chemical entity must undergo before a product license is granted(Park, Pirmohamed *et al.*, 1992).

Metabolites are known to cause unpredictable adverse effects: Reactive molecules formed spontanously or by phase I and phase II enzymes, bind to essential cellular macromolecules through covalent bond formation and interfere with normal physiological functions or even generate an immune response against the altered macromolecule.

Melarsen oxide, melarsoprol's metabolite is a typical example of a reactive metabolite.

Immune reactions:



In order to trigger an immune reaction the molecule must have a minimum molecular weight of 1,000 Da (Pohl, Satoh *et al.*, 1988). Thus, a drug once covalently bound can initiate a misdirected immune reaction. This immune reaction is directed against the drug (haptenic epitopes), the carrier protein (autoantigenic determinants), or against the whole complex. The altered macromolecule may provoke a humoral or cellular response, or a combination of both. Cellular activation includes the production of excitatory cytokines, clonal expansion and the recruitment of activated macrophages. A humoral response is elicited by antibody production through the interaction of B cells with T cells activated by antigen-presenting cells (Hess and Rieder, 1997). Important drugs for the production of toxic metabolites are butazones, chloramphenicol, anticonvul-

sants, sulphonamides, phenothiazines or antithyroid drugs(Pirmohamed, Kitteringham *et al.*, 1994).

Coomb's classification of the pathological processes involved in immunologic hypersensitivity reactions can be applied for drugs. Frequently a combination of all types is observed:

(1) Binding of IgE antibodies and antigen to mast cells stimulates the release of chemical mediators such as histamine.
(2) Antibody response directed against owncells lead to immune activation and to cell destruction.
(3) Formation of immune complexes of antibody and antigen deposited in tissue may induce local inflammatory response.
(4) A delayed hypersensitivity mediated by T-lymphocyte responses to antigen.

The clinical manifestation of hypersensitivity reactions is complex and any organ might be affected. Adverse effects like fever, hepatitis, and nephritis or tissue toxicity are often observed. Urticaria or dermatitis are frequent symptoms which are produced by the release of mediators, such as histamine or leukotrienes. The skin is immunologically functional and metabolically active, containing both phase I and phase II enzymes (Pirmohamed, Kitteringham *et al.*, 1994). Even reactions in the central nervous system have been reported. Hypersensitivity reactions to antidepressants, anesthetic agents, sulfonamides or penicillin in this compartment are well documented, but detailed mechanism remain unknown. All of these drugs contain a reactive molecule like an amine group, which is able to form covalent linkage, resulting in a hapten (Hess and Rieder, 1997).

An involvement of the immune system during adverse reactions of melarsoprol?

In treatment with the organo-arsenical drug melarsoprol unpredictable adverse effects are common: A drug-induced encephalopathy is a frequent complication. The reaction is clinically characterized by a convulsive status associated with high intracranial pressure, psychotic reactions and progressive coma, vomiting, diarrhoea and fever (WHO, 1998). Also hypersensitivity reactions, agranulocytosis, renal and hepatic dysfunctions have been noted. Skin reactions as maculopapular eruptions, exfoliative dermatitis or bullous eruptions are inadequately documented, they may be severe butoccur infrequently (Pepin and Milord, 1994).

The reason for the different individual susceptibility to adverse effects in the treatment with melarsoprol is not yet known. Our results indicate that a misdirected immune reaction initiated by the covalently bound derivative might trigger the adverse events.

An involvement of the immune system in adverse reactions was already discussed in the last years, but no final conclusion could be drawn: Hunter and colleagues suggested that parasite persisting in the CSF due to subcurative regimen may provoke a violent inflammatory response by activated astrocytes (Hunter, Jennings *et al.*, 1992). Another theory assumed that the massive killing of trypanosomes at treatment start releases antigens attracting an array of antibodies (Pepin and Milord, 1991). A drug related immune response by the arsenical or its bound derivatives to parasite proteins was also already discussed (Haller, Adams *et al.*, 1986).

The involvement of the immune system in the pathogenesis of adverse effects during melarsoprol treatment is supported by earlier findings:

Independence of dose, dependence of time and host

Typically, immunologic hypersensitivities are reported to be host-dependent but dose-independent. The reactions usually occur after repeated exposures independent from treatment schedules and after repeated doses of the drug.

Many authors described the time point of incidences in the treatment with melarsoprol: Major adverse effects were found to happen at the end of the first series of injections, during the interval between first or second, or during the second series (Pepin and Milord, 1991), but also 1 to 3 days after the third injection (Buyst, 1975), or generally after completion of a three days course (Robertson, 1963). It was reported that incidences could be avoided with the administration of only a single series of three doses of melarsoprol (Richet, Lotte *et al.*, 1959) or melarsen oxide (Jonchere, Gomer *et al.*, 1953). Furthermore, adverse effects were increased if a single application of drug was divided into several doses at consecutive days (Jonchere, Gomer *et al.*, 1953).

The independence from melarsoprol dosage of the occurrence of adverse effects, including the severe encephalopathy was demonstrated in the past using more than 20

treatment regimens with varying concentration of drug administered: Recently, the efficacy and safety of a new abriged treatment schedule was assessed in an open, randomised equivalence trial in Northern Angola. The new treatment schedule comprised ten daily injections of 2.2 mg/kg melarsoprol compared to the national Angolan schedule of 3 series of four daily injections of increasing doses from 1.2 to 3.6 mg/kg body weight: No difference was found in the rate of encephalopathy and the timepoint of the onset of the reactions between the new and the standard treatment regimen (Chr. Burri, personal communication). The identical temporal distribution of reaction under both treatment schedules discounts the hypothesis that non-curative treatment is the general cause of reaction. A currently published clinical study on the potential efficacy of melarsoprol in patients with refractory or resistant leukemia implies that not the parasite is involved in the mechanism of adverse effects, but favour a delayed immune response or direct toxic drug effect: 3/8 leukemia patients treated with melarsoprol, using the standard schedule experienced generalised seizures during the second week of therapy (Soignet, Tong *et al.*, 1999)

Interindividual susceptibilities

It is of interest to know why some patients are more susceptible to adverse effects in melarsoprol treatment.

Not only exogenous co-factors but also an abnormal pharmacodynamic response, abnormality in drug metabolism or idiosyncratic reactions could provide an explanation: Hypersensitivity reactions have a complex genetic basis, involving numerous processes that are independently regulated. Individual variation of metabolic processes might be one trigger since the drug or the drug conjugate are often metabolised before they are able to activate the immune system. In this regard, some drug-carrier conjugates may be more immunogenic than others. In addition, the immune system, consisting of a network of multicellular components, is regulated interindividually resulting in a different expression of antibodies and other modulators of the immune system. Halothane hepatitis, one example, can be regarded as a model for immune-mediated hepatoxicity. Although all patients exposed to the drug are able to generate the antigen, susceptibility to hepatitis is dependent on the immune response of the individual (Pirmohamed, Kitteringham *et al.*, 1994). Penicillins are another important group of drugs known for interindividual variation: Studies indicated that the drug induced IgE antibody response to the penicillolyl antigen is under genetic control (Park, Pirmohamed *et al.*, 1992).

The suggestion that an immunological process might be the trigger for adverse effects is supported by observations made during melarsoprol treatment of patients suffering from concomitant diseases: It was reported that the frequency of adverse effects is aggravated by concurrent viral infections such as influenza (Richet, Lotte *et al.*, 1959) and measles (Buyst, 1975) or parasitic infections such as malaria and onchocerciasis

(Apted, 1980). In addition, vaccinations for e.g. smallpox were not recommended (Buyst, 1975).

In general, seasonal and local variations have been noticed in the occurrence of adverse effects. An increase of melarsoprol reaction between June and October (Ancelle, Barret *et al.*, 1994) and a difference of mortality within regions was reported (Adriaenssens, 1960; Robertson, 1963; Buyst, 1975).

Reduction of adverse effects by immuno suppressive drugs

Pepin and colleagues clearly demonstrated a significant reduction of encephalopathy during concomitant prednisolone therapy. A univariate and multivariate analysis of more than 1,000 patients treated with melarsoprol showed that the fatality rate during melarsoprol treatment was reduced from 14.2% (patients without prednisolone) to 5.2% (patients with concomitant prednisolone therapy). The protective effect of prednisolone was very important: only 3.5 % of the patients developed an encephalopathy. Among patients who did not experience drug induced encephalopathy, prednisolone had no effect on mortality (Pepin, Milord *et al.*, 1995). Glucocorticoids like prednisolone effect the IL-1 synthesis by macrophages and IL-2 production by T-cells, that are necessary for the proliferation of activated T-cells (Rang and Dale, 1991). Two earlier trials that evaluated the use of prednisolone to reduce incidences of encephalopathy demonstrated no effect of the drug. However, the power of the first study was so low that even a huge effect of prednisolone would not have been detected (Foulkes, 1975), whereas the data sets of the second study included different treatment regimens applied during 25 years (Arroz, 1987).

A lowered mortality rate during melarsoprol treatment is reported not only from adjuvant corticosteroid therapy, but also in a few cases from the administration of the two drugs chloroquine and azathioprine (Buyst, 1975). Azathioprine is known to repress cell and antibody mediated reactions by inhibition of the polyclonal proliferation in the induction phase of the immune response. Chloroquine hinders phagocytic activity and the release of mediators during the inflammatory process (Rang and Dale, 1991). In contrast, the prescription of thiabendazole to treat strongyloidiasis during the melarsoprol cure was found to increase the risk of encephalopathic syndroms (Ancelle, Barret *et al.*, 1994). Thiabendazol is a drug, which is known to be given with caution to patients with a history of drug hypersensitivity,

The results of these studies and our own working hypothesis that an hapten induced immunological reaction might be responsible for the serious adverse effects, indicates that prednisolone or similar steroids should be administered during treatment to all patients. Until the detailed immunologic pathway of adverse events will be elucidated further investigations on the effectiveness of different immunosuppressive drugs should be carried out.

Continuation of treatment

Symptoms due to an immune reaction usually subside after discontinuation of the treatment. If the patient is rechallenged with the drug the same unfavorable condition is observed (with a much more rapid onset). However, if the reactions are not exclusively immune-mediated, adverse events do not occur immediately after rechallenge with the drug (Pirmohamed, Kitteringham *et al.*, 1994) or may proceed clinically silent (de Weck, 1991).

Sleeping sickness patients are often retreated with melarsoprol after a short treatment break without obvious symptoms. Buyst recommended "when the patient survives his adverse reactions the Mel B course should be continued, but the next injection has to be postponed for 1-3 days"(Buyst, 1981). In addition, Ginoux and colleagues observed that each time a patient has survived an encephalopathy the melarsoprol treatment could be continued without further incident (Ginoux, Bissadidi *et al.*, 1984). This practical experience is contradictory to the hypothesis of an immune involvement. However, it is not known whether the patients received steroids during the treatment break, whether the second immune response proceeds without clinical symptoms, wether the patients are desensibilised, or whether the toxicity reactions are not exclusively immune mediated (so that the remaining treatment period might be too short to induce further adverse reactions).

Conclusion

If specific antibodies or T cells can be detected a drug reaction is classified as a hypersensitive drug reaction. The individual susceptibility cannot be predicted so far for most drugs, which are known to cause unpredictable adverse effects. Lately, in the treatment with anticonvulsants the lymphocyte cytotoxicity assay, in treatment with penicillins prospective skin testing has been suggested to predict adverse reactions (Park, Pirmohamed *et al.*, 1992). However, not all predisposed individuals will be identified, especially if other mechanisms than imunologic factors are involved, and the tests are complex and expensive. An alternative strategy might be to take advantage of the knowledge of the functional group causing the toxicity. Oxide groups (as found in melarsen oxide) are known to be responsible for a covalent binding of drugs to proteins (Park, Pirmohamed *et al.*, 1992). This identification may allow the design of new compounds that retain therapeutic efficacy but do not cause toxicity. Toxic reactions are difficult to identify. In i*n vitro* systems a general toxicity is revealed rather than complex mechanisms occuring *in vivo*, and even preclinical safety evaluation in experimental animals does not identify drugs which cause idiosyncratic reactions in humans. Nevertheless, ti has to be investigated whether the arsenic oxide is essential for trypanocidal activity or whether it could be replaced.

Examples of this approach to prevent drug toxicity already exist: The nitro group of chloramphenicol has been replaced by the methylsulphonyl moiety to form thiamphen-

col, which possesses the same antibacterial spectrum but does not cause aplastic anaemia (Pirmohamed, Kitteringham *et al.*, 1994). In today's drug design the presence of sulphydryl groups or aromatic amino groups is generally avoided because of its toxic metabolites. Since more than 12,000 arsenicals were synthesized in the beginning of the century *in vitro* tests on their activity could be conducted. Pharmacologic properties like the long half-life of bound drugs have to be kept in mind. Compounds lacking the reactive group might have a short half-life and thus a low efficacy. In this respect, the irreversible binding of the active compound could act as a reservoir in the body. Interactions of the drug-protein complexes with hepatocytes lead to drug dissociation (Meijer and van der Sluijs, 1989). Since these endocytic processes in the liver are saturable, the free drug is available for the target for a period of time. In addition, the mode of action of melarsoprol is still not known. Following our results, it could be possible that the whole drug-protein complex of melarsoprol is active. One example is the active albumine complex of the trypanocidal drug suramin, which enters trypanosomes by endocytosis (Wang, 1995).

A combination of factors acting collectively in individuals may lead to the serious adverse effects in the treatment with melarsoprol and a direct arsenic toxicity can not be excluded. Since most arsenic derivatives are highly toxic and thus solutions far from optimal new compounds for the treatment of African sleeping sickness have to be discovered: For our structure activity relationship study compounds of the bacteriocidal class of quinolones, which mainly act via the inhibition of topoisomerases, were chosen. Topoisomerases mediate topological changes in the DNA, which are essential for cell survival. Trypanosomes possess a condensed, topologically interlocked circular type of mitochondrial DNA. The high demand for topoisomerase activity in combination with the rapid proliferation of trypanosomes makes the inhibition of topoisomerases an interesting drug target.

Evaluation of a risk benefit ratio for a drug associated with unpredictable adverse effects is complex. It is even difficult to decide in first world countries whether to withdraw a drug and protect a certain subgroup of the population and deny therefore the majority of population access to important therapy. Since we do not have any alternatives in the treatment of late stage trypanosomiasis we are not confronted with this discussion. However, it was important to study and elucidate the metabolic pathway of melarsoprol and its consequences in order to build a basis for future investigations. The presented results demonstrate that research efforts for new compounds for sleeping sickness treatment are urgently required.

References:

Adriaenssens, K. (1960). [Nota over de toxiciteit van Mel B in Trypanosoma rhodesiense slaapziekte]. *Ann. Soc. Belg. Med. Trop.*

Ancelle, T., B. Barret*, et al.* (1994). [2 epidemics of arsenical encephalopathy in the treatment of trypanosomiasis, Uganda, 1992-1993]. *Bull. Soc. Pathol. Exot.* 87(5): 341-346.

Apted, F. I. C. (1980). Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Eastern hemisphere. *Pharmac. Ther.* 11: 391-413.

Arroz, J. O. (1987). Melarsoprol and reactive encephalopathy in *Trypanosoma brucei rhodesiense*. *Trans. R. Soc. Trop. Med. Hyg.* 81: 192.

Buyst, H. (1975). The treatment of *T. rhodesiense* sleeping sickness, with special reference to its physio-pathological and epidemiological basis. *Ann. Soc. Belg. Med. Trop.* 55(2): 95-104.

Buyst, H. (1981). The treatment of subacute *T. rhodesiense* sleeping sickness in Zambia. *A Zambia National Trypanosomiasis Control Programme Circular*: 5p.

Ceccaldi, J., H. Arnoult*, et al.* (1949). Sur l'emploi du para (2-4 diamino-3-triazinyl-6-) arsino-oxyde par la voie intraveineuse dans le traitment de la trypanosomiase humaine. *Bull. Soc. Path. Ex.* 9- 10: 487- 498.

Danielli, J. F., M. Danielli*, et al.* (1946). BAL-INTRAV: A new non-toxic thiol for intravenous injection in arsenical poisoning. *Biochem. J.* 41: 325- 332.

de Weck, A. L. (1991). Pharmacologic and immunochemical mechanisms of drug hypersensitivity. *Drug Allergy* 11(3): 461-474.

Duggan, A. J. and M. P. Hutchinson (1951). The efficacy of certain trypanocidal compounds against *Trypanosoma gambiense* infection in man. *Trans. R. Soc.Trop. Med. Hyg.* 44(5): 535-544.

Flieger, K. (1993). FDA finds new ways to speed treatment to patients. *FDA Consum.* 27(8): 14-18.

Foulkes, J. R. (1975). An evaluation of prednisolone as a routine adjunct to the treatment of *T. rhodesiense*. *J. Trop. Med. Hyg.* 78: 72-74.

Friedheim, E. A. H. (1948). Melarsen oxide in the treatment of human trypanosomiasis. *Ann.Trop. Med.* 42: 357-363.

Friedheim, E. A. H. (1959). Some approaches to the development of chemotherapeutic compounds. *Ann. Trop. Med. Parasit.* 53: 1-9.

Ginoux, P. Y., N. Bissadidi*, et al.* (1984). [Complications observed in the treatment of trypanosomiasis in the Congo]. *Med. Trop. (Mars)* 44(4): 351-355.

Haller, L., H. Adams, *et al.* (1986). Clinical and pathological aspects of human African trypanosomiasis (*T. b. gambiense*) with particular reference to reactive arsenical encephalopathy. *Am. J. Trop. Med. Hyg.* 35: 94-99.

Hess, D. A. and M. J. Rieder (1997). The role of reactive drug metabolites in immune-mediated adverse drug reactions. *Ann. Pharmacother.* 31(11): 1378-1387.

Hunter, C. A., F. W. Jennings, *et al.* (1992). Subcurative chemotherapy and fatal post-treatment reactive encephalopathies in African trypanosomiasis. *Lancet* 339(8799): 956-958.

Jonchere, H., J. Gomer, *et al.* (1953). Contribution a l'etude de produits a radical melaminyl dans le traitement de la trypanosomiase humaine a *T. gambiense*. *Bull. Soc. Path. Exot.* 46: 386-396.

Laurence, D. R. and P. N. Bennett (1993). Clinical Pharmacology. Edinburgh, Churchill and Livingstone

Meijer, D. K. F. and P. van der Sluijs (1989). Covalent and noncovalent protein binding of drugs: Implication for hepatic clearance, storage, and cell-specific drug delivery. *Pharm. Research* 6(2): 105-118.

Mückter, H., B. Liebl, *et al.* (1997). Are we ready to replace dimercaprol (BAL) as an arsenic antidote? *Human & Experimental Toxicology* 16: 460-465.

Nodenot, L. (1947). Les derniers médicaments en expérimentation contre la maladie du sommeil. *Bull. Med. Afr. O. France.* 4: 299-302.

Park, B. K., M. Pirmohamed, *et al.* (1992). Idiosyncratic drug reactions: a mechanistic evaluation of risk factors. *Br. J. Clin. Pharmacol.* 34(5): 377-395.

Pepin, J. and F. Milord (1991). African trypanosomiasis and drug-induced encephalopathy; risk factors and pathogenesis. *Trans. R. Soc. Trop. Med. Hyg.* 85: 222-224.

Pepin, J. and F. Milord (1994). The treatment of human African trypanosomiasis. *Adv Parasitol.* 33: 1-47.

Pepin, J., F. Milord, *et al.* (1995). Risk factors for encephalopathy and mortality during melarsoprol treatment of *Trypanosoma brucei gambiense* sleeping sickness. *Trans. Roy. Soc. Trop. Med. Hyg.* 89: 92-97.

Peters, R. A. and L. A. Stocken (1946). Preparation and pharmacological properties of 4- hydroxymethyl-2- (3-amino-4-hydroxyphenyl)-1:3-dithia-2-arsacyclopentane (Mapharside- BAL compound). *Biochem. J.* 41: 53- 55.

Pirmohamed, M., N. R. Kitteringham, *et al.* (1994). The role of active metabolites in drug toxicity. *Drug. Saf.* 11(2): 114-144.

Pohl, L. R., H. Satoh, *et al.* (1988). The immunologic and metabolic basis of drug hypersensitivities. *Annu. Rev. Pharmacol. Toxicol.* 28: 367-387.

Rang, H. P. and M. M. Dale (1991). Pharmacology. Edinburgh, Churchill and Livingstone.

Richet, P., M. Lotte, *et al.* (1959). Resultats des traitments de la trypanosomiase humaine a *T. gambiense* par le Mel B ou l'arsobal. *Méd. Trop.* 19(3): 253- 265.

Robertson, D. H. H. (1963). The treatment of sleeping sickness (mainly due to *Trypanosoma rhodesiense*) with melarsoprol. *Trans. R. Soc. Trop. Med. Hyg.* 57(2): 122-133.

Rouzic, L. (1949). Nouveaux trypanocides en experimentation. *Bull. med. de l`Afrique Occidentale Francaise.* No. spec.: 63-77.

Rowland, M. and T. N. Tozer (1989). Clinical pharmacokinetics. 2nd ed. Philadelphia, Lea & Febiger.

Soignet, S. L., W. P. Tong *et al.* (1999). Clinical study of an organic arsenical melarsoprol in patients with advanced leukemia. *Cancer Chemother. Pharmacol*. 44: 417-421.

Trinquier, E. and A. Pellisier (1948). Emploi du 3177 RP par voie buccale dans le traitment de la maladie du somneil. *Bull. Soc. Path. Ex.* 3- 4: 161- 165.

Van Hoof, L. M. J. J. (1946). Observations on trypanosomiasis in the Belgian Congo (The second Royal Society of Tropical Medicine and Hygiene Chadwick Lecture). *Trans. R. Soc. Trop. Med. Hyg.* 40: 728-761.

Wang, C. C. (1995). Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. *Annu. Rev. Pharmacol. Toxicol.* 35: 93-127.

Webb, E. C. (1946). The action of British Anti lewisite on enzyme systems. *Biochem. J.* 41: 74- 82.

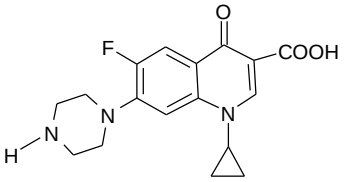
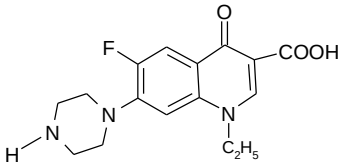
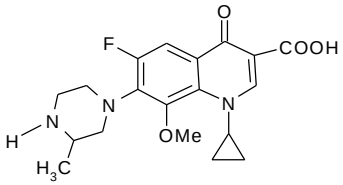
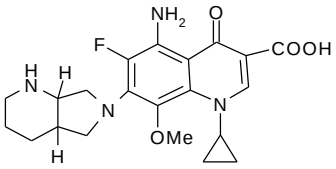
Weinmann, D. and K. Franz (1945). Early results of the treatment of African trypanosomiasis with two new arsenical preparations. *Amer. J. Trop. Med.* 25: 343-344.

WHO (1998). Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. *World Health Organ. Tech. Rep. Ser.* 881: I-VI, 1-114.

Appendix Table I

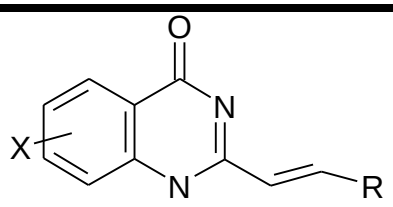
Structures of tested quinolone derivatives

Registered compounds

Compound	Structure	Formula	IC_{50}	$IC_{50\ (TOX)}$
Ciprofloxacin		$C_{17}H_{18}FN_3O_3$	Not active	n.d.
Norfloxacin		$C_{16}H_{18}FN_3O_3$	Not active	n.d.
Gatifloxacin		$C_{19}H_{22}FN_3O_3$	16	n.d.
Moxifloxacin		$C_{21}H_{25}FN_4O_4$	12	n.d.

Experimental compounds

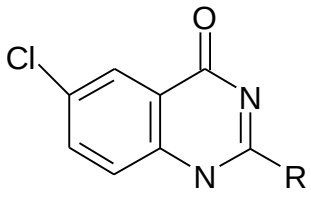
1. 2-Styrylquinazolin-4 (3H)-ones

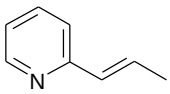


Compound	X	R	Formula	IC_{50}	$IC_{50\ (TOX)}$
DuPont 5a	6-Cl	Ph	$C_{16}H_{11}ClN_2O$	Not active	n.d.
DuPont 5b	5-Cl	Ph	$C_{16}H_{11}ClN_2O$	Not active	n.d.
DuPont 5c	7-Cl	Ph	$C_{16}H_{11}ClN_2O$	2.6	n.d.
DuPont 5d	8-Cl	Ph	$C_{16}H_{11}ClN_2O$	Not active	n.d.
DuPont 5e	6-Br	Ph	$C_{16}H_{11}BrN_2O$	Not active	n.d.
DuPont 5f	6-I	Ph	$C_{16}H_{11}IN_2O$	Not active	n.d.
DuPont 5g	6-F	Ph	$C_{16}H_{11}FN_2O$	6.2	n.d.
DuPont 5h	6-Cl	Me	$C_{11}H_9ClN_2O$	Not active	n.d.
DuPont 5i	6-NH_2	Ph	$C_{16}H_{13}N_3O$	Not active	n.d.
DuPont 5j	H	Ph	$C_{16}H_{12}N_2O$	Not active	n.d.
DuPont 5k	6-Me	Ph	$C_{17}H_{14}N_2O$	1.5	n.d.
DuPont 5l	6-F, 7-Cl	Ph	$C_{16}H_{10}ClFN_2O$	Not active	n.d.
DuPont 5m	6,7-$(MeO)_2$	Ph	$C_{18}H_{16}N_2O_3$	Not active	n.d.
DuPont 5n	5-Me	Ph	$C_{17}H_{14}N_2O$	Not active	n.d.
DuPont 5o	6-MeO	Ph	$C_{17}H_{14}N_2O_2$	Not active	n.d.
DuPont 5p	6-HO	Ph	$C_{16}H_{12}N_2O_2$	Not active	n.d.
DuPont 5q	6-F,7-piperidinyl	Ph	$C_{21}H_{20}FN_3O$	Not active	n.d.
DuPont 5r	6-EtO	Ph	$C_{18}H_{16}N_2O_2$	Not active	n.d.
DuPont 5s	6-F,7-N-Me-piperazinyl	Ph	$C_{21}H_{21}FN_4O$	**0.2**	**1.2**

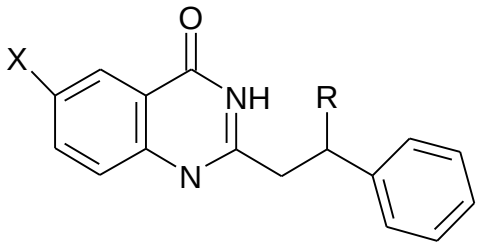
DuPont 5t	7-MeO	Ph	$C_{17}H_{14}N_2O_2$	3.3	n.d.
DuPont 5u	6-AcO	Ph	$C_{18}H_{14}N_2O_3$	4.6	n.d.
DuPont 5v	6-anthra	Ph	$C_{23}H_{17}N_3O_3$	**0.7**	**0.4**
DuPont 5w	H	Me	$C_{11}H_{10}N_2O$	1.5	n.d.

2. 2-Substituted Quinazolin-4 (3H)-ones

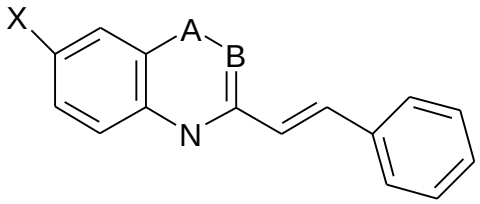


Compound	**R**	**Formula**	**IC_{50}**	**$IC_{50\ (TOX)}$**
DuPont 15a	Me	$C_9H_7ClN_2O$	22	n.d.
DuPont 15b	Phenylethynyl	$C_{16}H_9ClN_2O$	Not active	n.d.
DuPont 15c	$Ph(CH_2)_2$	$C_{16}H_{13}ClN_2O$	Not active	n.d.
DuPont 15d	$PhCH_2$	$C_{15}H_{11}ClN_2O$	Not active	n.d.
DuPont 15e		$C_{15}H_{10}ClN_3O$	Not active	n.d.

3. Michael Adducts of 2- Styrylquinazolinones

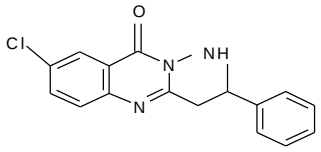
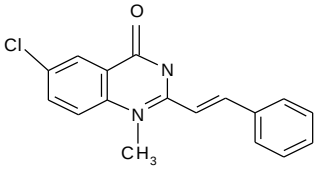
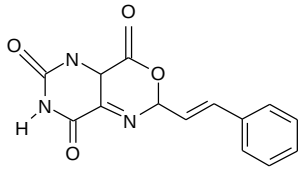
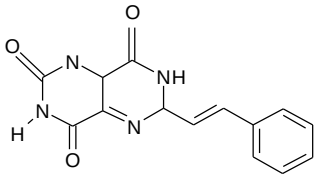
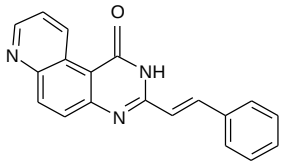
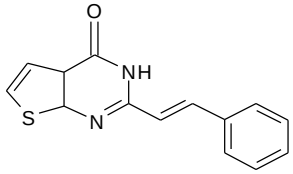
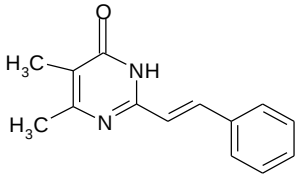
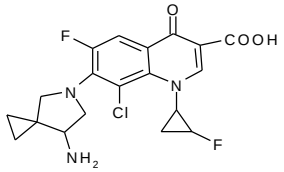
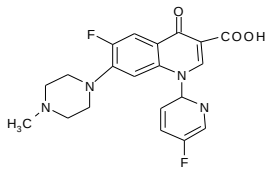


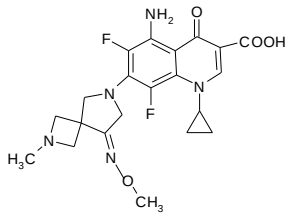
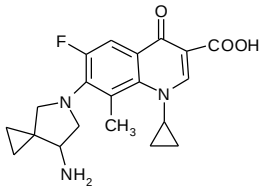
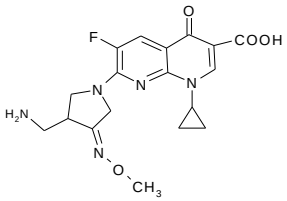
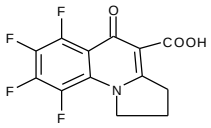
Compound	X	R	Formula	IC_{50}	$IC_{50\ (TOX)}$
DuPont 9a	Cl	N-Me-piperazinyl	$C_{21}H_{23}ClN_4O$	1.7	n.d.
DuPont 9b	Cl	N-morpholino	$C_{20}H_{20}ClN_3O_2$	**0.9**	**0.6**
DuPont 9c	MeO	N-piperidyl	$C_{22}H_{25}N_3O_2$	**0.7**	**0.4**



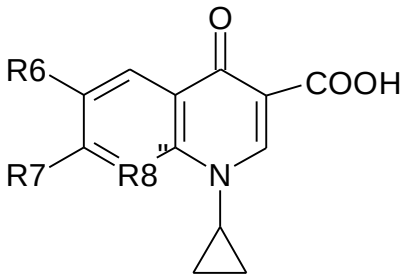
Compound	X	A / B	Formula	IC_{50}	$IC_{50\ (TOX)}$
DuPont 16b	Cl	CONNH	$C_{16}H_{12}ClN_3O$	**0.5**	**1.4**
DuPont 17	Cl	CSNH	$C_{16}H_{11}ClN_2S$	Not active	n.d.
DuPont 19	Cl	RNCN	$C_{25}H_{31}ClN_4$	**0.6**	**1.8**
DuPont 22	H	SO_2NH	$C_{15}H_{12}N_2O_2S$	Not active	n.d.

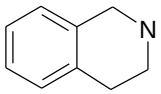
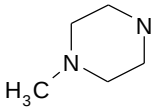
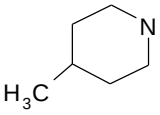
4. Other ring systems

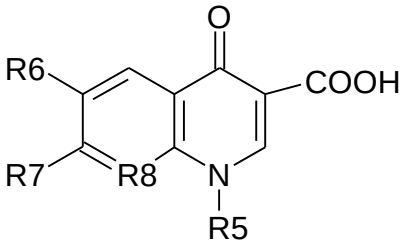
Compound	Structure	Formula	IC_{50}	$IC_{50\ (TOX)}$
DuPont 16c		$C_{16}H_{11}ClN_2O$	Not active	n.d.
DuPont 24		$C_{17}H_{13}ClN_2O$	**0.7**	**1.7**
DuPont 25		$C_{14}H_9N_3O_4$	Not active	n.d.
DuPont 26		$C_{14}H_{10}N_4O_3$	Not active	n.d.
DuPont 27		$C_{19}H_{13}N_3O$	4.6	n.d.
DuPont 28		$C_{14}H_{10}N_2OS$	4.0	n.d.
DuPont 29		$C_{14}H_{14}N_2O$	2.3	n.d.
DU 6859a		$C_{19}H_{19}F_2N_3O_3$	17.3	n.d.
DW 116		$C_{20}H_{18}F_2N_4O_3$	Not active	n.d.

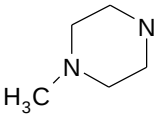
DW 271		$C_{21}H_{23}F_2N_5O_4$	**0.7**	**300**
HSR 903		$C_{20}H_{23}FN_4O_3$* CH_4O_3	1.75	n.d.
LB 20304		$C_{18}H_{20}FN_5O_4$* H_2O	1.4	n.d.
UM 36		$C_{13}H_7NF_4O_3$	Not active	n.d.
UM 35	Methylester of Compound 36	$C_{14}H_9NF_4O_3$	16.4	n.d.

4. Other ring systems (continued)

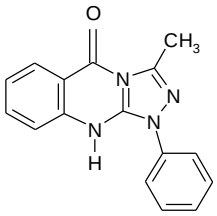
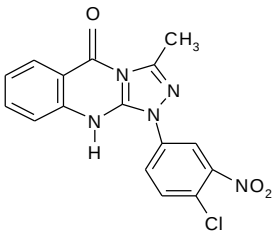


Compound	R6	R7	R8	Formula	IC_{50}	$IC_{50\ (TOX)}$
VG 6/1	NO_2		CCH_3	$C_{23}H_{21}N_3O_5$	6.1	n.d.
MF 5137	NH_2		CCH_3	$C_{23}H_{23}N_3O_3$	7.8	n.d.
MF 5168	NO_2		CH	$C_{18}H_{19}N_4O_5$	Not active	n.d.
MF 5101	NH_2		CH	$C_{18}H_{21}N_4O_3$	11.6	n.d.
MF 5142	NH_2		CH_3	$C_{20}H_{24}N_3O_3$	16.5	n.d.

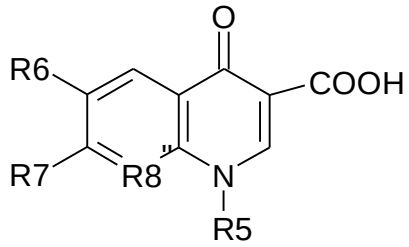


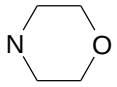
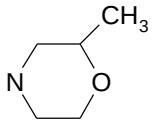
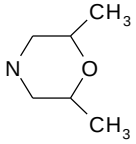
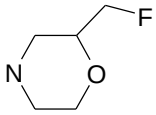
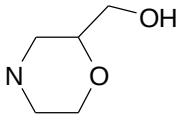
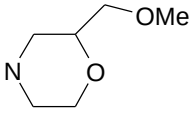
Compound	R5	R6	R7	R8	Formula	IC_{50}	$IC_{50\ (TOX)}$
MF 5124	C_4H_9	NH_2		CH	$C_{19}H_{25}N_4O_3$	Not active	n.d.
WF1	C_6H_5F	NH_2		CH	$C_{21}H_{21}FN_4O_3$	Not active	n.d.

4. Other ring systems (continued)

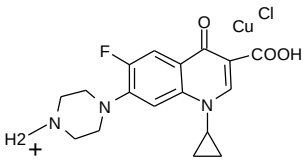
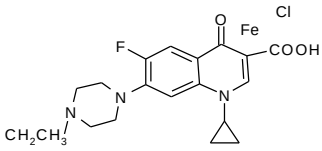
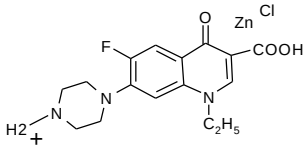
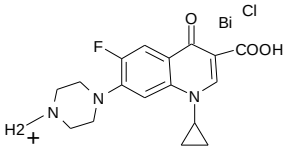
Compound	Structure	Formula	IC_{50}	$IC_{50\ (TOX)}$
V 1		$C_{16}H_{13}N_4O$	2.3	n.d.
V 2		$C_{16}H_{11}ClN_5O_3$	2.7	n.d.

5. Thiazolopyrazine incorporated tetracyclic quinolones

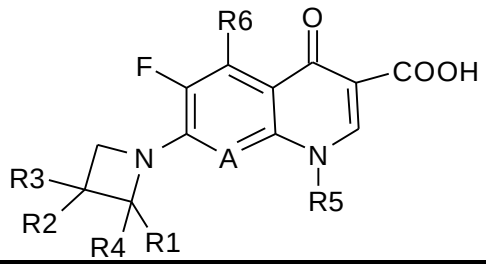


Compound	X	Formula	$IC_{50(Tryp)}$	$IC_{50\ (TOX)}$
Kanebo 8a		$C_{18}H_{16}FN_3O_4S$	8.8	n.d.
Kanebo 8b		$C_{19}H_{18}FN_3O_4S$	1.6	n.d.
Kanebo 8c		$C_{20}H_{20}FN_3O_4S$	14.6	n.d.
Kanebo 8f		$C_{19}H_{17}F_2N_3O_4S$	1.3	n.d.
Kanebo 8g		$C_{19}H_{18}FN_3O_5S$	**0.8**	**7.3**
Kanebo 8h		$C_{20}H_{20}FN_3O_5S$	**0.3**	**12.6**

6. Metal - ion quinolones

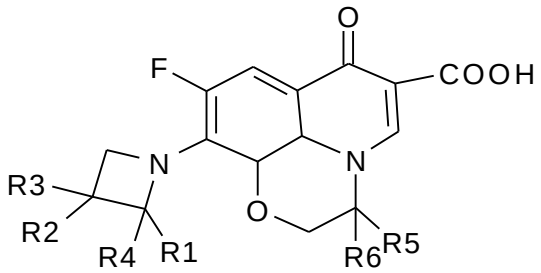
Compound	Structure	Formula	IC_{50}	$IC_{50\ (TOX)}$
UL 1		$[Cu(cf)_2]Cl_2 * 6\ H_2O$	5.6	n.d.
UL 2	Not known	$(CfH_2)(cfH)[CuCl_4]Cl*H_2O$	3.6	n.d.
UL 3		$ErxH_2[FeCl_4]Cl$	21	n.d.
UL 4		$(NfH_2)(nfH)[ZnCl_4]Cl*H_2O$	13.7	n.d.
UL 5		$(CfH_2)(cf)(BiCl_6)*2\ H_2O$	**0.1**	**33**

7. Azetidinyl fluoroquinolones

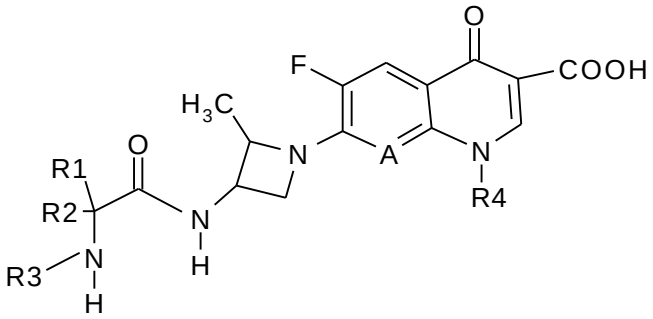


Comp.	R1	R2	R3	R4	R5	R6	A	Formula	IC_{50}	IC_{50TOX}
E-4535	CH_3	H	NH_2	H	c-C_3H_5	H	CH	$C_{17}H_{17}FN_3O_3$	Not active	n.d.
E-4534	CH_3	H	NH_2	H	c-C_3H_5	H	CF	$C_{17}H_{16}F_2N_3O_3$	Not active	n.d.
E-4528	H	CH_3	NH CH_3	H	c-C_3H_5	H	CH	$C_{18}H_{19}FN_3O_3$	Not active	n.d.
E-4527	H	CH_3	NH CH_3	H	c-C_3H_5	H	CF	$C_{18}H_{18}F_2N_3O_3$	2.2	n.d.
E-4696	H	NH_2	H	CH_3	c-C_3H_5	H	CH	$C_{17}H_{18}FN_3O_3$	5.0	n.d.
E-4629	CH_3	H	NH_2	H	c-C_3H_5	H	CH	$C_{17}H_{18}FN_3O_3$	3.0	n.d.
E-4628	CH_3	H	NH_2	H	c-C_3H_5	H	CF	$C_{17}H_{17}F_2N_3O_3$	3.0	n.d.
E-4591	CH_3	NH_2	H	H	c-C_3H_5	H	CF	$C_{17}H_{17}F_2N_3O_3$	4.7	n.d.
E-4811	H	H	NH_2	CH_3	c-C_3H_5	H	CF	$C_{17}H_{17}F_2N_3O_3$	Not active	n.d.
E-4555	CH_3	H	NH_2	H	c-C_3H_5	H	N	$C_{17}H_{17}F_2N_3O_3$	4.0	n.d.
E-4695	CH_3	H	NH_2	CH_3	c-C_3H_5	H	N	$C_{16}H_{17}FN_4O_3$	4.7	n.d.
E-4630	CH_3	H	NH_2	H	c-C_3H_5	H	N	$C_{16}H_{17}FN_4O_3$	3.3	n.d.
E-4715	H	NH_2	H	CH_3	c-C_3H_5	H	CCl	$C_{17}H_{17}ClFN_3O_3$	3.3	n.d.
E-5091	CH_3	H	NH_2	H	c-C_3H_5	H	CCl	$C_{17}H_{17}ClFN_3O_3$	Not active	n.d.
E-4619	H	NH_2	H	CH_3	C_2H_5	H	CF	$C_{17}H_{19}FN_4O_3$	5.2	n.d.
E-5112	H	NH_2	H	CH_3	C_2H_5	NH_2	CF	$C_{16}H_{18}F_2N_4O_3$	1.8	n.d.
E-4625	H	NH_2	H	CH_3	4-FPh	H	CF	$C_{20}H_{16}F_3N_3O_3$	2.3	n.d.
E-4626	H	NH_2	H	CH_3	2,4-F_2Ph	H	CF	$C_{20}H_{15}F_4N_3O_3$	3.5	n.d.
E-4724	CH_3	NH_2	H	CH_3	2,4-F_2Ph	H	CF	$C_{20}H_{15}F_4N_3O_3$	6.5	n.d.

E-4685	H	NH_2	H	CH_3	2,4-F_2Ph	H	N	$C_{19}H_{15}F_3N_4O_3$	1.3	n.d.
E-4936	CH_3	H	NH_2	H	2,4-F_2Ph	H	N	$C_{19}H_{15}F_3N_4O_3$	6.0	n.d.
E-4868	H	NH_2	H	CH_3	2,4-F_2Ph	H	CH	$C_{20}H_{16}F_3N_3O_3$	Not active	n.d.
E-4935	CH_3	H	NH_2	H	2,4-F_2Ph	H	CH	$C_{20}H_{16}F_3N_3O_3$	6.2	n.d.
E-5100	H	NH_2	H	CH_3	2,4-F_2Ph	NH_2	CF	$C_{20}H_{16}F_4N_4O_3$	Not active	n.d.

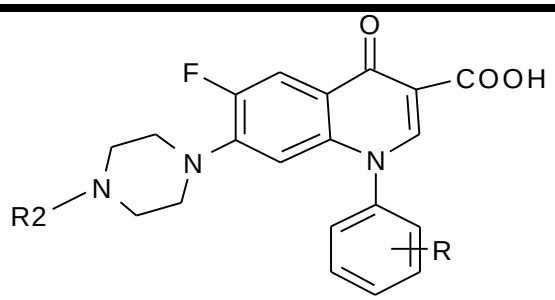


Compound	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**Formula**	**IC_{50}**	**IC_{50} (TOX)**
E-4548	H	H	NH_2	H	H	CH_3	$C_{16}H_{16}F_3N_3O_4$	Not active	n.d.
E-4487	H	CH_3	NH_2	H	CH_3	H	$C_{17}H_{18}FN_3O_4$	Not active	n.d.
E-4497	H	CH_3	NH_2	H	H	CH_3	$C_{17}H_{18}FN_3O_4$	Not active	n.d.
E-4654	H	CH_3	NHMe	H	H	CH_3	$C_{18}H_{20}FN_3O_4$	Not active	n.d.
E-4549	H	H	NMe_2	H	H	CH_3	$C_{18}H_{20}FN_3O_4$	5.8	n.d.
E-4550	H	CH_3	NMe_2	H	H	CH_3	$C_{19}H_{22}FN_3O_4$	Not active	n.d.
E-4572	H	CH_3	CH_2NHEt	H	H	CH_3	$C_{20}H_{24}FN_3O_4$	Not active	n.d.
E-4627	CH_3	H	NH_2	H	H	CH_3	$C_{17}H_{18}FN_3O_4$	8.8	n.d.



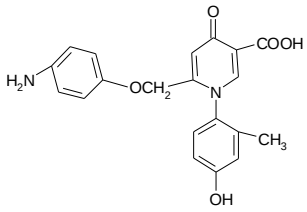
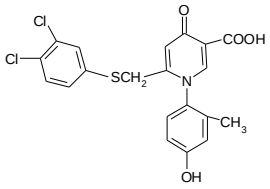
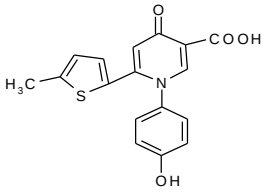
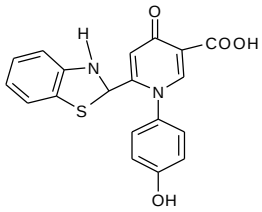
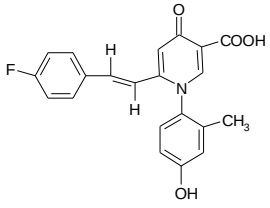
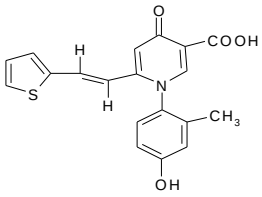
Compound	A	R1	R2	R3	R4	Formula	IC_{50}	IC_{50} (TOX)
E-4889	N	H	CH_3	H	c-C_3H_5	$C_{19}H_{23}ClFN_5O_4$	Not active	n.d.
E-4947	N	CH_3	H	H	c-C_3H_5	$C_{19}H_{23}ClFN_5O_4$	Not active	n.d.
E-4972	N	CH_3	H	H	c-C_3H_5	$C_{19}H_{23}ClFN_5O_4$	Not active	n.d.
E-4898	N	H	Isobutyl	H	c-C_3H_5	$C_{22}H_{19}ClFN_5O_4$	3.8	n.d.
E-4996	N	H	CH_3	l-alanine	c-C_3H_5	$C_{22}H_{28}ClFN_6O_5$	6.2	n.d.
E-5008	CH	H	CH_3	H	2,4-F_2Ph	$C_{30}H_{29}F_3N_4O_7S$	Not active	n.d.
E-5019	CH	H	n-propyl	H	2,4-F_2Ph	$C_{32}H_{33}F_3N_4O_7S$	Not active	n.d.

8. N-1 Aryl substituted quinolones

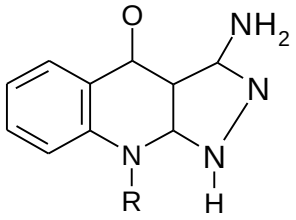


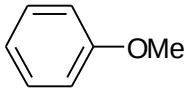
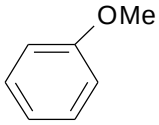
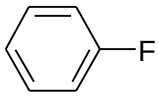
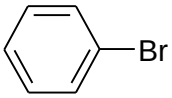
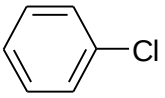
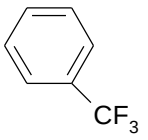
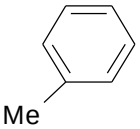
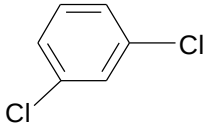
Compound	R2	R	Formula	IC_{50}	$IC_{50\ (TOX)}$
H 1	H	H	$C_{20}H_{18}FN_3O_3$	8.7	n.d.
H 2	H	o-OCH_3	$C_{21}H_{20}FN_3O_4$	Not active	n.d.
H 3	H	o-CH_3	$C_{21}H_{20}FN_3O_3$	Not active	n.d.
H 4	H	p-CH_3	$C_{21}H_{20}FN_3O_3$	1.1	n.d.
H 5	H	p-CF_3	$C_{21}H_{17}F_4N_3O_3$	5.2	n.d.
H 6	H	o-F	$C_{20}H_{17}F_2N_3O_3$	Not active	n.d.
H 7	H	p-NO_2	$C_{20}H_{17}FN_4O_5$	Not active	n.d.
H 8	H	m-OH	$C_{20}H_{18}FN_3O_4$	22.4	n.d.
H 9	CH_3	p- OCH_3	$C_{22}H_{22}FN_3O_4$	Not active	n.d.
H 10	CH_3	m-OCH_3	$C_{22}H_{22}FN_3O_4$	Not active	n.d.

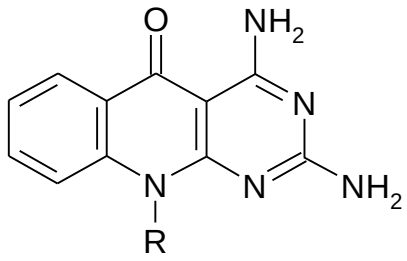
9. Dihydronicotine acids derivatives

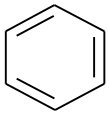
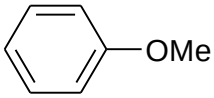
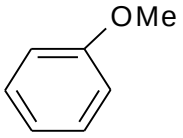
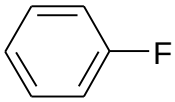
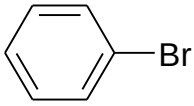
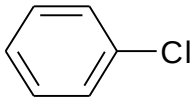
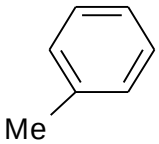
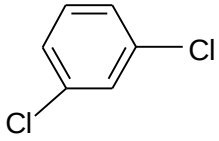
Compound	Structure	Formula	IC_{50}	$IC_{50\ (TOX)}$
G-64070		$C_{20}H_{18}N_2O_5$	Not active	n.d.
G-67012		$C_{20}H_{15}Cl_2NO_4S$	Not active	n.d.
G-71258		$C_{17}H_{13}NO_4S$	Not active	n.d.
G-59030		$C_{19}H_{14}N_2O_4S$	Not active	n.d.
G-72800		$C_{21}H_{16}FNO_4$	Not active	n.d.
G-71286		$C_{19}H_{15}NO_4S$	Not active	n.d.

10. R-2/3 Derivatives

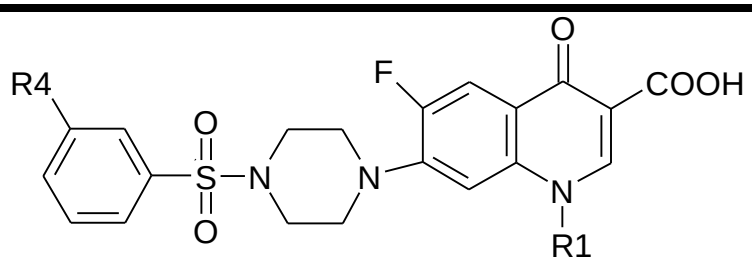


Compound	R	Formula	IC_{50}	$IC_{50(TOX)}$
UV 1		$C_{16}H_{12}N_4O$	Not active	n.d.
UV 3		$C_{17}H_{14}N_4O_2$	Not active	n.d.
UV 5		$C_{17}H_{14}N_4O_2$	4.9	n.d.
UV 7		$C_{16}H_{11}FN_4O$	Not active	n.d.
UV 9		$C_{16}H_{11}BrN_4O$	Not active	n.d.
UV 11		$C_{16}H_{11}ClN_4O$	Not active	n.d.
UV 13		$C_{17}H_{11}F_3N_4O$	Not active	n.d.
UV 14		$C_{17}H_{14}N_4O$	Not active	n.d.
UV 16		$C_{16}H_{10}Cl_2N_4O$	Not active	n.d.



Compound	R	Formula	IC_{50}	$IC_{50(TOX)}$
UV 2		$C_{17}H_{13}N_5O$	7.0	n.d.
UV 4		$C_{18}H_{15}N_5O_2$	3.4	n.d.
UV 6		$C_{18}H_{15}N_5O_2$	**1.1**	n.d.
UV 8		$C_{17}H_{12}FN_5O$	2.0	n.d.
UV 10		$C_{17}H_{12}BrN_5O$	5.9	n.d.
UV 12		$C_{17}H_{12}ClN_5O$	4.4	n.d.
UV 15		$C_{18}H_{15}N_5O$	4.0	n.d.
UV 17		$C_{17}H_{11}Cl_2N_5O$	6.9	n.d.

11. Sulfanilyl Fluoroquinolones



Compound	R1	R4	Formula	IC_{50}	$IC_{50\ (Tox)}$
NSFQ-104	C_2H_5	NH_2	$C_{22}H_{23}FSN_4O_5$	2.2	n.d.
NSFQ-105	C_3H_5	NH_2	$C_{23}H_{23}FSN_4O_5$	2.35	n.d.
CH_3HNSNx	C_2H_5	$NHCH_3$	$C_{23}H_{25}FSN_4O_5$	1.6	n.d.
CH_3HNScx	C_3H_5	$NHCH_3$	$C_{24}H_{25}FSN_4O_5$	1.6	n.d.
NO_2SNx	C_2H_5	NO_2	$C_{22}H_{21}FSN_4O_7$	3.3	n.d.
NO_2SCx	C_3H_5	NO_2	$C_{23}H_{21}FSN_4O_5$	4.6	n.d.
HSNx	C_2H_5	H	$C_{22}H_{22}FSN_2O_5$	1.8	n.d.
HSCx	C_3H_5	H	$C_{23}H_{22}FSN_2O_5$	1.9	n.d.
$N(CH_3)_2$SNx	C_2H_5	$N(CH_3)_2$	$C_{24}H_{27}FSN_4O_5$	**0.9**	**>250**
$N(CH_3)_2$SCx	C_3H_5	$N(CH_3)_2$	$C_{25}H_{27}FSN_4O_5$	Not active	n.d.
CH_3SNx	C_2H_5	CH_3	$C_{23}H_{24}FSN_3O_5$	2.1	n.d.
CH3SCx	C_3H_5	CH_3	$C_{24}H_{24}FSN_3O_5$	Not active	n.d.
OCH_3SNx	C_2H_5	OCH_3	$C_{23}H_{24}FSN_3O_6$	**0.9**	**>250**
OCH_3SCx	C_3H_5	OCH_3	$C_{24}H_{24}FSN_3O_6$	Not active	n.d.

Legend to Table 1-11: n.d. = not determined

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