Treatment of African Trypanosomosis with DB 75: Pharmacokinetics, relapses and cross resistance

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Prof. H. J. Wirz
Dekan

dedicated to my parents

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Abbrevations

α-AGP	Alpha-1-acidic glycoprotein
ANOVA	Analysis of variance
ASPT	Adenosine sensitive pentamidine transporter
AUC	Area under the curve
AUMC	Area under the first moment curve
BBB	Blood brain barrier
bp	Base pair
BVET	Swiss Federal Veterinary Department
CASY	Cell counter and analysing system
cDNA	Complementary DNA
C_{max}	Peak concentration
CNS	Central nervous system
CSF	Cerebro spinal fluid
CYP P 450	Cytochrome P 450
DEAE	2-[(Diethylamino)ethyl]cellulose
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FPIX	Ferriprotoporphyrin IX
HAPT	High affinity pentamidine transporter
HAT	Human African trypanosomosis
HEPES	N-2-Hydroxyethylpiperazine-N`-2-ethanesulfonic acid
HPLC	High pressure liquid chromatography
IC_{50}	50% inhibitory concentration
ILRAD	International Laboratory for Research on Animal Diseases – Kenya
k_{el}	Constant of elimination
LAPT	Low affinity pentamidine transporter
LILIT	Long incubation low inoculation test
MDR	Multiple drug resistance
MEM	Minimum essential medium
MIC	Minimal inhibitory concentration
mRNA	Messenger RNA
MRT	Mean residence time
MS	Mass spectrometry
NPP	New permeability pathway
P, log P	Partition coefficient
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PSG	Phosphate-buffered saline with glucose
RNA	Ribonucleic acid
RT	Reverse transcription
SCID	Severe combined immune deficiency
SL	Spliced leader
spp	Species
$t_{1/2}$	Half-life
TFA	Trifluoroacetic acid
t_{max}	Time, when C_{max} occurs
UNC	University of North Carolina

SUMMARY

Resurgent human African trypanosomosis (HAT) displays a major burden for people living in Sub-Saharan Africa, although it had been nearly eradicated in the 50`s, due to selective ground spraying with DDT and an extensive vector control. Today, treatment of HAT relies only on a few drugs at hand. The use of these drugs is restricted, since they show an unacceptable toxicity, complicated administration, emerging and spreading of resistance, shortness of availability and/or show only an impact on gambiense or rhodesiense trypanosomes. Therefore, new drugs are urgently needed.

A consortium, the UNC lead consortium to discover new drugs for the treatment of parasitic diseases, was founded with the aim to improve treatment of HAT. The ideal profile of a new drug is an uncomplicated synthesis, easy administration, high efficacy, cost effectiveness and no toxicity. Series of compounds were synthesised in analogy to pentamidine, the first line drug in the treatment of first stage trypanosomosis. The so called DB compounds showed promising results in vitro and in vivo, not only to trypanosomes but also to *Leishmania spp, Mycobacterium tuberculosis, Plasmodium spp* and *Pneumocystis carinii*. Lead compound of this project is the diamidine DB 75 and its oral applicable prodrug DB 289.

Although the prodrug had entered an open label phase IIa clinical trial already when this thesis started, several questions referring to the interplay between DB 75, trypanosomes and mice had been unanswered. To elucidate development of parasitaemia in first infection, first and second relapse in mice, studies have been undertaken in immunocompetent and –deficient mice, with two different trypanosome strains and various treatment schedules for DB 75.
At the same time, parasite clearance after drug application was studied. Since relapse trypanosomes did not show any differences in sensitivity to selected drugs, observed relapses were not due to resistant trypanosomes but rather due to re-invasion from extravascular sites. Identification of these sites was a second goal of this project. A method based on the detection of trypanosomal mRNA by nested PCR was established and tested. Results showed that more time has to be invested to increase the sensitivity of this method. However, one organ was found positive for trypanosomal mRNA, indicating the spleen to be one of the niches.
A system was established to check DB 75 for cross resistance in melarsoprol and pentamidine resistant laboratory strains. With the aid of this system, further upcoming drugs may be tested

for their cross resistance. For DB 75, only a low cross resistance was obtained in both resistant strains.

Additionally, the pharmacokinetics for DB 75, DB 820 and the their corresponding prodrugs DB 289, DB 844 in trypanosome infected and uninfected mice was studied. Differences in plasma levels of uninfected compared to infected mice were obtained. Mainly, two effects were shown to influence plasma levels considerably: accumulation of active drug in case of DB 75 and DB 289, and inhibition of metabolism for DB 820 and DB 844.
The extent of DB 75 accumulation in trypanosomes was determined in vitro, to assess that the differences between plasma levels of DB 75 after prodrug application obtained in infected mice compared to uninfected mice were due to accumulation. In a further study, indications of the presence of possible active metabolites were obtained when activity of plasma samples was assessed by bioassay and compared to levels determined by HPLC/MS/MS.
In another pharmacokinetic study, it was shown that conversion of DB 289 to DB 75 in *P. berghei* infected mice was poor, leading to subtherapeutic doses of active compound, which explained that no activity in the in vivo model was obtained for the prodrug compared to a moderate to good activity for the active compound.

ZUSAMMENFASSUNG

Die wiederauftretende Afrikanische Schlafkrankheit stellt eine grosse Bürde für die Menschen, die südlich der Sahara leben dar, obschon die Krankheit während der 50-iger Jahren aufgrund des Gebrauchs von DDT und einer umfassenden Vektorkontrolle fast ausgerottet worden war. Heute basiert die Behandlung der Afrikanischen Schlafkrankheit auf wenigen zur Verfügung stehenden Medikamenten. Der Einsatz dieser Medikamente wird durch eine inakzeptable Toxizität, eine komplizierte Verabreichung, das Auftreten und die Ausbreitung von Resistenzen, die limitierte Verfügbarkeit und/oder die ausschliessliche Wirkung gegenüber gambiense oder rhodesiense Trypanosomen eingeschränkt. Neue Medikamente werden deshalb dringend benötigt.

Ein Konsortium, das von der UNC geführte Konsortium zur Entwicklung neuer Wirkstoffe für die Behandlung parasitärer Erkrankungen, wurde gegründet um die Behandlung der Afrikanischen Schlafkrankheit zu verbessern. Das Anforderungsprofil eines solchen Wirkstoffes sieht folgendermassen aus: unkomplizierte Synthese, einfache Verabreichung, hohe Wirksamkeit, Kosteneffizienz und keine Toxizität. Ganze Serien von Verbindungen wurden in Analogie zu Pentamidine, dem Medikament der Wahl bei Afrikanischer Schlafkrankheit im Erststadium, synthetisiert. Die so genannten DB Verbindungen zeigten vielversprechende Wirkungen in vitro und in vivo, nicht nur gegen Trypanosomen, sondern auch gegen Leishmanien, *Mycobacterium tuberculosis*, Plasmodien und *Pneumocystis carinii*. Die beste Verbindung (lead compound) in diesem Projekt ist DB 75 und ihr oral applizierbarer Prodrug DB 289.

Obwohl sich der Prodrug bereits in der Phase IIa einer offenen klinischen Studie befand als diese Doktorarbeit begann, waren einige Fragen bezüglich der Wechselwirkungen zwischen DB 75, Trypanosomen und Mäusen unbeantwortet. Studien wurden in immunokompetenten und immundefizienten Mäusen, mit zwei verschiedenen Stämmen von Trypanosomen und unterschiedlichen Behandlungsschemata mit DB 75 durchgeführt, um die Entwicklung einer Parasitaemia bei einer Erstinfektion, beim ersten und zweiten Rückfalls zu klären. Gleichzeitig wurde die Elimination der Parasiten nach Behandlung untersucht. Da die Trypanosomen nach einem Rückfall keinen Unterschied bezüglich der Sensitivität gegenüber ausgewählten Wirkstoffen zeigten, wurden die Rückfälle weniger den resistenten Trypanosomen sondern eher der Wiedereinwanderung von Trypanosomen aus

extravaskulären Orten zugeschrieben. Die Identifikation dieser Orte war ein zweites Ziel dieser Arbeit. Eine Methode, die auf dem Nachweis der trypanosomalen mRNA durch nested PCR basiert, wurde entwickelt und getestet. Die Ergebnisse zeigten, dass mehr Zeit zur Erhöhung der Sensitivität dieser Methode investiert werden müsste. Trotzdem wurde in einem Organ trypanosomale mRNA nachgewiesen, was darauf hinwies, dass die Milz eine dieser Nischen ist.

Ein System wurde erstellt, mit dem die Kreuz-Resistenz von DB 75 gegenüber Melarsoprol und Pentamidine resistenten Laborstämmen geprüft werden konnte. Mit Hilfe dieses Systems können weitere Verbindungen auf ihre Kreuz-Resistenz hin überprüft werden. Im Falle von DB 75 konnte nur eine geringe Kreuz-Resistenz gegenüber beiden resistenten Stämmen nachgewiesen werden.

Zusätzlich wurde die Pharmakokinetik von DB 75, DB 820 und den dazugehörigen Prodrugs DB 289, DB 844 in mit Trypanosomen infizierten und nicht infizierten Mäusen untersucht. Unterschiede in den Plasmaspiegeln von nicht infizierten und infizierten Mäusen wurden erhalten. Hauptsächlich beeinflussen zwei Effekte den Plasmaspiegel beträchtlich: die Anreicherung der aktiven Verbindung im Falle DB 75, DB 289 und die Hemmung des Metabolismus im Falle von DB 820, DB 844.

Das Ausmass der Anreicherung von DB 75 in Trypanosomen wurde in vitro bestimmt um zu ermitteln, ob die DB 75 Plasmaspiegelunterschiede, die nach der Verabreichung des Prodrugs in infizierten Mäusen im Vergleich zu nicht infizierten erhalten wurde, auf dieser Anreicherung basierten. In einer weiteren Studie wurden Hinweise auf die Anwesenheit möglicher aktiver Metabolite erhalten, als die Aktivität der Plasmaproben durch den Bioassay bestimmt und mit den Spiegeln, die durch HPLC/MS/MS ermittelt wurden, verglichen wurde.

In einer anderen Pharmakokinetikstudie konnte gezeigt werden, dass die Umwandlung von DB 289 zu DB 75 in *P. berghei* infizierten Mäusen gering war und zu subtherapeutischen Dosen an aktivem Wirkstoff führte, was erklärte, dass man im in vivo Model keine Aktivität für den Prodrug im Vergleich zu einer mässigen bis guten Aktivität für die aktive Verbindung erhielt.

General Introduction

Sleeping sickness – the disease

One of the most severe diseases in Sub-Saharan Africa is human African trypanosomosis (HAT). It is estimated that 60 million people are at risk to be infected and that 300 000 new cases occur every year [WHO Geneva (1998)]. The causative agents are trypanosomes, protozoans belonging to the order Kinetoplastida, family Trypanosomatidae. Infection by two subspecies of this family, *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) and *Trypanosoma brucei gambiense* (*T. b. gambiense*) result in HAT. *T. b. rhodesiense* mainly occurs in Eastern Africa, whereas *T. b. gambiense* is present in Central and West Africa. Due to the limited distribution of the vector, the tsetse fly, which transmits the pathogens while biting, sleeping sickness occurs only in defined regions (foci). A distinction between an infection caused by *T. b. rhodesiense* and *T. b. gambiense* is possible based on pathogenesis and by immunological and molecular markers but not on morphological appearance. Generally, *T. b. rhodesiense* causes acute symptoms, appearing within a short time after infection, whereas the appearance of clinical symptoms in a *T. b. gambiense* infection can emerge months or years after the infectious bite. Once infected, people suffer from unspecific symptoms like fever, severe headache, joint pain, muscle aches, and others. These symptoms represent the first stage, the haemolymphatic stage. When trypanosomes invade the central nervous system (CNS) and cause neurological symptoms like sleep disturbances, alterations of mental state or sensory disorders, the second stage or meningoencephalitic stage is reached [WHO Geneva (1998)]. If untreated, patients always die.

Developmental cycle of *T. brucei*

Trypanosomes undergo different stages within their life cycle in both the mammalian host and the vector. Changes in morphology, metabolism and gene expression are the basis of the transformation into the different stages. The advantage of this multiple appearance is the adaptation to survival and the better evasion of the host´s immune system. In mammals and in the tsetse flies trypanosomes alter between proliferative phases, to establish themselves in the new environment, and non-proliferative phases, which serve as transition stages. These transition stages are important for the parasite, since within this stage of the life cycle the

organism prepares itself for the new host. The most striking changes occur within the mitochondrial system and at the surface of the flagellate. In the tsetse fly, variable surface glycoproteins (VSG) of bloodstream forms are replaced by procyclins (GPEET, EP). Energy metabolism has to change from one based on glucose (bloodstream forms) to one depending mainly on proline (procyclic forms in the tsetse fly) and vice versa.
In the mammalian blood the long slender (LS) form represents the proliferative stage, whereas the short stumpy (SS) form is non-proliferative and only able to survive when taken up by the vector where transformation into the procyclic stage is possible [Vickerman, K. (1985)]. Whether the short stumpy forms are susceptible to drugs is not yet known.

Control of HAT

Two approaches are carried out to improve the situation concerning HAT:

a) Vector control

A very important tool to reduce HAT is the elimination of the vector. Populations of tsetse flies can be reduced successfully with special traps and/or by using insecticides. In former days selective ground spraying with DDT was the method of choice, but has been discontinued due to environmental concerns. In October 2001, the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) was launched to eradicate the tsetse fly population by spreading sterile male tsetse flies. Such an approach was carried out successfully in a 4 year campaign (1994-98) on the Island of Zanzibar. Whether PATTEC will be successful remains uncertain, due to the extended study area and the vast diversity of tsetse fly species present.

Applying repellents directly to the skin and clothes is another possibility to prevent infection [Barrett, J. (1997)].

b) Drug treatment

Currently, five drugs are in use for the treatment of HAT. To cure patients in the first stage of infection, pentamidine or suramin are administered. Both drugs are highly protonated at physiological pH, so they are not able to cross the blood brain barrier (BBB).

Suramin, synthesised as dye 1916, shows a naphthalene like structure. It strongly binds to plasma proteins like albumin and low-density lipoproteins from where it is

slowly released. The mode of action is not fully elucidated, but interaction with enzymes due to its anionic structure is assumed to play a major role. [Denise, H. (2001)]. Suramin is mainly restricted to the treatment of early stage trypanosomosis caused by *T. b. rhodesiense* in Eastern Africa.

Pentamidine, an aromatic diamidine, was developed in analogy to synthalin, a hypoglycaemic agent. Parasites were found up to 48 hours after treatment in the blood which shows that this drug is slow acting [Pepin, J. (1994)]. Administration is performed to treat first stage sleeping sickness in West and East Africa. In contrast to suramin, pentamidine is reported to be actively accumulated in trypanosomes via specific transporters [de Koning, H.P. (2001)]. The mode of action of pentamidine is not fully understood. Interaction with the DNA at AT rich sites of the minor groove is discussed to be important for its anti-parasitic effect [Sands, M. (1985)], [Denise, H. (2001)], [Nok, A.J. (2003)].

Drugs like melarsoprol, eflornithine (both registered) and nifurtimox (not registered for HAT, but clinically used as add-on therapy with melarsoprol) cross to some extent the blood brain barrier and are used to treat 2nd stage infections.

Melarsoprol, a melaminophenyl arsenical, is the drug most frequently used in the treatment of second stage sleeping sickness despite its high toxicity. A rapid clearance of trypanosomes from blood within 30-120 min is reported for this drug [Pepin, J. (1994)]. Uptake into the trypanosome is performed by a nucleobase transporter: the P2 transporter [Carter, N.S. (1993)]. The mode of action is not yet revealed completely, but melarsoprol was shown to bind irreversibly to thiol groups. Therefore, all thiol-containing molecules or enzymes like trypanothione or glycerol-3-phosphate dehydrogenase are possible targets [Nok, A.J. (2003)], [Docampo, R. (2003)].

DMFO (D,L-α-difluoromethylornithine)/Eflornithine, is an ornithine analogue, which acts as inhibitor of the ornithine decarboxylase, an enzyme in the polyamine synthesis. It was developed as an anticancer agent and was found to be active also against *T. b. gambiense* infections [Denise, H. (2001)]. The affordability and the large doses required for treatment limits its use, apart from the specific action only against gambiense trypanosomes [Barrett, M.P. (2003)].

Nifurtimox, a synthetic nitrofuran, was developed for the treatment of American trypanosomosis (Chagas disease) caused by *T. cruzi*. Its use in HAT is mainly restricted

to patients which were refractory to melarsoprol [Barrett, M.P. (2003)], [Denise, H. (2001)].

Another drug, which has to be mentioned in this context is diminazene. Diminazene, also an aromatic diamidine as pentamidine, is used to treat animals infected with trypanosomes. This drug plays an important role in the control of trypanosomes in domestic livestock [Peregrine, A.S. (1993)]. Clinical use in humans was reported from several sites for first stage trypanosomosis, despite it has never been registered for human use [Pepin, J. (1994)].

Since all mentioned drugs are old, show severe adverse reactions, lack efficacy and are expensive, new drugs are urgently needed. However, development of new drugs to treat sleeping sickness was stopped due to economic interests in the 80`s and 90`s. A few years ago, several public-private-partnerships were founded and started to screen for drugs against parasitic diseases occurring in the developing countries.
Within the same period, new regimens for DMSO and melarsoprol were suggested and tested to improve the unsatisfactory situation in the treatment of HAT [Burri, C. (2000)], [Pepin, J. (2000)].
Today, supply with drugs already on the market is guaranteed since the drug companies Aventis and Bayer signed agreements in 2001 with the WHO to donate melarsoprol, pentamidine, DMFO, Suramin and Nifurtimox for 5 years [Barrett, M.P. (2003)]. In total U$25 million to support WHO`s activities in the field of African trypanosomosis for a five-year period was offered by Aventis. This donation comprises the production of the three key drugs pentamidine, melarsoprol and eflornithine, funds for the management and control of the disease, as well as funds for research [www.who.int/tdr/publications/ tdrnews/news65/default.htm, TDR news, No.65 (June 2001)]. Even though production of drugs is ensured, major efforts have to be made to improve the delivery of the drugs to places where they were urgently needed.

Problem of resistance

Resistance to drugs used for the treatment of HAT was reported in various high transmission areas and for most established drugs, but mainly for melarsoprol. Resistance is likely to occur when only a few drugs are available to treat the disease over a long period of time and when a

sufficient plasma level of drug cannot be reached (under-dosing) due to compliance problems or failure in treatment schedules. This favours selection of less susceptible trypanosomes. Reports on occurring relapses often do not distinguish whether these relapses were due to drug resistant trypanosomes or if they were due to patient related problems (e.g. metabolism, pharmacokinetics).

Different mechanisms are involved in development of resistance by the trypanosome:

1) Regulation of drug transport across the cell membrane by
   a) decrease of drug transport in the trypanosome caused by alteration of transporter proteins,
   b) increase of expression of an efflux pump like the P-glycoprotein (PGP), an ABC-transporter.

2) Alteration of target enzymes so that the drug is not able to bind and show its effect
   The DMFO resistance, for example, is believed to be caused by alteration of ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis [Matovu, E. (2001)].

Dicationic compounds

One of those mentioned new public private partnership is the “UNC lead consortium to discover new drugs for the treatment of parasitic diseases”, founded in 1999. The aim of this consortium is to improve treatment for certain parasitic diseases. Universities like Georgia State University, University of North Carolina at Chapel Hill, Ohio State University, London School of Hygiene and Tropical Medicine, Kenya Trypanosomiasis Research Institute and the Swiss Tropical Institute are involved in a project to improve the situation concerning HAT. Support is obtained by companies like Immtech International, MediChem, Applied Analytical International, Northwest Bioanalytical and Parexel. Funding is provided by the Bill and Melinda Gates foundation. The aim of this consortium is to develop a drug for first stage treatment, which may be produced very easily, cost efficient and preferably shows oral bioavailability. Thereto, series of new compounds were synthesised in analogy to the well efficient and well documented first line drug pentamidine. Since the active principle of pentamidine depends on two diamidine groups, which are separated by the so called spacer region, drugs were designed in structural similarity. Thereto, compounds showing variations

as well in the spacer regions as in the diamidine groups at either end were synthesised. Activity screening against parasites revealed that the distance between the diamidines is of major importance. The spacer region was shown to be necessary to keep the distance between the diamidine moieties. By chemical modelling of the spacer region, properties of the molecule like for example binding affinities to AT rich sequences within the DNA may be optimised.

Generally, aromatic diamidines, like DB 75, showed an effect in vivo against *Pneumocystis carinii* [Trent, J.O. (1996)], [Tidwell, R.R. (1990)], *Cryptosporidium parvum* [Trent, J.O. (1996)], [Blagburn, B.L. (1991)], and trypanosomes [Steck, E.A. (1981)] and in vitro against *Leishmania mexicana amazonensis* [Bell, C.A. (1990)], *Plasmodium falciparum* [Bell, C.A. (1990)], *Giardia lamblia* [Bell, C.A. (1991)], *Candida albicans* [Del Poeta, M. (1998b)], *Cryptococcus neoformans* [Del Poeta, M. (1998a)], and *Mycobacterium tuberculosis* [Tidwell R. R., personal communication].

DB compounds, analogues of pentamidine, were synthesised by Dr. David Boykin at Georgia State University, USA [Das, B.P. (1977)]. DB 75 is a diphenylfuran derivative with diamidine moieties at either end. This dicationic molecule interferes with the DNA by a binding to the minor groove with AT specificity. As a result of this binding, selective inhibition of DNA-dependent enzymes is achieved.
A remarkable feature of this molecule is the intensive fluorescence, with a maximum excitation wavelength at 365 nm and maximum emission wavelength at 465 nm [Taylor, Y. (2001)], which can be exploited in various qualitative and quantitative analyses, such as determination of distribution patterns, uptake studies and many others.

Another characteristic which DB 75 shares with its “ancestor” pentamidine is the accumulation in trypanosomes and plasmodia. Accumulation in mM concentration was obtained in trypanosomes [Bray, P.G. (2003)], and pentamidine concentration in plasmodia infected erythrocytes was 500x higher than in uninfected erythrocytes [Stead, A.M. (2001)]. Accumulation of DB 75 was found to occur preferably at sites where DNA is present as fluorescence microscopy reveals.
Due to the limited oral bioavailability of DB 75, the bis-O-methylamidoxime prodrug was synthesised (DB 289). The proposed metabolic pathways of DB 289 metabolised by liver

enzymes leads via two sequential O-demethylation and two amidoxime reduction steps to the active drug DB 75.

Other DB compounds like DB 820 and its prodrug DB 844 also showed promising results in vitro and in vivo. In the case of DB 820/DB 844 one phenyl ring is replaced by a pyridyl ring compared to the structure of DB 75/DB 289. This minor change in structure had a big effect on the uptake of compound through membranes. The more lipophilic compounds DB 820/DB 844 reached higher levels in plasma, and DB 844 was able to cross the blood brain barrier to a higher extent compared to DB 289. This was shown in the chronic mouse model where only 3 out of 5 mice were cured after application of DB 289, whereas total cure was achieved after DB 844 application.

DB 75 and Malaria

As already mentioned above, DB 75 is accumulated in plasmodia infected erythrocytes. Data obtained in the in vitro screening was very promising with an IC_{50} of 5.8 ng/ml for DB 75 in the strain K1 (chloroquine/pyrimethamine resistant) and of 17 ng/ml in the strain NF54 [Wenzler T., Scheurer C., personal communication]. In vivo data revealed moderate to good activity when DB 75 was applied to *P. berghei* infected mice, and almost no activity when DB 289 was administered. The reason for this discrepancy should be revealed within this Ph. D. project.

Another observation made, when in vivo testing is performed with DB compounds, is that relapses were obtained 20-30 days after the treatment period when mice were infected with trypanosomes of the *T. b. rhodesiense* strain STIB 900. Such relapses were not observed in mice infected with the trypanosome strain STIB 795 (*T. b. brucei*). The reasons for the re-appearance of trypanosomes in the blood is not known. Two possibilities are conceivable:

1. The applied drug is able to eliminate most of the trypanosomes. However, some trypanosomes may be resistant to drug pressure. Within a certain time, trypanosomes proliferated and it is possible to detect them in the blood, since the number of trypanosomes exceeded the detection limit when tail blood is inspected microscopically.

2. Re-appearance of trypanosomes in the blood is a due to a re-invasion process from extravascular sites which had served as niche. It is assumed that each organ creates a unique milieu, where drug levels different from the blood concentration may be found. Supposed, drug levels were lower in some organs, these organs may serve as niches, where proliferation independent of the amount of drug in the blood may take place and from where re-invasion after cessation of the drug is possible.

To find out more about these relapses was another aim of this thesis.

Goal of the thesis

A first goal is to gain a better understanding of the kinetics of parasitaemia after drug treatment in mice, disappearance and recrudescence, and the origin of relapses. A second goal is the assessment of cross resistance of DB 75 to melarsoprol and pentamidine resistant trypanosome strains. A third aim is to gain knowledge on the pharmacokinetics of DB 75 and DB 289 in infected and uninfected mice.

Objectives

A. To determine the kinetics of parasitaemia in control animals and animals treated with DB compounds and to assess the influence of the immune system on pathogenesis and cure of trypanosomosis
B. To elucidate the origin of relapses after subcurative treatment with DB compounds
C. To determine if cross resistances between melarsoprol and pentamidine resistant trypanosome strains and DB 75 exists
D. To determine the pharmacokinetics of the active compounds DB 75, DB 820 and their prodrugs DB 289, DB 844 in trypanosome infected and uninfected mice
E. To determine the accumulation factor for DB 75 in trypanosomes in vitro

F. To test for active metabolites in plasma samples of uninfected and trypanosome infected mice treated with DB 75, DB 820 and their corresponding prodrugs DB 289, DB 844

G. To determine the pharmacokinetics of DB 75 and its prodrug DB 289 in *P. berghei* infected mice and uninfected mice

References:

1. Barrett J. Control Strategies for African Trypanosomiasis: Their Sustainability and Effectiveness. In: Hide G, Mottram JC, Coombs GH, Holmes PH, editors. Trypanosomiasis and Leishmaniasis - Biology and Control. 1997. p. 347-59.

2. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ et al. The trypanosomiases. Lancet 2003; 362:1469-80.

3. Bell CA, Cory M, Fairley TA, Hall JE, Tidwell RR. Structure-activity relationships of pentamidine analogs against *Giardia lamblia* and correlation of antigiardial activity with DNA-binding affinity. Antimicrob Agents Chemother 1991; 35:1099-107.

4. Bell CA, Hall JE, Kyle DE, Grogl M, Ohemeng KA, Allen MA et al. Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. Antimicrob Agents Chemother 1990; 34:1381-6.

5. Blagburn BL, Sundermann CA, Lindsay DS, Hall JE, Tidwell RR. Inhibition of *Cryptosporidium parvum* in neonatal Hsd:(ICR)BR Swiss mice by polyether ionophores and aromatic amidines. Antimicrob Agents Chemother 1991; 35:1520-3.

6. Bray PG, Barrett MP, Ward SA, de Koning HP. Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. Trends Parasitol 2003; 19:232-9.

7. Burri C, Nkunku S, Merolle A, Smith T, Blum J, Brun R. Efficacy of new, concise schedule for melarsoprol in treatment of sleeping sickness caused by *Trypanosoma brucei gambiense*: a randomised trial. Lancet 2000; 355:1419-25.

8. Carter NS, Fairlamb AH. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature 1993; 361:173-6.

9. Das BP, Boykin DW. Synthesis and antiprotozoal activity of 2,5-bis(4-guanylphenyl)thiophenes and -pyrroles. J Med Chem 1977; 20:1219-21.

10. de Koning HP. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol Pharmacol 2001; 59:586-92.

11. Del Poeta M, Schell WA, Dykstra CC, Jones S, Tidwell RR, Czarny A et al. Structure-in vitro activity relationships of pentamidine analogues and dication-substituted bis-benzimidazoles as new antifungal agents. Antimicrob Agents Chemother 1998a; 42:2495-502.

12. Del Poeta M, Schell WA, Dykstra CC, Jones SK, Tidwell RR, Kumar A et al. In vitro antifungal activities of a series of dication-substituted carbazoles, furans, and benzimidazoles. Antimicrob Agents Chemother 1998b; 42:2503-10.

13. Denise H, Barrett MP. Uptake and mode of action of drugs used against sleeping sickness. Biochem Pharmacol 2001; 61:1-5.

14. Docampo R, Moreno SN. Current chemotherapy of human African trypanosomiasis. Parasitol Res 2003; 90 Supp 1:S10-S13.

15. Matovu E, Seebeck T, Enyaru JC, Kaminsky R. Drug resistance in *Trypanosoma brucei* spp., the causative agents of sleeping sickness in man and nagana in cattle. Microbes Infect 2001; 3:763-70.

16. Nok AJ. Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. Parasitol Res 2003; 90:71-9.

17. Pepin J, Khonde N, Maiso F, Doua F, Jaffar S, Ngampo S et al. Short-course eflornithine in Gambian trypanosomiasis: a multicentre randomized controlled trial. Bull World Health Organ 2000; 78:1284-95.

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

19. Peregrine AS, Mamman M. Pharmacology of diminazene: a review. Acta Trop 1993; 54:185-203.

20. Sands M, Kron MA, Brown RB. Pentamidine: a review. Rev Infect Dis 1985; 7:625-34.

21. Stead AM, Bray PG, Edwards IG, DeKoning HP, Elford BC, Stocks PA et al. Diamidine compounds: selective uptake and targeting in *Plasmodium falciparum*. Mol Pharmacol 2001; 59:1298-306.

22. Steck EA, Kinnamon KE, Rane DS, Hanson WL. *Leishmania donovani, Plasmodium berghei, Trypanosoma rhodesiense*: antiprotozoal effects of some amidine types. Exp Parasitol 1981; 52:404-13.

23. Taylor, Y. The antimicrobial agent 2,5-Bis(4-amidinophenyl)furan binds to acidocalcisomes in African trypanosomes. 2001. University of North Carolina, School of Pharmacy. Ref Type: Thesis/Dissertation

24. Tidwell RR, Jones SK, Geratz JD, Ohemeng KA, Bell CA, Berger BJ et al. Development of pentamidine analogues as new agents for the treatment of *Pneumocystis carinii* pneumonia. Ann N Y Acad Sci 1990; 616:421-41.

25. Trent JO, Clark GR, Kumar A, Wilson WD, Boykin DW, Hall JE et al. Targeting the minor groove of DNA: crystal structures of two complexes between furan derivatives of berenil and the DNA dodecamer d(CGCGAATTCGCG)2. J Med Chem 1996; 39:4554-62.

26. Vickerman K. Developmental cycles and biology of pathogenic trypanosomes. Br Med Bull 1985; 41:105-14.

27. WHO Geneva. Control and Surveillance of African Trypanosomiasis: report of a WHO Expert Commitee. 1998.

Studies of kinetics of parasitaemia and trypanosome disappearance after drug treatment of first infection and relapses

Abstract

Basic knowledge about a developing parasitaemia, clearance of trypanosomes after drug treatment and reappearance of trypanosomes of first infection and relapse was gained in immunocompetent and immunodeficient mice. Additionally, subpassaged trypanosomes were monitored, paying attention to the development of the parasitaemia, clearance of parasites after drug treatment and reappearance of trypanosomes in both immunocompetent and in immunosuppressed mice. This procedure was performed with the trypanosome strain STIB 900 (*T. b. rhodesiense*) and with trypanosomes of the strain STIB 795 (*T. b. brucei*). The latter strain was monitored only in immunocompetent mice. Trypanosome populations, isolated from first infection, first relapse and second relapse, were checked for changes in size distribution, morphological appearance and sensitivity to four selected compounds (DB 75, diminazene, melarsoprol and pentamidine) due to host factors and/or drug treatment.
It was shown that an intact immune system decreases the time for clearance of parasites from blood after drug treatment and prolongs the kinetics of a reappearance of parasites. Additionally, parasite free periods took longer when an intact immune system was present. Infections with STIB 795 could be cured totally when treated with DB 75, whereas treatment of mice infected with STIB 900 did not lead to complete cure. Relapse populations of trypanosomes did not show any differences in size distribution, morphological appearance and sensitivity to the selected drugs. These data indicated that relapses are not a problem of resistant parasites but rather of invasion of extravascular sites from where re-invasion of the blood is possible.

1. Introduction

One of the most life-threatening diseases in Africa still is human African trypanosomosis (HAT). The causative agents of HAT are the parasitic protozoans *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) which are transmitted by the bite of the tsetse fly. Two stages of infection can be distinguished according to clinical progression of the disease: the first or haemolymphatic stage, when

parasites are found in blood or lymphatic organs and the second or meningoencephalitic stage, when parasites have crossed the blood brain barrier and can be detected in the cerebrospinal fluid. Clinical symptoms of the haemolyphatic (first) stage are unspecific symptoms like fever, headache and others due to propagation of the parasites in blood and lymphatic fluids. Invasion of the central nervous system (CNS) leads to neurological symptoms (second stage) and if not treated to death. Treatment depends on whether CNS invasion has occurred or not. The drugs used to treat HAT are stage specific; however, they are old, expensive, lack efficacy and show severe adverse effects.

An international consortium founded by the Gates Foundation was launched in 1999 with the aim to improve the treatment of HAT. New drugs were designed modelling pentamidine, a first stage drug, which was applied against sleeping sickness in 1939 for the first time. The obtained analogues were tested in vitro and in vivo for their anti-trypanosomal activity. While testing these compounds in the mouse model an important observation was made: mice infected with STIB 795 (*T. b. brucei*) could be cured totally, whereas all mice infected with STIB 900 (*T. b. rhodesiense*.) relapsed after an aparasitic period. Interestingly, both strains showed a similar in vitro sensitivity to DB 75 when tested in the hypoxanthine incorporation assay with an incubation period of 40 h, showing an IC_{50} value of 6.48 ng/ml for STIB 795 (also named S427.1) and 9.28 ng/ml for STIB 900. Why these relapses were obtained only in the STIB 900 mouse model but not in the STIB 795 mouse model remains to be clarified. To better understand this phenomenon, basic knowledge about a developing parasitaemia and disappearance of trypanosomes after drug treatment in both mouse models (STIB 795 and STIB 900) is a pre-requisite. Thereto, trypanosome populations were isolated from mice after having undergone various treatment schedules and possible changes in morphology and in drug sensitivity were monitored.

By comparing the course of parasitaemia of the trypanosome populations established in immunocompetent vs. immunocompromised mice, the role of the immune system in a developing parasitaemia and its role in the clearance of parasites after drug treatment is assessable.

Additionally, knowledge about relapses may be gained. After having performed this study it will be possible to assess if these relapses are due to acquired resistance of the trypanosomes to the applied drug. Otherwise, it would be possible that trypanosomes invade extravascular sites which are less accessible to the drug and re-invade the blood from there after drug pressure decreased.

2. Materials and methods

In the following, the word line always refers to the different mouse strains, whereas the word strain refers to the different trypanosome sub-species.

2.1. Trypanosomes

2.1.1. *Trypanosoma brucei rhodesiense* (STIB 900)

STIB 900 is a derivative of STIB 704. Pathogens were isolated from a male patient at St. Francis Hospital in Ifakara/Tanzania in 1982. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenic growth in vitro.

2.1.2. *Trypanosoma brucei brucei* (STIB 795)

Trypanosomes were isolated by ILRAD -International Laboratory for Research on Animal Diseases, Kenya- (strain S427.1) from *Glossina pallidipes* in Uganda in 1960. After a passage in a sheep, a tsetse fly and several passages in mice, a clone was adapted to axenic cultivation in vitro.

2.2. Immunocompetent and immunodeficient mice

As immunocompetent mice, female NMRI mice, aged 4-6 weeks with a weight of 24-34 g were used. They were obtained from RCC, Ittingen, Switzerland.
Female SCID (Cb17/lcr-Prkdc^{scid}/Crl) mice, representing the immunodeficient mice, aged 4 weeks, with a weight of 17-20 g were purchased from Charles River, Sulzfeld, Germany.

Animal use adhered to the guidelines issued by the Swiss Federal Veterinary Department (BVET) for laboratory animals.

2.3. Drugs (Fig. 1)

The diamidine compound DB 75 was synthesised at Georgia State University, Atlanta, USA by the group of D. W. Boykin. Diminazene aceturate was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Melarsoprol was obtained from Specia France, Rhone-Mérieux, Toulouse, France, and Pentamidine isethionate (Pentacarinat) from Rhone-Poulenc, Thalwil, Switzerland.

2.4. Cultivation and in vitro screening

2.4.1. Culture medium

Bloodstream forms of trypanosomes were cultivated in Minimum Essential Medium (MEM) with Earle`s salts (powder, GIBCO, Invitrogen, Basel, Switzerland), supplemented with 25 mM HEPES (GIBCO, Invitrogen, Basel, Switzerland), 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM nonessential amino acids (100x, GIBCO, Invitrogen, Basel, Switzerland). The medium was further supplemented with 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine and 15% heat inactivated horse serum [Baltz, T. (1985)].

2.4.2. Screening medium (without hypoxanthine and thymidine)

For drug sensitivity assays the same medium as mentioned above was used but without the addition of hypoxanthine and thymidine.

2.4.3. Hypoxanthine solution

A solution containing 50 μCi/ml of ^{3}[H]-Hypoxanthine (Amersham Biosciences, Buckinghampshire HP7 9NA, UK) in screening medium was prepared.

2.5. Infection and treatment of mice

Mice were divided into 5 groups (A-E), each consisting of 5 mice. Groups A-C were infected at day 0 with trypanosomes. Group A served as untreated control group, whereas groups B and C each underwent a treatment period with DB 75 for 3 days starting at day 3 post infection (p.i.). After an aparasitic period, mice of group C were treated again with DB 75 for 3 days, at day 3 after the first trypanosome could be detected in the tail blood or immediately, when parasitaemia was classified as + (categories: see below: 2.6.1. Tail blood examination). Trypanosomes of a randomly chosen mouse of group B were subpassaged into mice of groups D and E. Mice of group D were left untreated, whereas mice of group E were treated with DB 75 for 3 days starting 3 days after the first trypanosome was detected in the blood or the day the mouse showed a + parasitaemia.

To characterise the trypanosome populations, cardiac puncture was performed with 3 out of 5 mice, the day parasitaemia was classified as +++ (or as ++ when mice showed severe symptoms of an acute trypanosome infection). For further investigations as morphological

examinations and drug sensitivity, trypanosomes were separated from blood by DEAE column [Lanham, S.M. (1970)].

The whole infection and treatment procedure (2.5.) was performed with 2 different strains of trypanosomes (STIB 795 and STIB 900) and within 2 different lines of mice (immuno-competent NMRI and immunodeficient SCID mice).

2.5.1. Infection of mice

Cryopreserved stabilates containing bloodstream forms of trypanosomes (STIB 795, STIB 900) were thawed and diluted with phosphate-buffered saline with glucose (PSG) buffer to a final concentration of $1*10^5$ trypanosomes/0.25 ml. Administration of parasites was performed intraperitoneally at day 0 into mice of groups A, B and C.

2.5.2. Subpassage of trypanosomes

Blood of mice, obtained from cardiac puncture of a parasitaemic mouse, was diluted with PSG to a final concentration of $2.5*10^4$ trypanosomes/0.25 ml and inoculated intraperitoneally into mice of groups D and E.

2.5.3. Treatment of mice

A drug solution containing 1 mg/ml DB 75 in 10% DMSO was prepared. 10 mg/kg body weight was administered intraperitoneally for 3 days once a day.

2.6. Monitoring the course of parasitaemia

2.6.1. Tail blood examination

Tail blood was checked daily in parasitic periods and twice a week in aparasitic periods. Thereto, one drop of blood obtained by tail snip was diluted with one drop of sodium citrate 3.2% in sterile water and observed under the microscope at a magnification of 200x. To classify the grade of parasitaemia, trypanosomes per field were counted and categorised using the table below:

Trypanosomes per field	**Category**
no trypanosome within 20 fields	((-))
1-20 trypanosomes within 20 fields, (x) representing the number of parasites	((X))
1-10 trypanosomes in 1 field	+
10-20 trypanosomes in 1 field	++
More than 20 trypanosomes in 1 field	+++

2.6.2. Count in Neubauer haemocytometer

Tail blood was diluted 1:10 with PSG buffer and stored at –80° C. After thawing, the number of trypanosomes were determined by a count in the Neubauer haemocytometer.

2.7. Harvesting trypanosomes

2.7.1. Cardiac puncture

Mice showing a medium or high grade parasitaemia (++ - +++), were anaesthetised with CO_2. Cardiac puncture was performed using a 1 ml syringe containing 0.05 ml PSG to which heparin (100 I.U., diluted 1: 50, Fresenius, Fresenius Medical Care Schweiz AG, Stans) had been added.

2.7.2. DEAE column

Trypanosomes were separated from blood components by anion exchange chromatography using DEAE cellulose [Lanham, S.M. (1970)].

2.7.3. Cryopreservation

Stabilates containing 10% glycerol in PSG were prepared from extracted trypanosomes and stored at –80° C.

2.8. Characterisation of trypanosomes

2.8.1. Morphological examination

2.8.1.1. Morphological appearance

Blood smears were made, fixed in methanol and stained with Giemsa solution (9% for 10 min). Trypanosome morphology was compared microscopically at a magnification of 500x and 1000x.

2.8.1.2. Size distribution

The size distribution of the trypanosomes was determined using the CASY (Cell Counter and Analysing System, Schärfe System GmbH, Reutlingen, Germany). A 1000-fold dilution of the trypanosome suspension (pre-diluted 1:5, 1:10 or 1:100, depending on the trypanosome density) was measured three times using a 60 μm capillary. Obtained data was analysed visually and an analysis of variance was performed using the statistic program SAS System. To determine any differences in the size, average volumes were analysed. The GLM Procedure (three-way ANOVA- **An**alysis **o**f **va**riance-) using the least squares analysis was chosen to test for differences.

2.8.2. Drug sensitivity

A drug sensitivity profile of each population was generated based on the IC_{50} values for DB 75, diminazene, melarsoprol and pentamidine obtained in the ^{3}[H]-hypoxanthine incorporation assay [Brun, R. (1989)].

To determine the IC_{50} of the trypanosome populations of each group, 3 mice per group were selected and their trypanosomes were separated from blood by DEAE column. The extracted parasites were used directly, or from cryopreserved stabilates. When using the cryopreserved stabilates, trypanosomes underwent several passages in BMEM medium until they showed a stable growth rate, before the assay was performed.

Hypoxanthine assay

Drug sensitivity was determined by assessing the viability of trypanosomes incubated with drug for 40 hours at 37° C and 5% CO_2. A serial drug dilution in screening medium was prepared in a 96-well plate and trypanosomes of a given concentration ($5*10^5$ trypanosomes/ml) were added to each well. Some wells served as control containing

trypanosomes and screening medium without drug, others as background consisting of screening medium without drug and without trypanosomes (see plate layout Fig. 2). After an incubation period of 24 hours, radioactively labelled hypoxanthine was added to each well for another 16 hours. Incorporation of radioactive hypoxanthine was determined for each well. Thereto, the contents of the wells were transferred onto a glass fibre filter (Filtermat A, Wallac, Perkin Elmer, Schwerzenbach, Switzerland) using a cell harvester (Wallac, Berthold Technologies GmbH, Regensdorf, Switzerland). Filtermats were dried and sealed into a plastic foil containing scintillation fluid (Betaplate Scint, Wallac, Perkin Elmer, Schwerzenbach, Switzerland) which amplifies the radioactive signal of the radiolabelled hypoxanthine when read by a liquid scintillation counter (1205_021 Betaplate, Wallac, Perkin Elmer, Schwerzenbach, Switzerland). To assess the IC_{50} value, the ratios of the obtained signal of incorporated radioactivity of each well to the signals of incorporated radioactivity of the control wells were calculated using EXCEL software. The ratios were plotted in a graph from which the IC_{50} could be assessed. The obtained IC_{50} values represent the drug concentrations at which 50% of the incorporated hypoxanthine was obtained compared to the control, and therefore the concentration which kills 50% of the parasites. This determination was performed with each trypanosome population extracted from mice 3 times, each time in duplicate.

Data based analysis

To assess influences of any parameter (various trypanosome strains, lines of mice and treatment schedules) on the IC_{50} of DB 75, diminazene, melarsoprol and pentamidine a three way analysis of variance was performed using the mixed procedure (PROC MIXED) of the statistic program SAS System. As estimation method REML (Restricted Maximum Likelihood) was used.

3. Results

3.1. Kinetics of different populations

In the following, the courses of parasitaemia will be described for each group separately. To facilitate the understanding, mice which show a different behaviour compared to the group were mentioned showing their coding in brackets.

3.1.1. Kinetics of first infection parasitaemia in untreated mice (control)

A NMRI 900: (Fig. 3, 4, 5)

4 days after inoculation with $1*10^5$ trypanosomes 4 out of 5 mice showed a high parasitaemia. 24 hours later all mice were infected heavily. Based on the counts in the Neubauer haemocytometer, the 5^{th} mouse (St) developed the parasitaemia less rapidly than the other four.

A SCID 900: (Fig. 6, 7, 8)

In SCID mice development of a full parasitaemia took slightly less time than in NMRI mice, on average 3.8 days vs. 4 days in NMRI. Analysing the data obtained from the counts in the Neubauer haemocytometer, the infection was established with a similar velocity in all five SCID mice.

A NMRI 795: (Fig. 9, 10, 11)

In an infection with STIB 795, the time to reach the same high level of parasitaemia (+++) is prolonged compared to the STIB 900 infections (4.5 days vs. 4 days in STIB 900 infected NMRI mice). One mouse (K) showed a completely different parasitaemia pattern, decreasing the parasite density of + at day 3 and 4 to ((-)) within 1 day. Parasitaemia rose and decreased once more, before a high density was reached at day 12. As the counts in the Neubauer haemocytometer showed, the course of infection varied within this group.

3.1.2. Kinetics of first infection parasitaemia, trypanosome disappearance of first infection after DB 75 treatment and parasitaemia of the relapse population

B NMRI 900: (Fig. 12, 13, 14)

3 days after inoculation a heavy parasite load could be detected in only one mouse (St), the others showed a low grade parasitaemia. Under treatment all mice could reduce their number of trypanosomes immediately and no parasites were detectable within 2 days, except the mouse showing the high grade parasitaemia. This mouse responded with delay to drug treatment and clearance took 5 days after beginning of the drug treatment. Average time to clear blood from parasites was 2 days.

The time period between total clearance and re-appearance of trypanosomes took 9 to 20 days, with an average of 16.2 days. Interestingly the mouse, that reacted with delay to drug treatment got the relapse first.

Clearly, it took more time to develop a high level parasitaemia after having undergone a first treatment period compared to first infection.

B SCID 900: (Fig. 15, 16, 17)
Clearance of trypanosomes after drug treatment took more time compared to clearance in NMRI mice (3.2 days vs. 2 days). The first trypanosomes re-appeared already 2 to 7 days (average: 4.4 days) after their elimination under drug treatment. It could be monitored that in SCID mice the development of the relapse-parasitaemia was slowed down also. Counts in the Neubauer haemocytometer showed a small increase of parasites over time for 4 mice. One mouse (KSt) behaved differently, reaching a high grade parasitaemia quickly (see growth rate: B SCID 900 Neubauer count). Although no daily observation of the tailblood was performed, it seemed that the development of the relapse parasitaemia occurred the faster, the longer the elimination of parasites after the treatment period took.

B NMRI 795: (Fig. 18, 19, 20)
Within 1 day after DB 75 treatment no parasites could be detected in the blood anymore. Screening for parasites was performed up to the end of the study period at day 185, without detecting any trypanosomes.

3.1.3. Kinetics of first infection parasitaemia, disappearance of trypanosomes of first infection after drug treatment, relapse parasitaemia, disappearance of relapse parasitaemia after second drug treatment and second relapse parasitaemia

C NMRI 900: (Fig. 21, 22, 23)
On average it took 1.8 days to clear the first infection and 11 days (between 8 and 18 days) till the trypanosomes re-emerged. The average time between the second treatment period and the second relapse was with 17.6 days (11 - 27 days) longer compared to the time between first treatment and first relapse.
Three mice out of five were able to reduce their parasite load of the second relapse without further drug treatment for a certain time (R, KR, KSt). One of those mentioned mice (KR) was able to reduce the parasites of the second relapse without any further treatment for 14 days. Then trypanosomes re-appeared for 24 days, before another parasite-free period lasting 4 days was monitored. Even in an additionally performed haematocrit no trypanosome could

be detected. After 4 days the parasitaemia rose rapidly (from 0 to +++ in 4 days), leading to death after 15 days.

C SCID 900: (Fig. 24, 25, 26)

The data of one mouse was rejected, because this mouse was suspected not to be a SCID mouse (R) due to her resistance to relapse (1st relapse on day 66, death on day 111).
First parasite clearance averaged 2 days, whereas 3.2 days were obtained for B SCID 900 mice. After treatment trypanosomes re-appeared in SCID mice in almost half of the time compared to trypanosomes in NMRI mice (5.25 days vs. 11.6 days). The time between clearance of first relapse and second relapse was surprisingly short: 1.5 days (compared to 17.6 days in NMRI mice) and also shorter than between first treatment and first relapse.

C NMRI 795: (Fig. 27, 28, 29)

Total cure was achieved in 3 out of 5 mice already after the first treatment day (based on the Neubauer counts), one day later the other 2 mice were trypanosome-negative, too. Up to the end of the study period (day 185) no trypanosome was detected anymore.

3.1.4. Kinetics of a first infection parasitaemia of a subpassaged relapse trypanosome population

D NMRI 900: (Fig. 30, 31, 32)

The development of a trypanosome infection obtained from parasites of mouse B NMRI R inoculated into mice of group D took more time, due to the lower number of parasites applied with the inoculum ($2.5*10^4$ vs. $1*10^5$ trypanosomes). A parasitaemia pattern typical for a first infection could be monitored.

D SCID 900: (Fig. 33, 34, 35)

Development of parasitaemia did not differ from the one of group D NMRI 900. The inoculated trypanosomes derived from mouse B SCID KR.

3.1.5. Kinetics of first infection, disappearance of trypanosomes after treatment and relapse parasitaemia of a subpassaged relapse trypanosome population

E NMRI 900: (Fig. 36, 37, 38)

The average time till trypanosomes were eliminated after treatment lasted 3.4 days. 7 to 11 days (mean: 8.2 days) after clearance trypanosomes could be found in the blood again.
In this group, one mouse (K) responded with delay to drug treatment, too. As already observed for other groups, this mouse relapsed first. Another mouse (KSt) showed the phenomenon of self-cure and died within the parasite-free period.

E SCID 900: (Fig. 39, 40, 41)

Clearance as well as re-appearance took little time, on average 2.4 days (2 - 3 days) for the elimination of the parasites and 5.6 days (5 - 8 days) till the relapse trypanosomes could be detected.

3.2. Comparing groups

3.2.1. Time to develop a +++ parasitaemia after first infection

Infections with STIB 900 were established faster (A NMRI: 4, A SCID 3.8 days) than infections with the strain STIB 795 (4.5 days). It was not possible to determine this period in group B, C and E since treatment was performed at day 3 after the first trypanosome was detected independent of the grade of parasitaemia, which led to a reduction of parasites before a +++ parasitaemia was developed.

3.2.2. Time period from high grade parasitaemia to death of mice

3.2.2.1. Mice without any treatment period (groups A and D)

NMRI mice endured a high parasitaemia longer than SCID mice (on average 4.5 days vs. 2 days). The day the mice died, a +++ parasitaemia was detectable in all animals.

3.2.2.2. Mice which underwent at least one treatment period (groups B, C, and E)

A comparison was not possible, because some mice died with a low level parasitaemia. Possibly, these mice were weakened by the antecedent first infection.

3.2.3. Time for clearance of parasites after treatment

The time to eliminate the parasites varied between the different trypanosome strains in the same line of mice. Generally, elimination of trypanosomes of strain STIB 795 was performed faster than elimination of STIB 900 trypanosomes in NMRI mice.

group B:	STIB 900:	2 days	STIB 795:	1 day
group C:	STIB 900:	1.8 days	STIB 795:	1.2 days

Differences between clearance of STIB 900 in NMRI compared to clearance in SCID mice were also observed.

		period	average number of trypanosomes before treatment
group B:	NMRI mice:	2 days	$2.20*10^6$ per ml (w/o St)$^{\otimes}$
	SCID mice:	3.2 days	$2.49*10^6$ per ml
group C:	NMRI mice:	1.8 days	$6.04*10^6$ per ml
	SCID mice:	1.8 days	$2.19*10^6$ per ml
group E:	NMRI mice:	3.4 days	$1.92*10^7$ per ml
	SCID mice:	2.4 days	$1.24*10^6$ per ml

[$^{\otimes}$ data of St mouse was neglected, since mouse reacted with delay to treatment]

Taking the counts in the Neubauer haemocytometer into consideration, the same number of parasites was eliminated faster in NMRI than in SCID mice (see group B). In group C, elimination of parasites took as long in NMRI mice as in SCID mice, but the number of trypanosomes in the blood differed, being approximately 3 times higher in NMRI mice than in SCID mice. In group E, the time to clear parasites from the blood was prolonged in NMRI mice compared to SCID mice. Here also, the number of trypanosomes varied considerably: around 10 times more parasites were detectable in blood of NMRI mice than in the one of SCID mice.

3.2.4. Time period between clearance and relapse

Independent of the treatment schedules, aparasitic periods were longer in NMRI mice compared to SCID mice.

	NMRI		SCID	
group B:	16.2	vs.	4.4	days
group C:	11	vs.	5.25	days (first clearance – first relapse)
group C:	17.6	vs.	1.5	days (second clearance - second relapse)
group E:	8.2	vs.	5.6	days

3.3. Morphological examination

3.3.1. Morphological appearance

Visually no differences in morphological appearance were observed between trypanosomes separated from the different groups of mice when examined at a magnification of 500x and 1000x (Fig. 42).

3.3.2. Size distribution

3.3.2.1. Visual analysis

All graphs of the size distribution obtained from CASY showed consistently the same distribution pattern with a steep rise on the left hand side of the curve and a less steeper fall on the right hand side (Fig. 43).

3.3.2.2. Data based analysis

The mean volumes of trypanosomes of all groups were compared, ranging from 32.6 – 40.7 fl. Performing an analysis of variance with this data no significant differences within the size distribution could be detected, neither between trypanosomes proliferating in immunocompetent or in immunodeficient mice ($F_{1,28} < 0.01$, $p = 0.97$), nor between the different trypanosome strains ($F_{1,28} = 1.1$, $p = 0.3$), nor between trypanosome populations which underwent different treatment schedules ($F_{2,28} = 0.07$, $p= 0.9$).

3.4. Drug sensitivity (Fig. 44), (Chapter 10 Appendix A Table 1-5)

3.4.1. Sensitivity to DB 75

The IC_{50} values for all STIB 900 populations were consistently similar, ranging on average from 11.84 ng/ml to 16.44 ng/ml independent of the strain of mouse, or treatment schedule.

Interestingly, the IC_{50} value for STIB 795 was decreased being 7 ng/ml on average (3 ng/ml to 10 ng/ml).

3.4.2. Sensitivity to diminazene

Comparing the IC_{50} of the various populations to diminazene, a trend to lower values was obtained in SCID mice, and for STIB 795.

3.4.3. Sensitivity to melarsoprol

No difference was detectable in melarsoprol IC_{50} values of trypanosomes of different strains, nor when isolated from different lines of mice, nor when the trypanosome populations underwent different treatment schedules.

3.4.4. Sensitivity to pentamidine

IC_{50} values were similar, independent of the strain of trypanosome, the line of mice from which they got extracted, or the treatment schedule which was performed.

Data based analysis

A three-way analysis of variance was used to test the effects of treatment schedules, mouse line and strain of parasite on the IC_{50} of each of the four drugs: DB 75, diminazene, melarsoprol and pentamidine.

DB 75

The IC_{50} of DB 75 was not significantly affected by different treatment schedules ($F_{4,66} = 0.5$, $p = 0.72$) nor by different lines of mice ($F_{1,66} = 0.1$, $p = 0.75$), but was lower in strain STIB 795 (mean: 7 ng/ml, stand. dev. 2.6), than strain STIB 900 (mean: 14.6 ng/ml, stand. dev. 4.8) ($F_{1,66} = 13.1$, $p = 0.0006$).

Diminazene

All parameters affected the IC_{50} value of diminazene significantly: the treatment schedule ($F_{4,66} = 4.57$, $p = 0.0025$), the line of mice ($F_{1,66} = 14.17$, $p = 0.0004$), and the used trypanosome strain ($F_{1,66} = 15.56$, $p = 0.0002$).

Melarsoprol

Different treatment schedules ($F_{4,66} = 1.54$, $p = 0.2$), or different line of mice ($F_{1,66} = 0.33$, $p = 0.57$), or different trypanosome strains ($F_{1,66} = 2.59$, $p = 0.11$) had no significant effect on the IC_{50} value of melarsoprol.

Pentamidine

The same result as for melarsoprol was observed for the IC_{50} value of pentamidine: neither a statistically significant influence of the treatment schedule ($F_{4,66} = 0.77$, $p = 0.55$), nor of the different line of mice ($F_{1,66} = 1.43$, $p = 0.24$), nor of the different trypanosome strain ($F_{1,66} = 0.44$, $p = 0.51$) could be assessed.

IC_{50} of reference trypanosomes (STIB 795 and STIB 900) vs. IC_{50} of trypanosomes extracted from mice

Trypanosomes of the strain STIB 795 extracted from mice did not show any differences in the IC_{50} compared to reference trypanosomes of the strain STIB 795 (IC_{50} (reference): 6.48 ng/ml, IC_{50} (extracted from mice): 7 ng/ml). Whereas slightly higher IC_{50} values were obtained for STIB 900 trypanosomes extracted from mice (IC_{50} 14.6 ng/ml) compared to an IC_{50} of 9.28 ng/ml for reference trypanosomes of the strain STIB 900.

4. Discussion

4.1. General aspects

As the study design implied, 3 mice were sacrificed to further investigate their trypanosomes and therefore population of mice of each group was reduced from 5 to 2 individuals when mice showed a +++ parasitaemia. Therefore, statements concerning the time after having reached this parasitaemia just show the behaviour of two animals and should be interpreted with caution.

Tail blood examination is a rapidly performed examination method to determine the grade of parasitaemia. The accurate number of parasites is not assessable by this method, but a rough estimation of the density of the trypanosome population is possible. When the course of parasitaemia determined by tail blood examination is compared to the course of parasitaemia determined by counts in the Neubauer haemocytometer, a good correlation of these curves is clearly visible. The advantage of the determination of the trypanosome density in the Neubauer haemocytometer is, that the velocity can be assessed with which the population grows. A comparison between different individuals is therefore possible, even if a constant parasite level is determined by tail blood examination.
Freezing and thawing facilitated the determination of the numbers of trypanosomes in the blood samples, since erythrocytes but not trypanosomes get lysed by this technique.

The number of trypanosomes was diminished to one fourth when trypanosomes from one mouse were subpassaged to other mice compared to the number of trypanosomes inoculated straight from a stabilate. This was done to take the loss of trypanosomes obtained by the freezing process into consideration. This reduction in the number of inoculated trypanosomes was too high, as the course of parasitaemia showed. Mice of groups D and E, which were inoculated with subpassaged trypanosomes, had less parasites per ml blood at any given time before the first treatment period started compared to mice which were inoculated with trypanosomes from the stabilate.

Apparently, infections with trypanosomes of the strain STIB 795 proceeded milder than infections with trypanosomes of the strain STIB 900. Although the same number of trypanosomes had been injected, the course of parasitaemia differed already within the first days. A high level parasitaemia was reached more rapidly in mice infected with STIB 900 compared to mice infected with STIB 795. After drug treatment, mice infected with the more virulent strain STIB 900 needed more time to clear the blood from trypanosomes compared to mice infected with STIB 795, although in vitro sensitivity to DB 75 was about the same, with an IC_{50} of 6.48 ng/ml for STIB 795 and 9.28 ng/ml for STIB 900.

In this study the immunocompetent mice were represented by Hanlbm: NMRI mice. These albino white mice were raised in a so called outbreeding system. The aim of this breeding system is to obtain the highest possible degree of heterozygousity of alleles. This leads to genetically undefined animals. Therefore it was not surprising, that some animals behaved differently from the average when subjected to parasites and also to treatment (A NMRI STIB 795 K, B NMRI STIB 900 St, C NMRI STIB 900 KR, E NMRI STIB 900 KSt).

SCID mice show a **s**evere **c**ombined **i**mmune **d**eficiency (SCID) caused by a spontaneous occurring autosomal recessive mutation. Primary immune defects of these mutations are B and T cell deficiency, which are expressed in a developmental rest at a preliminary stage of B and T cells. Additionally, there are deficits in the CD3+ cells and in T cell receptor ab+ cells [Bosma, G.C. (1983)], [Bosma, M.J. (1991)]. Macrophages, antigen presenting cells (APC), natural killer cells and the activity of the complement are not affected by this mutation [Czitrom, A.A. (1985)]. SCID mice show an increased susceptibility to certain murine pathogens as trypanosomes, which is the reason for the proliferation of *Trypanosoma brucei*

gambiense in SCID mice. Even if immunosuppressed with cyclophosphamide, it is almost impossible to establish a *T. b. gambiense* infection in the NMRI mice.
This genetic defect is not stable, since “leakiness” was reported for these mice. This means that depending on the original line of the immunodeficient mouse, its environment and its age, SCID mice may produce some functional B and T cells. There are several mice lines on the market showing severe combined immune deficiency. In this study mice of the strain Cb17 were used. Leakiness is not expected, because very young mice were used in this experiment.

4.2. Expectations

When comparing immunocompetent vs. immunodeficient mice in this study, differences are expected.
The first infection parasitaemia, which represents the first challenge of the animal by the parasite, will develop with the same velocity in the immunocompetent as in the immunodeficient mice. At this time the immune system is naive, and therefore reasons for any differences occurring must lie within the elements of the unspecific immune response. Since the unspecific immune response is not affected by the genetic defect of the SCID mice, no differences are expected to show up.
As it is known from literature, the aid of the immune system is required to clear the blood stream from parasites after drug treatment (see below: immune-dependency of chemotherapy). Therefore, the clearance of parasites from the blood stream is expected to be prolonged in immunodeficient mice.
The development of the relapse parasitaemia will take longer in NMRI mice than in SCID mice, since memory cells were produced after the first contact which may control the parasite growth for a certain time when challenged the next time.
Additionally, it is expected that SCID mice will not survive an infection as long as NMRI mice since the immune system is weakened by the genetic defect.

4.3. Influence of the immune system on the development of an infection, on clearance after drug treatment and on relapses

Differences in the development of a STIB 900 parasitaemia

As expected, no big differences were detectable within the first 4 days after infection referring to the velocity with which the numbers of trypanosomes increased in SCID mice compared to

NMRI mice. Also, the survival time of mice showing a high parasitaemia behaved as expected. No SCID mouse survived longer than five days (p.i.), whereas one of the NMRI mice survived up to day 9 (p.i.). From day 5 to 9 this mouse was infected heavily (more than $1*10^7$ trypanosomes per ml). Within this period the numbers of parasites increased very little.

Differences after drug treatment

Clearance of parasites from the blood stream was prolonged in SCID mice compared to NMRI mice. The exact mechanisms of this elimination process are not known to date, but many components of the immune system were shown to play an important role in the past. Depending on the drug used to treat the trypanosome infection, different factors of the immune system were discussed to be responsible for the clearance of the parasite. In all studies the so called immune-dependence of chemotherapy was observed to be elementary: showing that a close relationship between the drug therapy and the immune system is necessary for clearance.

1934, Von Jancso N. et al. showed that the reticulo-endothelial (monocyte/macrophage) system is involved in removal of the parasites after suramin treatment. Rodents which were splenectomised and/or treated with colloidal copper to suppress phagocytosis by reticulo-endothelial cells, cleared trypanosomes slower than immunocompetent mice. Changes in the morphology of trypanosomes could be detected only in these immunosuppressed rodents, which led to the hypothesis, that suramin damages trypanosomes in a way, that they were recognised and opsonised by the reticulo-endothelial cells, which are only present in immunocompetent animals.

Anti-trypanosomal antibodies play an important role after treatment with DMFO. Removal of trypanosomes after DMFO treatment in immunocompromised rodents (cyclophosphamide, dexamethasone) was inefficient or prolonged compared to immunocompetent counterparts. These chemically suppressed rodents were shown to have very low levels of anti-trypanosomal antibodies [de Gee, A.L. (1983)], [Bitonti, A.J (1986)].
Athymic mice, which lack T cells, cleared parasites as immunocompetent mice, which indicated that the anti-trypanosomal antibody response is T cell independent [Campbell, G.H. (1977)].

Curative doses of melarsoprol were ineffective in cyclophosphamide or gamma irradiation suppressed mice [de Gee, A.L. (1983)]. The same applied for cortisone suppressed mice after treatment with arsenicals. Corticosteroids are known to affect antibody and cell mediated immune responses, affecting inhibition of antigen presentation as well as inhibition of T cell independent antibody responses.

Immunodeficient mice in this study lack B and T cells. Therefore no T cell dependant antigens, nor a proliferation from B cells to antigen producing B cells, nor T cells which may present antigens are present. The only mechanism of immune defence in these mice is the unspecific immune response, based on the reticulo-endothelial system, the natural killer cells, complement and T cell independent antibodies. As the graphs show, these components are able to clear the blood from trypanosomes, even though the time is prolonged compared to mice with an intact immune system.

Differences in the duration of the aparasitic period between clearance and relapses

The aparasitic period after drug treatment and before the occurrence of the relapse was longer in NMRI mice than in SCID mice. SCID mice were probably more susceptible to the relapse because of their weakened general condition. An additional immunosuppression occurs naturally in trypanosome infected hosts [Sacks, D.L. (1980)]. In NMRI mice, the production of specific antibodies may control the growth of the parasites on a low level for a certain time. This ability is lacking in SCID mice. The production of specific antibodies may be the reason for the prolongation of the establishment of the parasitaemia of a relapse population compared to the one of a first infection. A certain role in the prolonged establishment of an infection must play also the unspecific antibodies since a similar prolongation was detected when SCID mice relapse.

An indirect correlation of the density of trypanosomes to the duration of the aparasitic period between infection and relapse could be detected. In all mice, independent of the status of their immune system, relapses occurred the faster the higher the parasite load was at the day treatment started. The reason for this could be that the slower the drug acts the higher the chance that trypanosomes invade tissues to which the drug has less access to and from where they could re-invade the blood. Additionally, the more trypanosomes are present in the blood the less drug per trypanosome is at disposal. Trypanosomes are also known to accumulate

diamidines as DB 75 and therefore subcurative plasma levels of the drug may be possible (see chapter 6: Accumulation of DB 75 in trypanosomes in vitro).

This experiment showed clearly that there is a difference between SCID and NMRI mice concerning the survival time with a high parasitaemia. Generally, NMRI mice can endure a high parasite load significantly longer than SCID mice.

	first infection	relapse		second relapse	
	group A	group B	/group C	group C	/group E
NMRI:	2-5 days	13-15	/5-6 days	13+12$^{\otimes}$	/13 days
SCID:	1 day	n.d.*	/2-3 days	9	/10-11 days

(n.d.* = not determinable: all mice were sacrificed, $^{\otimes}$ one mouse could reduce parasitaemia for some days (12) within recurrent +++ periods (13 days), the other mouse died with a low grade parasitaemia)

4.3. Morphological characterisation

Morphological appearance between smears obtained from the different trypanosome populations was compared. No significant differences between the populations could be detected, neither at magnification of 500x nor at 1000x. Smears of the trypanosomes looked identical to smears obtained of reference populations of STIB 795 and STIB 900 trypanosomes. No differences referring to increased number of abnormal looking cells could be detected.

Size distribution was determined by using the CASY. The obtained profiles did not differ significantly in their appearance within the different groups of mice nor to reference trypanosomes of the corresponding trypanosome strain, neither did the average volume.

4.4. Drug sensitivity

To find out whether the various treatment schedules in different mouse strains had an effect on the sensitivity of the parasites to different drugs, the IC_{50}s of each trypanosome population from the different study groups of mice to 4 different drugs (DB 75, diminazene, melarsoprol and pentamidine) were determined. This was done to find out if the impairment of the host immune system leads to a rapid development of drug resistance, as was reported

for *Trypanosoma evansi* to mel Cy, diminazene aceturate and isometamidium chloride in immunosuppressed mice [Osman, A.S. (1992)].

The hypoxanthine incorporation assay was used, because this assay delivers reliable and reproducible results, even for a low number of trypanosomes or if the proliferation time is prolonged, which is expected for these trypanosome populations since their environment changes drastically when transferred from mice to the in vitro system. The separation from the blood by the DEAE column will stress the trypanosomes additionally, which is expected to result in a slower growth rate.
By using a scintillation cocktail, the signal of the incorporated radioactive hypoxanthine will be converted to a photo emission. Detection of this converted signal is more sensitive than the radioactive signal itself. Samples containing low-energy beta emitters, like Tritium (^{3}[H]), are placed in a scintillation cocktail, which consists of an organic solvent and a fluor molecule (scintillant). Solvent molecules become excited when an emitted beta particle passes through the scintillation fluid and strikes the solvent molecules. Energy is transferred from the solvent molecules to the fluor molecules, which emit photons when decay from the excited to the ground state takes place. Direct excitation of the fluor molecule by the emitted beta radiation is also possible. The emission is detected by a photomultiplier and is registered as a count. The number of counts correlates to the incorporated radioactivity and represents therefore the viability of the trypanosomes.

Results of the data analysis showed no differences within the IC_{50} of the different groups after various treatment schedules in different mice for DB 75, melarsoprol and pentamidine. Interestingly, the IC_{50} of diminazene was found to be influenced by all parameters: strain of trypanosomes, line of mouse and various treatment schedules. Standard deviations of the performed diminazene assays showed a great variability. The reason for this lies probably in the stability of the drug itself. Diminazene is reported to be stable in aqueous solution only for 2-3 days at room temperature [Peregrine, A.S. (1993)]. Since trypanosomes were incubated at 37° C for 40 hours in BMEM when the hypoxanthine assay is performed, the activity of the drug will decline, since the drug could be degraded partly to that time or may have interacted with ingredients of the medium. This would be a minor problem if diminazene would be a fast-acting drug, which kills parasites within the first hours, but from other studies, diminazene is known to exhibit a moderately fast activity.

Diminazene for veterinary use is marketed in combination with the stabiliser phenyldimethyl pyrazolone, what prolongs the duration of stability to up to 10-15 days when stored at room temperature.

Problems with instability in aqueous solutions are not reported in literature for melarsoprol and pentamidine. Melarsoprol is only slightly water soluble, but this is not expected to be a problem since the concentrations which were needed when performing the assay are very low and therefore below the limit of solubility.

IC_{50} of DB 75 from reference trypanosomes vs. IC_{50} of trypanosomes extracted from mice

Differences in the IC_{50} did not occur when reference trypanosomes of the strain STIB 795 were compared to trypanosomes of the same strain extracted from mice.
In contrast, trypanosomes of the strain STIB 900 showed a trend to a slight increase of the IC_{50} when extracted from mice compared to STIB 900 reference trypanosomes, independent of the treatment schedule performed and from which line of mouse trypanosomes were extracted. Since this effect is very small and only detectable for the STIB 900 trypanosomes, one could speculate about an interaction between this strain and the host.

5. Conclusion

Immune system and trypanosome infections

This study showed that an intact immune system is important for the control of a developing parasitaemia and for the clearance of parasites after drug treatment. However, an intact immune system is not able to eliminate the parasites without the support of anti-trypanosomal drugs.

Effect of hosts, trypanosome strains and treatment schedules on morphology and sensitivity to anti-trypanosomals

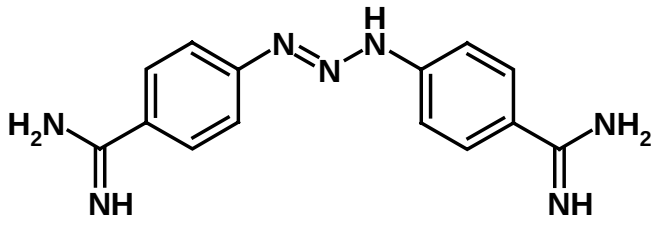
Trypanosomes did not alter their size distribution, morphological appearance or sensitivity to drugs significantly, when various treatment schedules were performed or when trypanosomes were raised in various lines of mice. Probably, no effect could be shown, since the influence of the drug on trypanosomes was too short to induce resistance to the drug applied and also to develop cross resistance to other drugs. Alike, the time was too short for the trypanosomes for major alterations in any of the inspected criteria when proliferated in various mouse lines.

Another reason that no changes in any of the chosen criteria could be found, could be that the sensitivity of the determination methods is too insensitive to detect small changes which could have been possible in that short time.

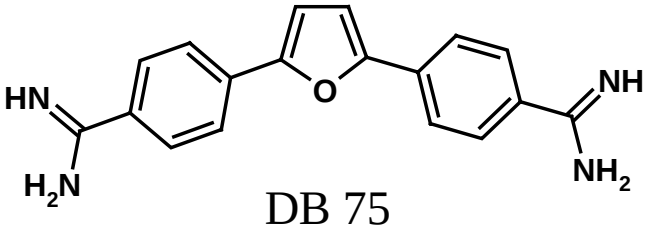
This experiment clearly showed the reproducibility and predictability of the relapses in STIB 900 infected animals after DB 75 treatment. Knowledge gained from this studies was important for the work described in Chapter 3 “An attempt to identify organs in mice which may serve as niches for trypanosomes for relapse development”.

However, the most important conclusion that can be drawn from this experiment is, that relapse populations in this mouse model are not less sensitive to drugs than the first infection trypanosomes. Therefore, relapses are not phenomena of resistance to applied drugs, but rather of invasion of sites which were less accessible to the drug and from where re-invasion of the blood is possible.

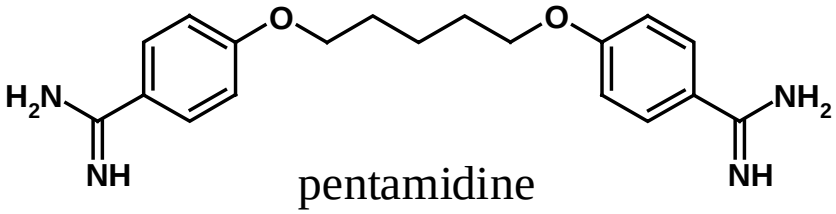
Fig. 1: Chemical structures of compounds to treat trypanosomosis



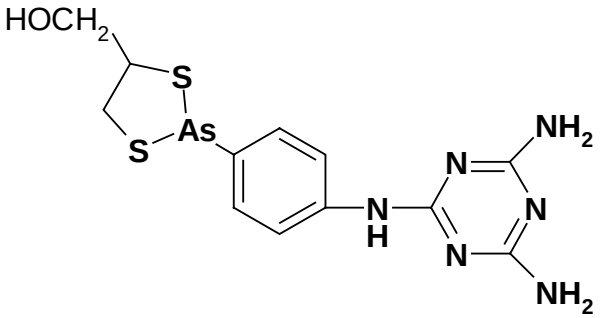
diminazene



DB 75

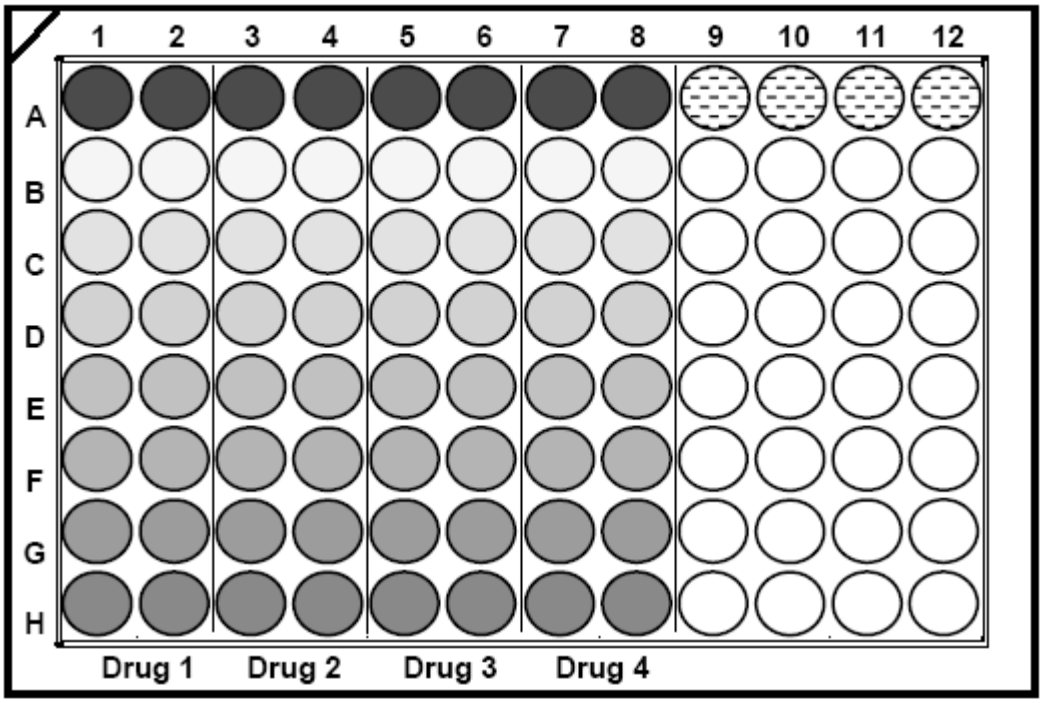


pentamidine



melarsoprol

Fig. 2: Plate layout hypoxanthine assay



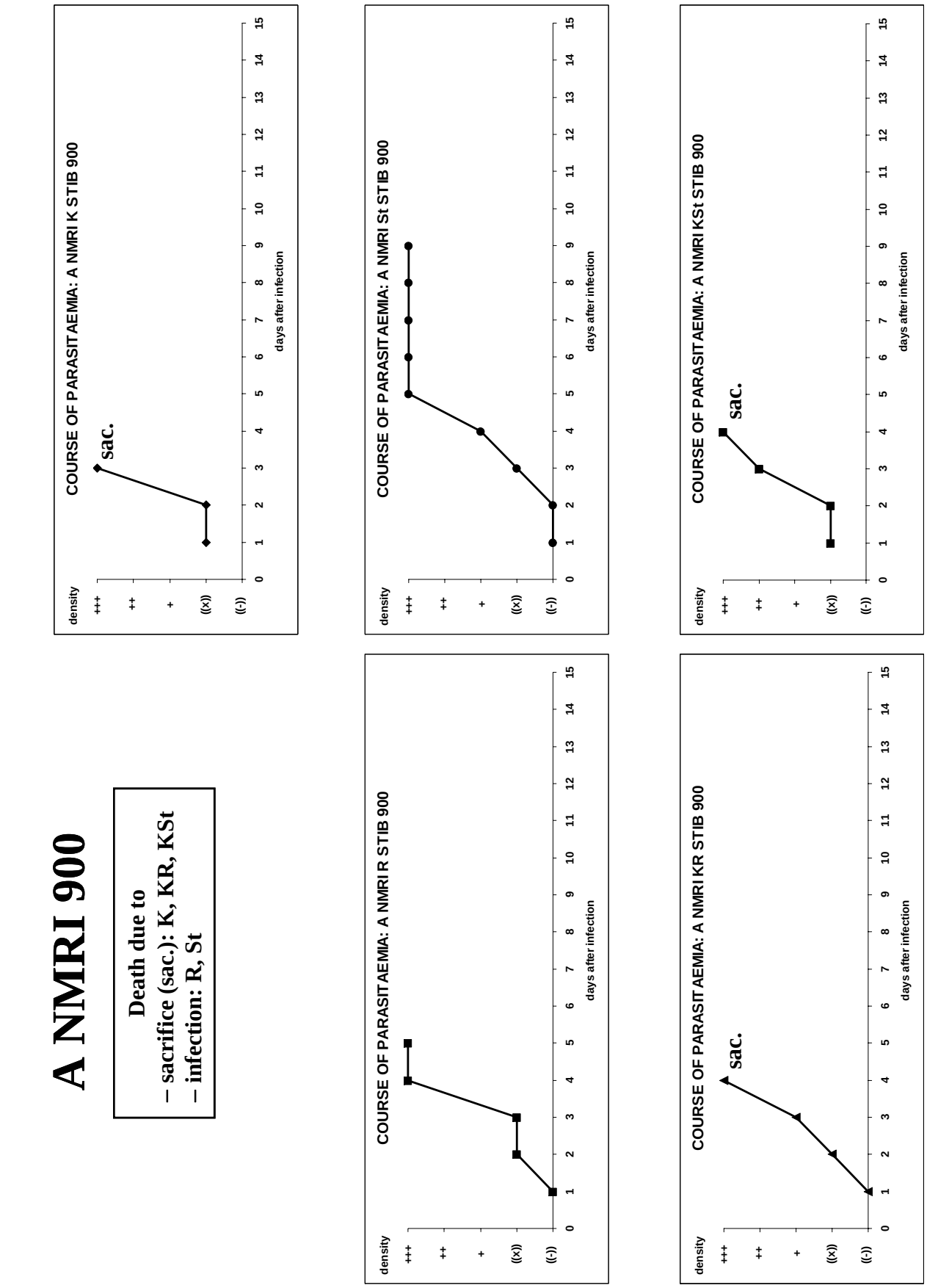
control

background

drug dilutions

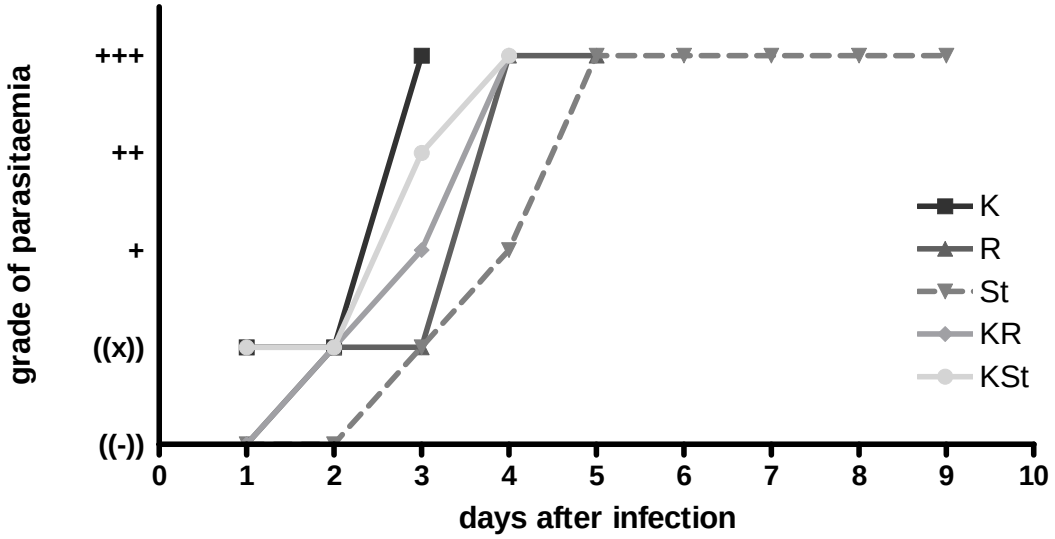
Drug 1: DB 75
Drug 2: diminazene
Drug 3: melarsoprol
Drug 4: pentamidine

Fig. 3: Course of parasitaemia: untreated STIB 900 infected NMRI mice



Group A:

Fig. 4: Course of parasitaemia: untreated STIB 900 infected NMRI mice



death ·due to sacrifice: K, KR, KSt ·due to infection: R, St

Fig. 5: Counts on Neubauer: untreated STIB 900 infected NMRI mice

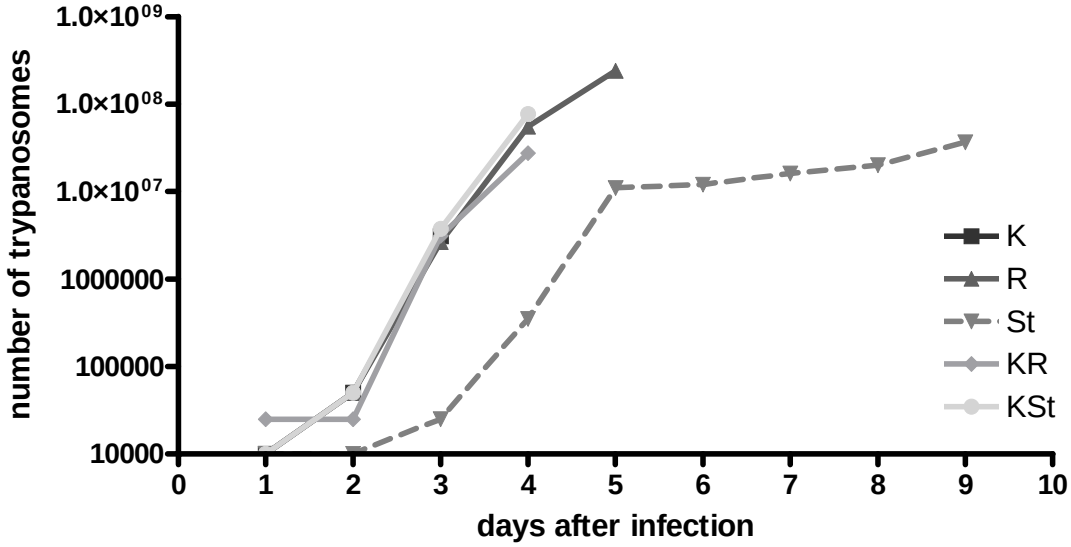
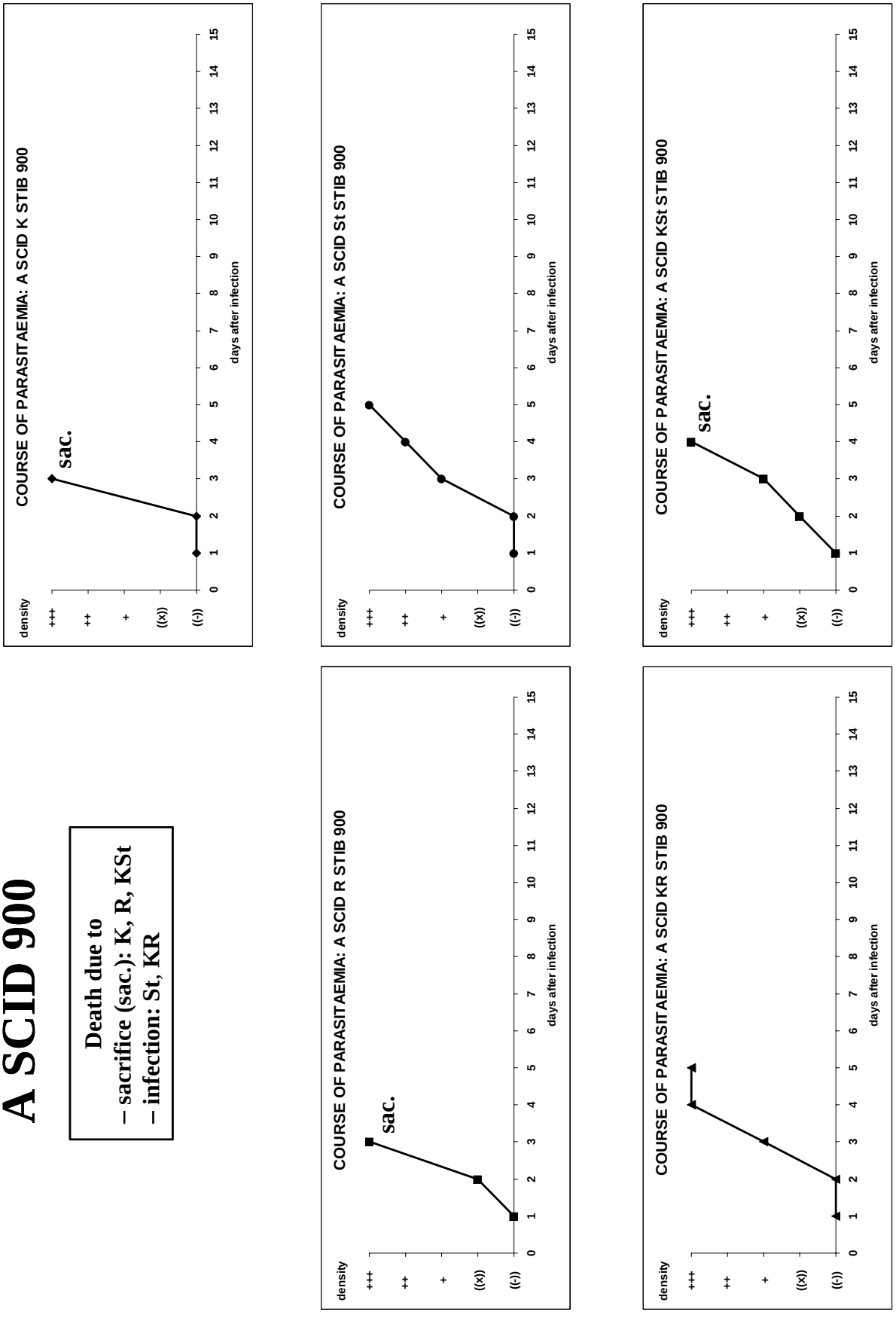
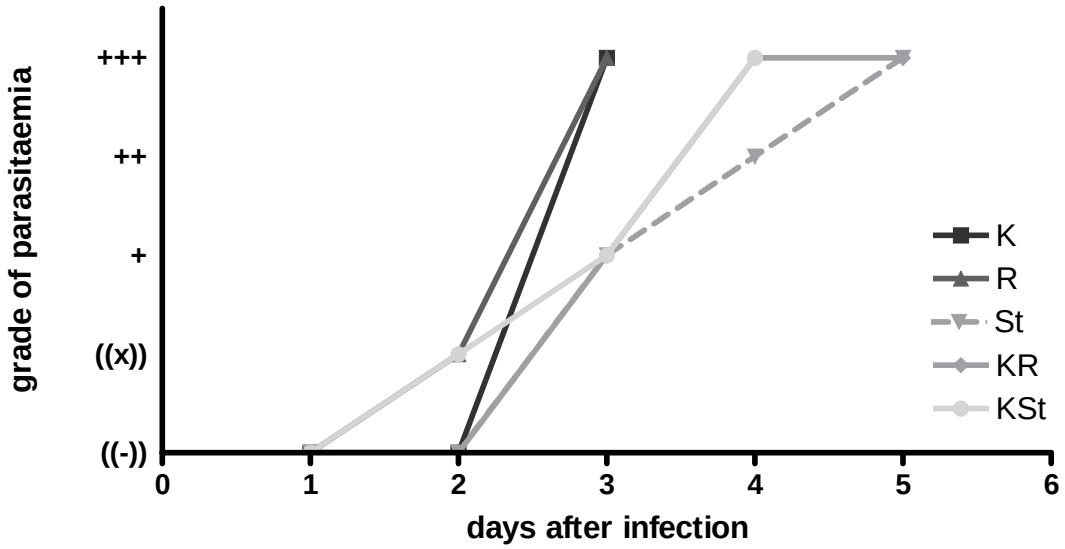


Fig. 6: Course of parasitaemia: untreated STIB 900 infected SCID mice



Group A:

Fig. 7: Course of parasitaemia: untreated STIB 900 infected SCID mice



death ·due to sacrifice: K, R, KSt ·due to infection: St, KR

Fig. 8: Counts on Neubauer: untreated STIB 900 infected SCID mice

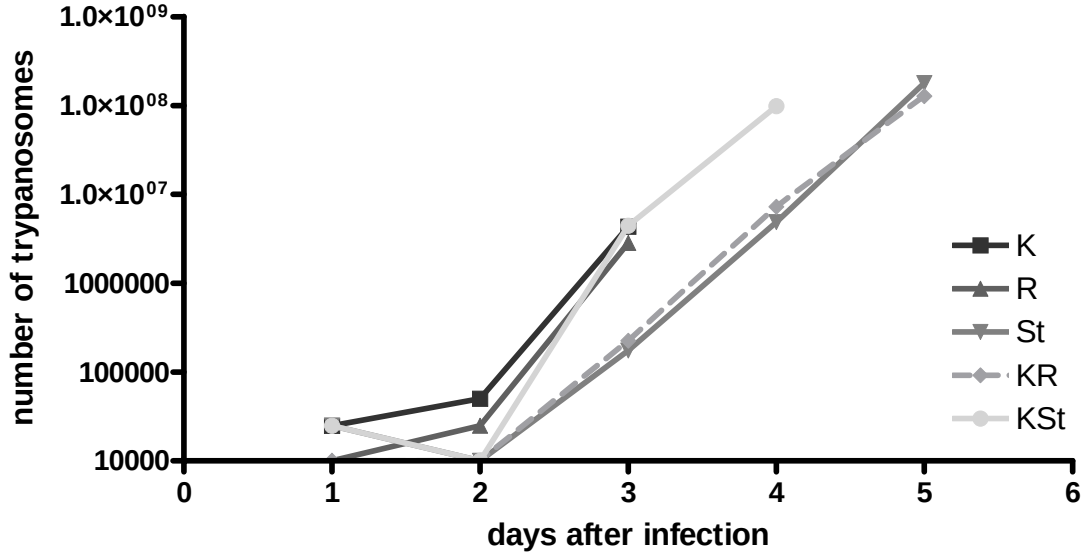
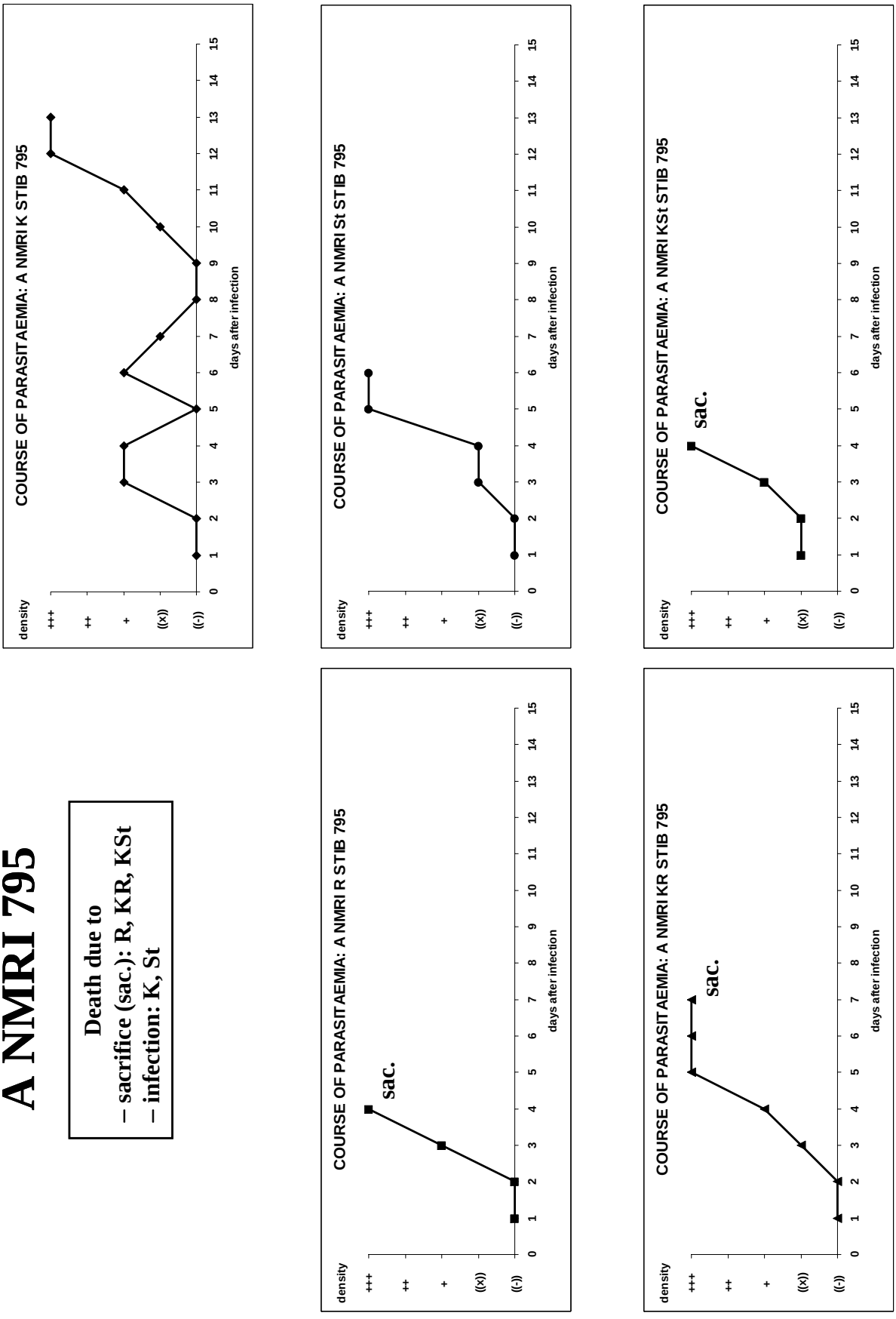
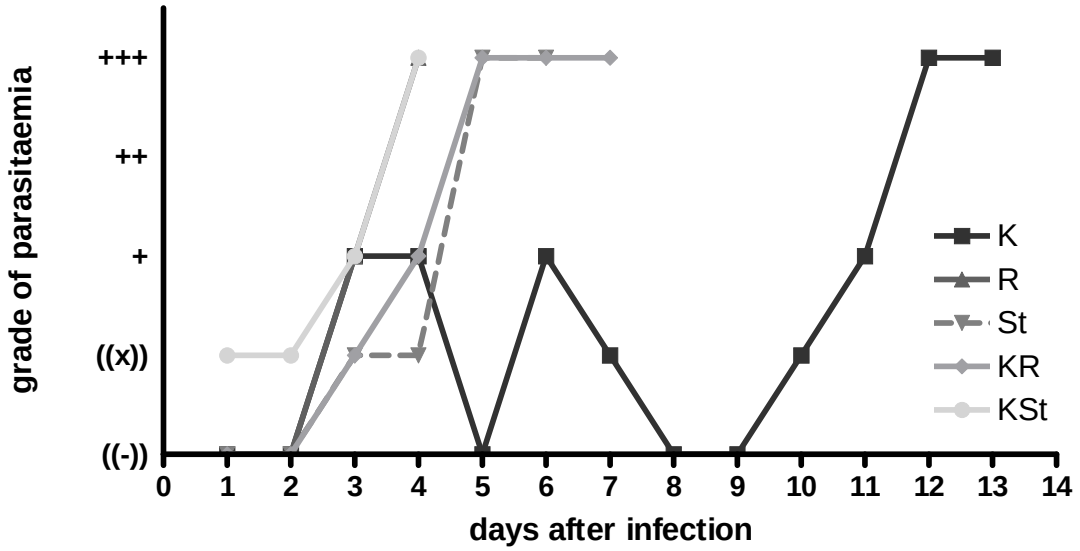


Fig. 9: Course of parasitaemia: untreated STIB 795 infected NMRI mice



Group A:

Fig. 10: Course of parasitaemia: untreated STIB 795 infected NMRI mice



death ·due to sacrifice: R, KR, KSt ·due to infection: K, St

Fig. 11: Counts on Neubauer: untreated STIB 795 infected NMRI mice

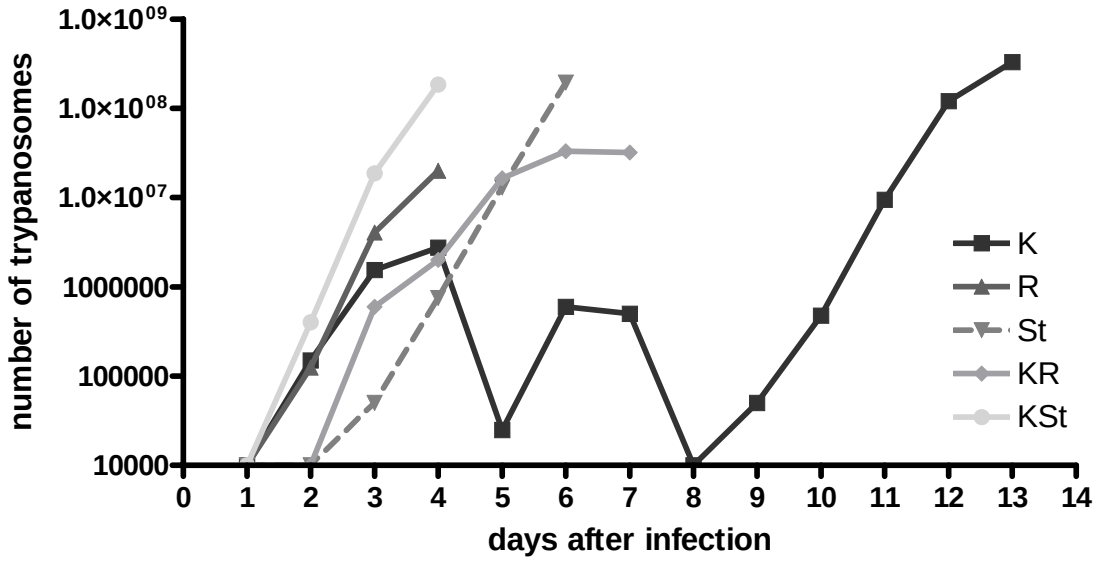
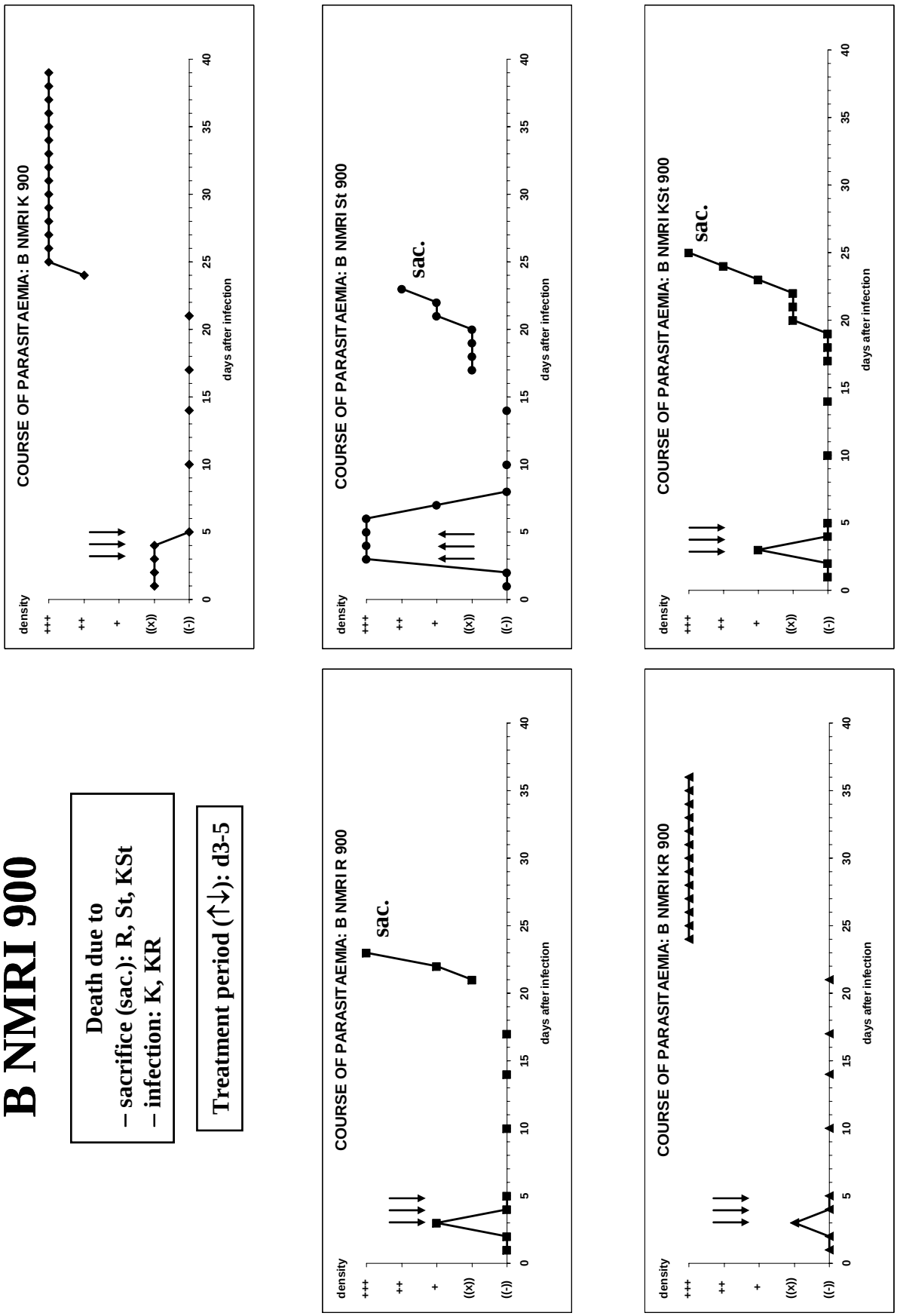
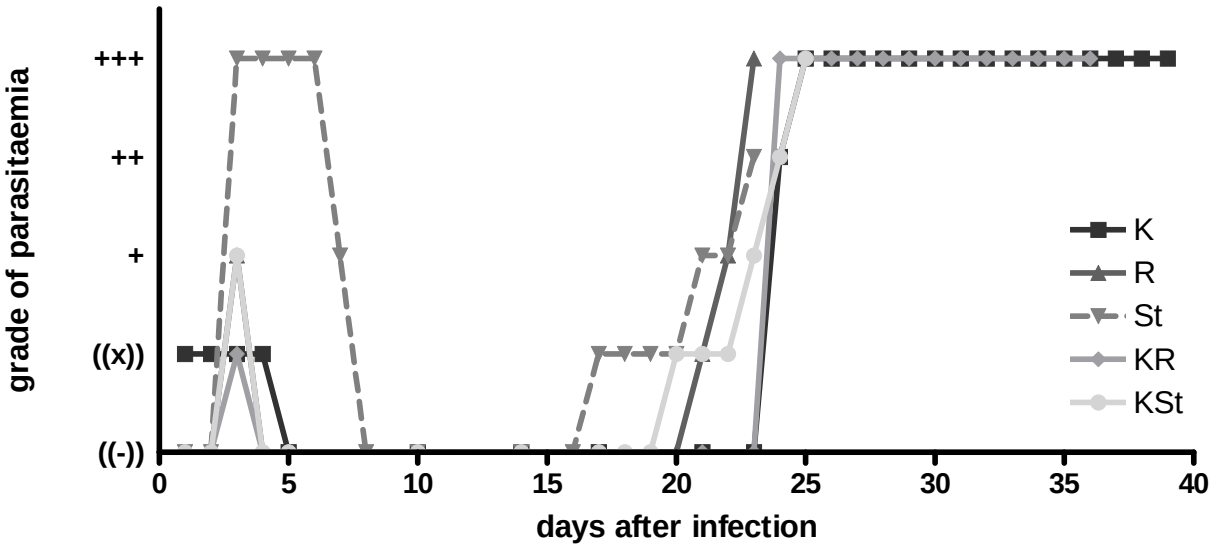


Fig. 12: Course of parasitaemia: STIB 900 infected NMRI mice with one treatment period



Group B:

Fig. 13: Course of parasitaemia:
STIB 900 infected NMRI mice with one treatment period



death ·due to sacrifice: R, St, KSt ·due to infection: K, KR

Fig. 14: Counts on Neubauer:
STIB 900 infected NMRI mice with one treatment period

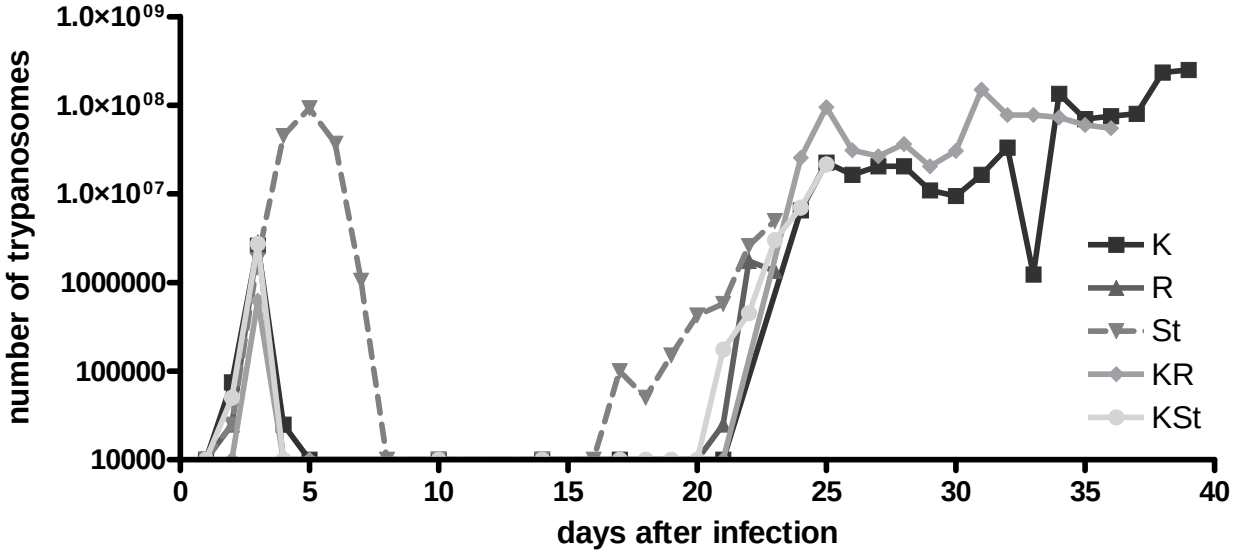
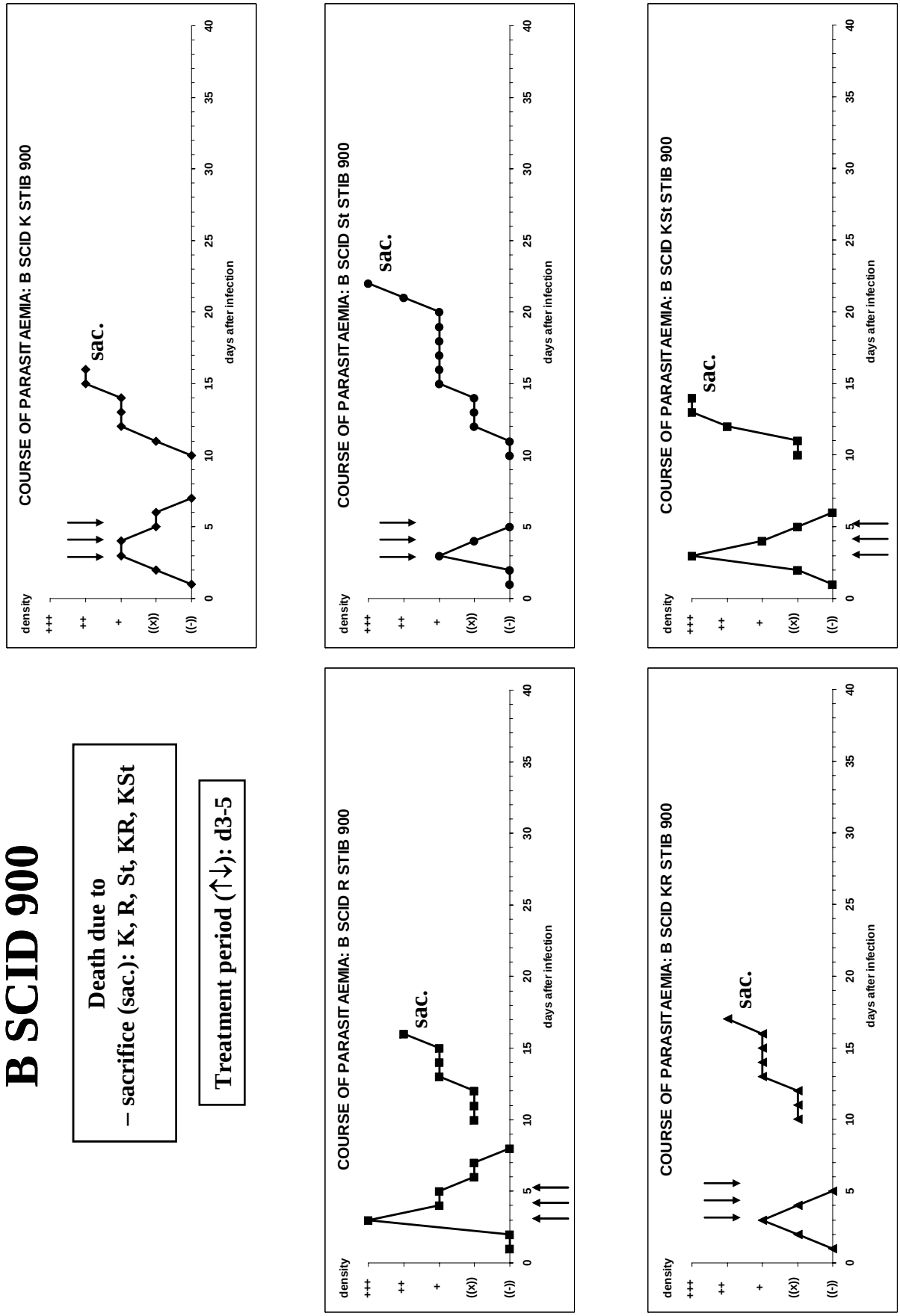
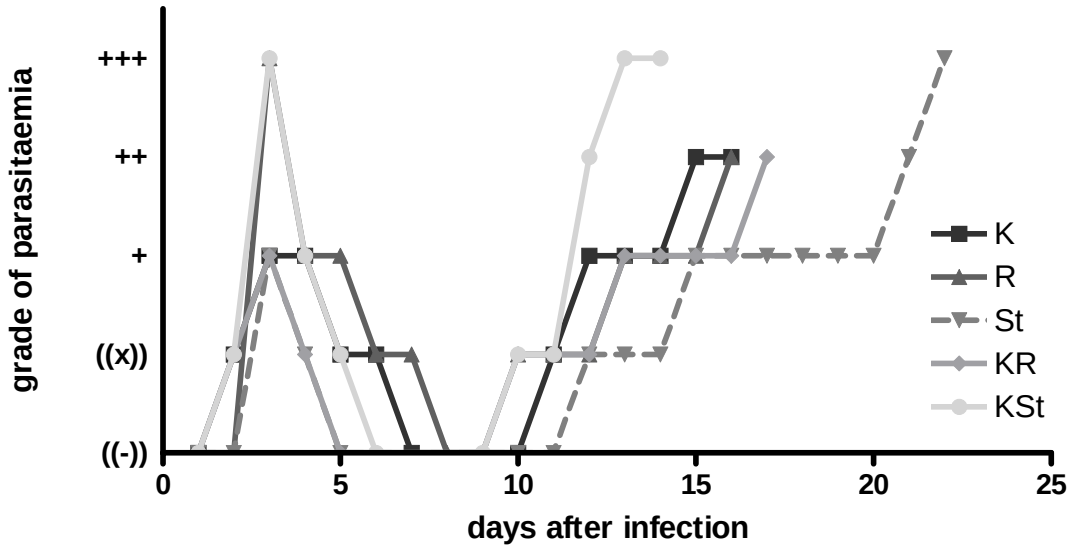


Fig. 15: Course of parasitaemia: STIB 900 infected SCID mice with one treatment period



Group B:

Fig. 16: Course of parasitaemia:
STIB 900 infected SCID mice with one treatment period



death ·due to sacrifice: K, R, St, KR, KSt

Fig. 17: Counts on Neubauer:
STIB 900 infected SCID mice with one treatment period

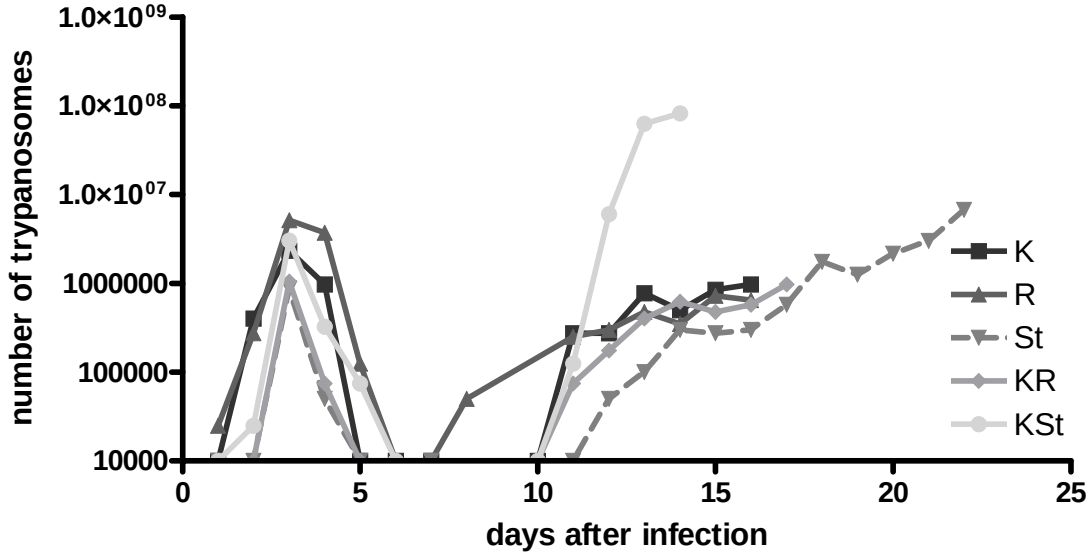
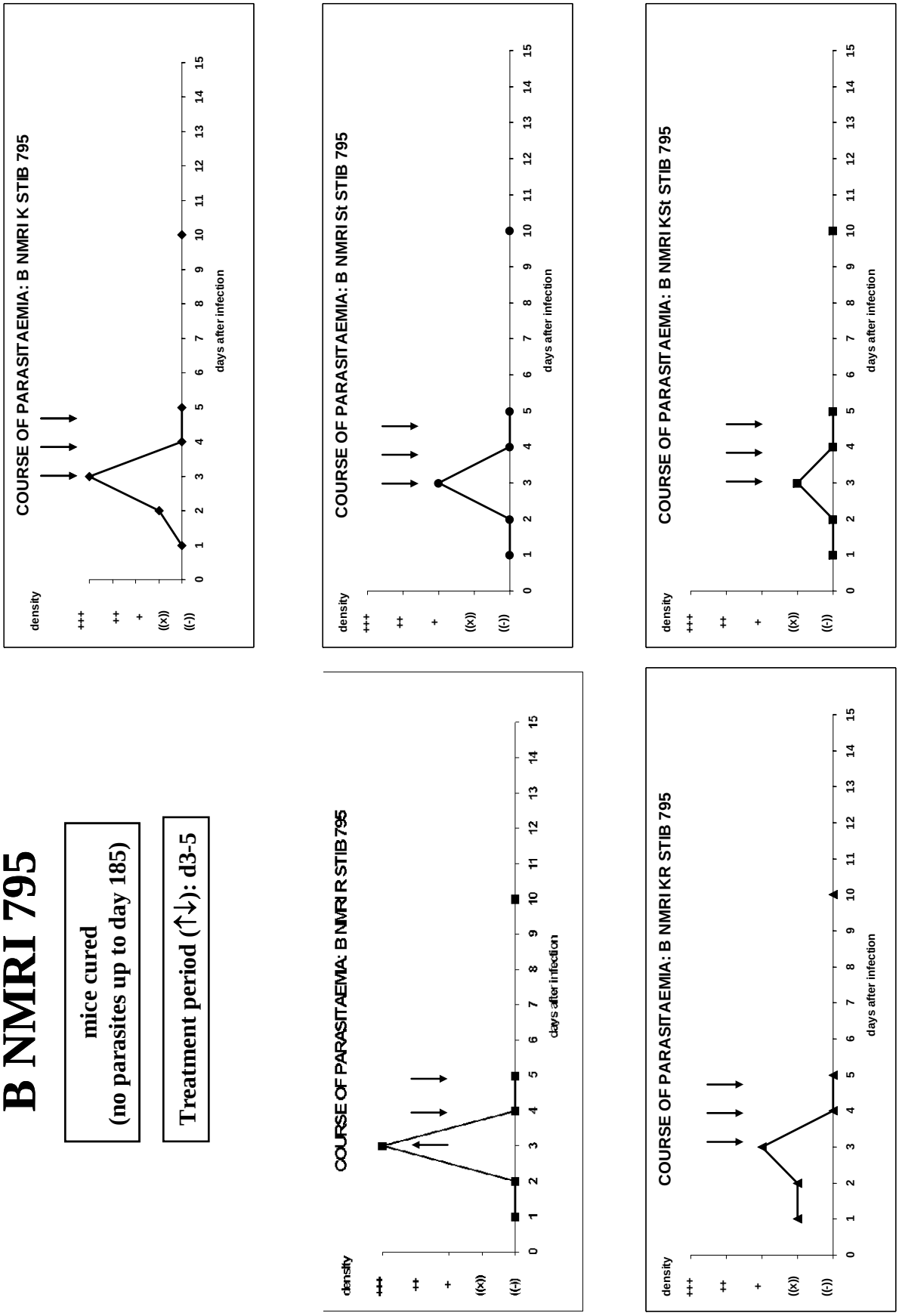
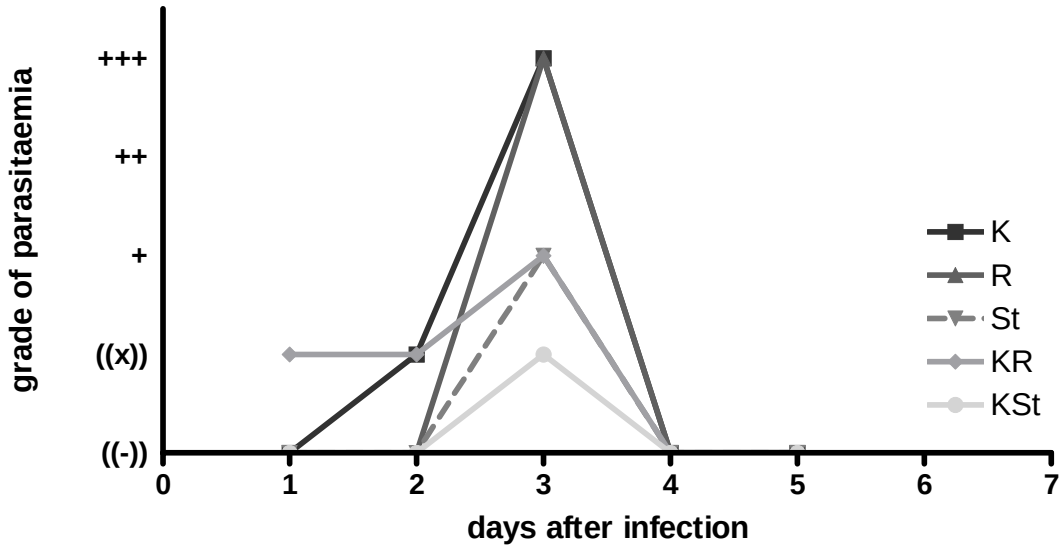


Fig. 18: Course of parasitaemia: STIB 795 infected NMRI mice with one treatment period



Group B:

Fig. 19: Course of parasitaemia:
STIB 795 infected NMRI mice with one treatment period



parasite free up to end of study at day 185

Fig. 20: Counts on Neubauer:
STIB 795 infected NMRI mice with one treatment period

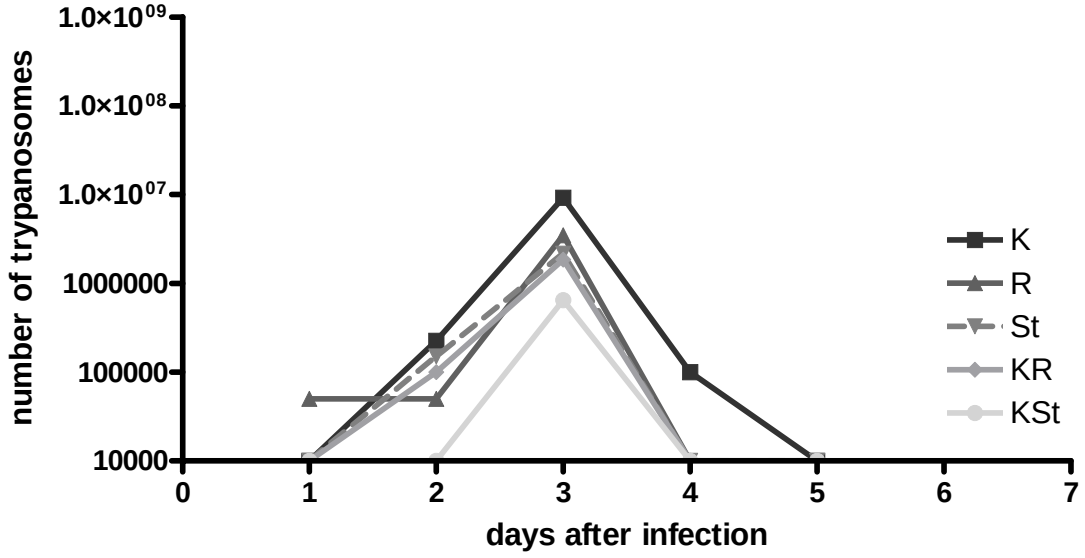
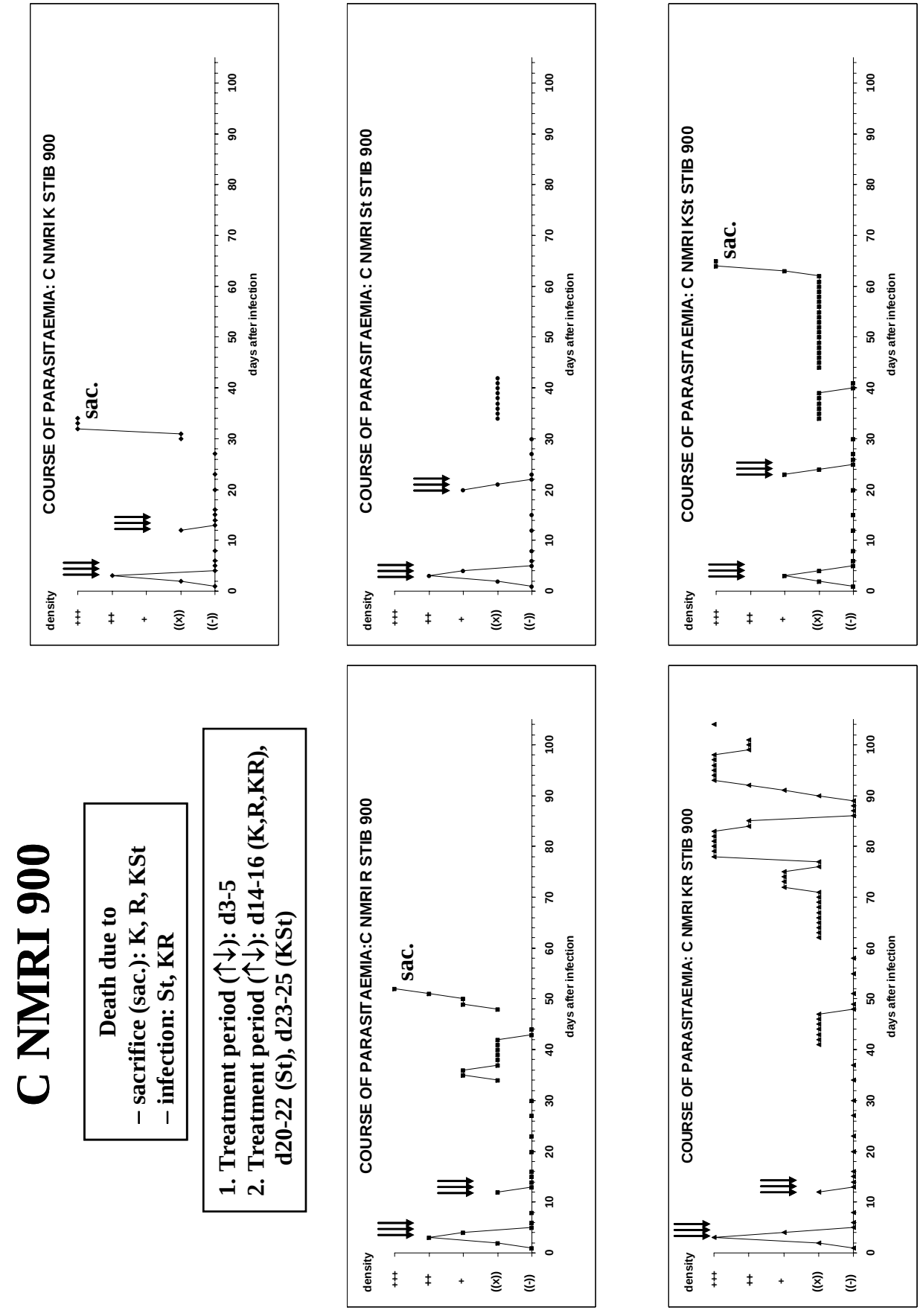
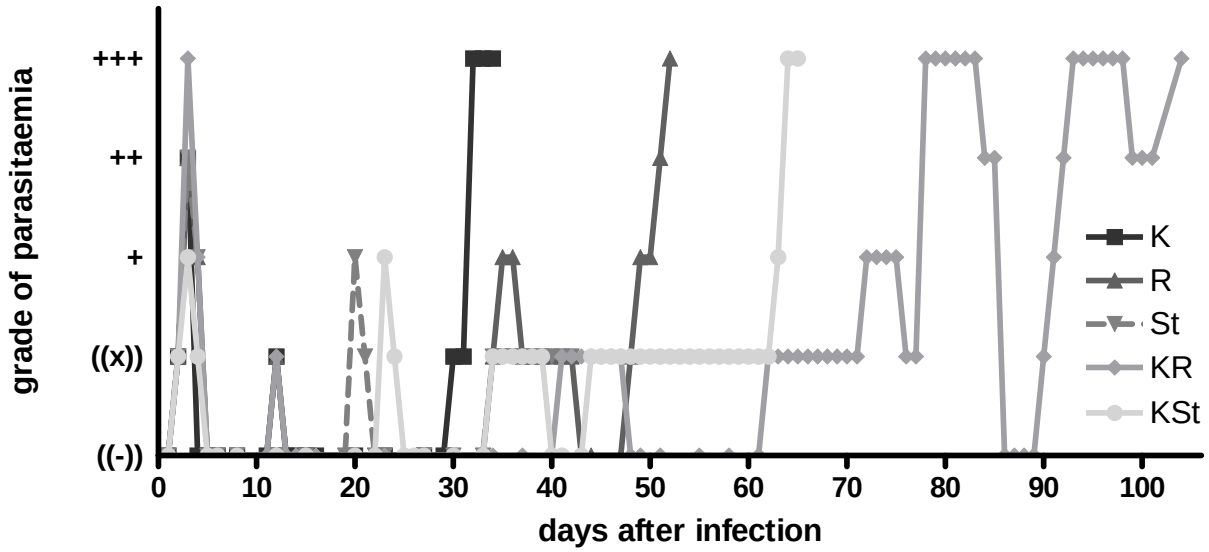


Fig. 21: Course of parasitaemia: STIB 900 infected NMRI mice with two treatment periods



Group C:

Fig. 22: Course of parasitaemia:
STIB 900 infected NMRI mice with two treatment periods



death ·due to sacrifice: K, R, KSt ·due to infection: St, KR

Fig. 23: Counts on Neubauer:
STIB 900 infected NMRI mice with two treatment periods

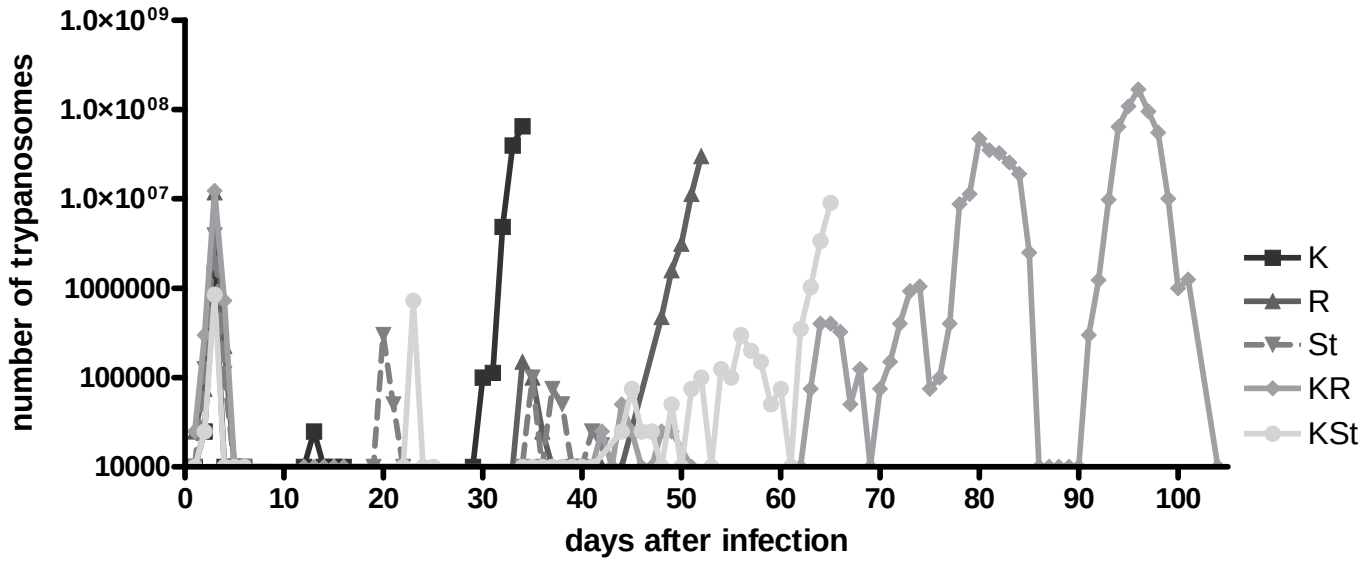
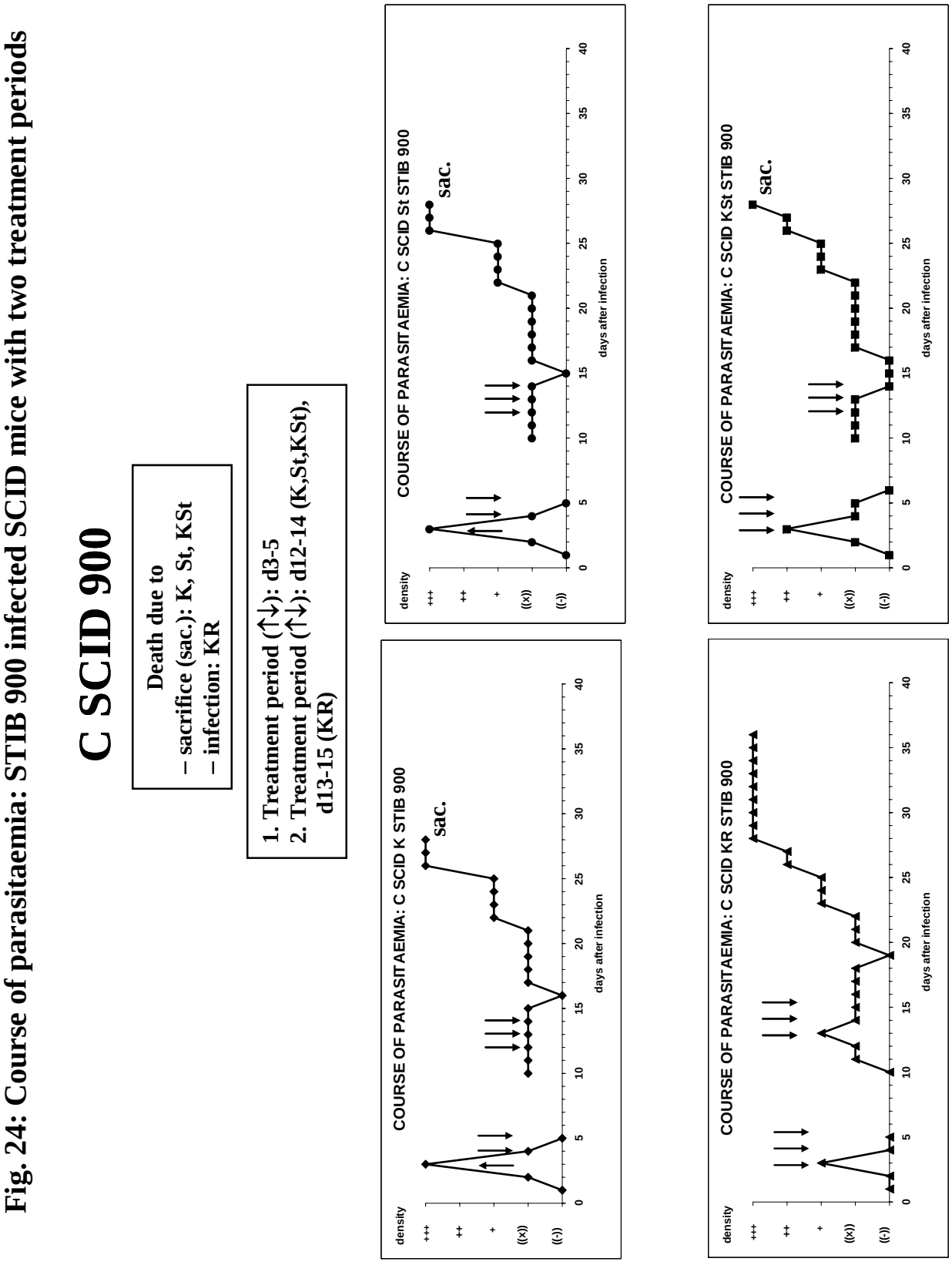
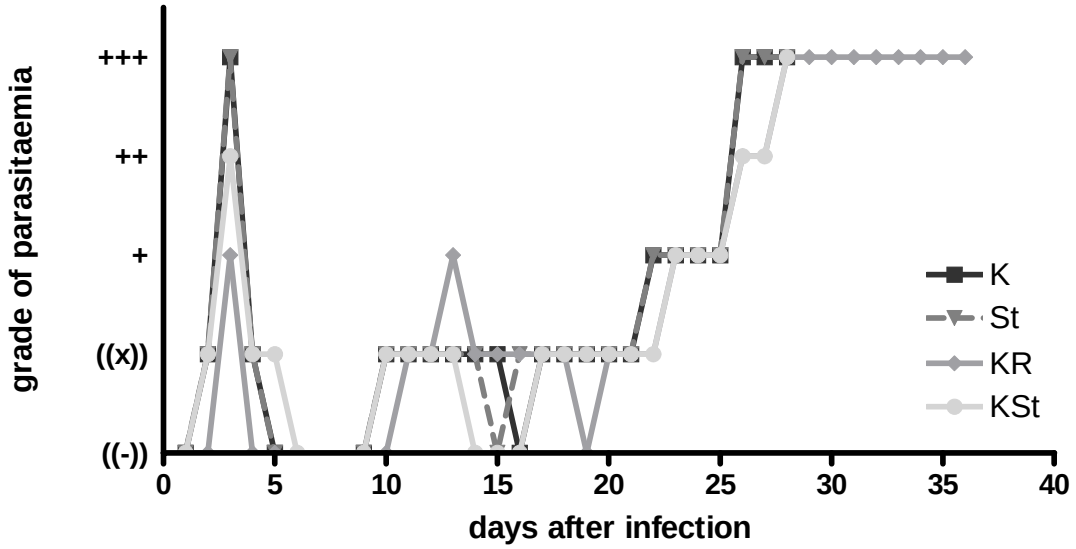


Fig. 24: Course of parasitaemia: STIB 900 infected SCID mice with two treatment periods



Group C:

Fig. 25: Course of parasitaemia:
STIB 900 infected SCID mice with two treatment periods



death ·due to sacrifice: K, St, KSt ·due to infection: KR

Fig. 26: Counts on Neubauer:
STIB 900 infected SCID mice with two treatment periods

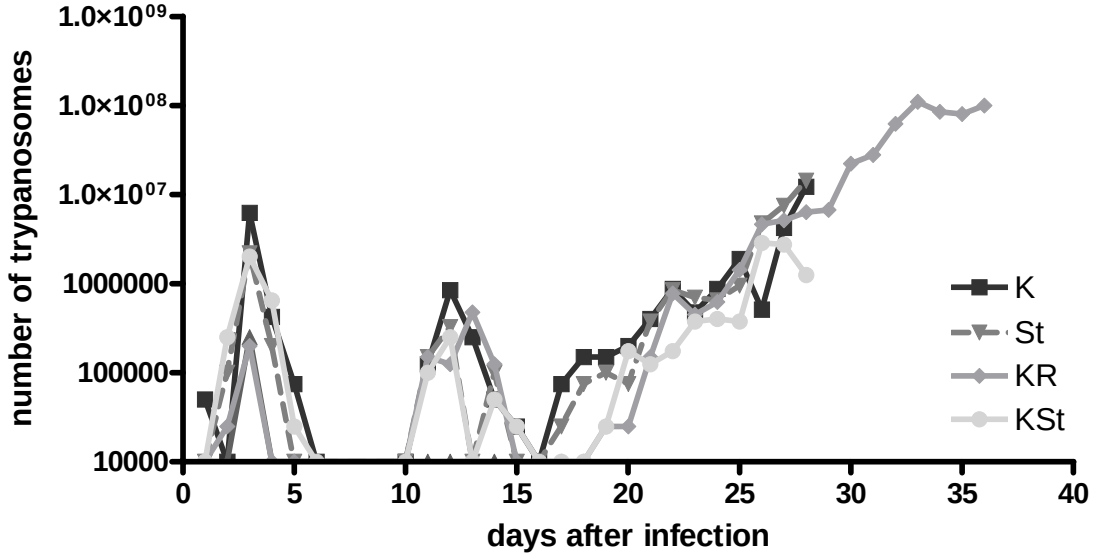
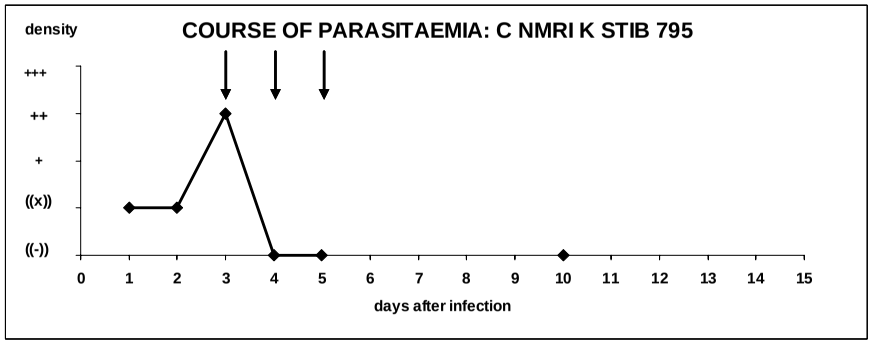


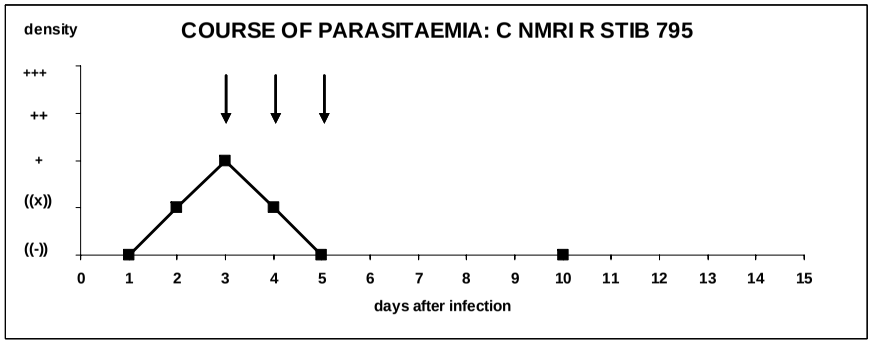
Fig. 27: Course of parasitaemia: STIB 795 infected NMRI mice with one treatment period

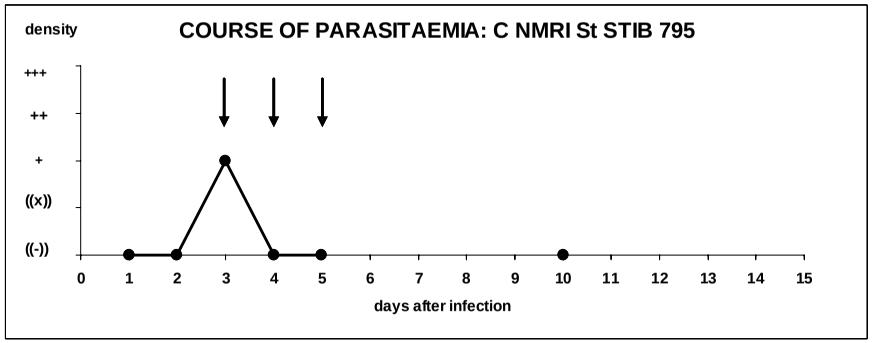
C NMRI 795

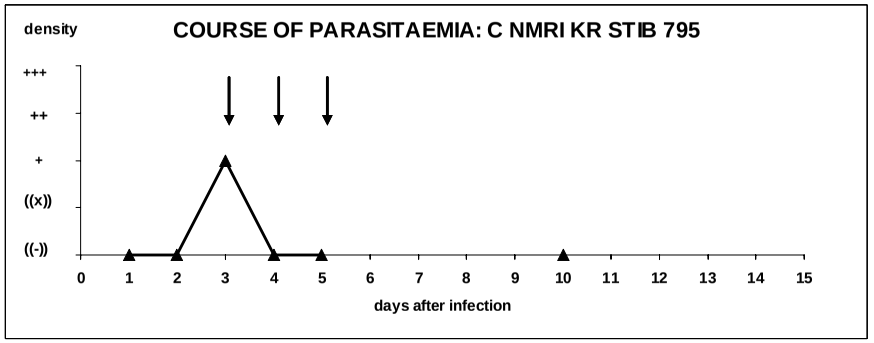
mice cured (no parasites up to day 185)

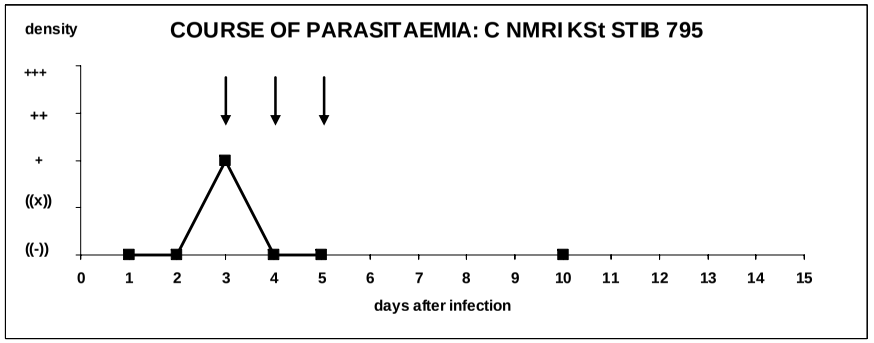
Treatment period (↑↓): d3-5





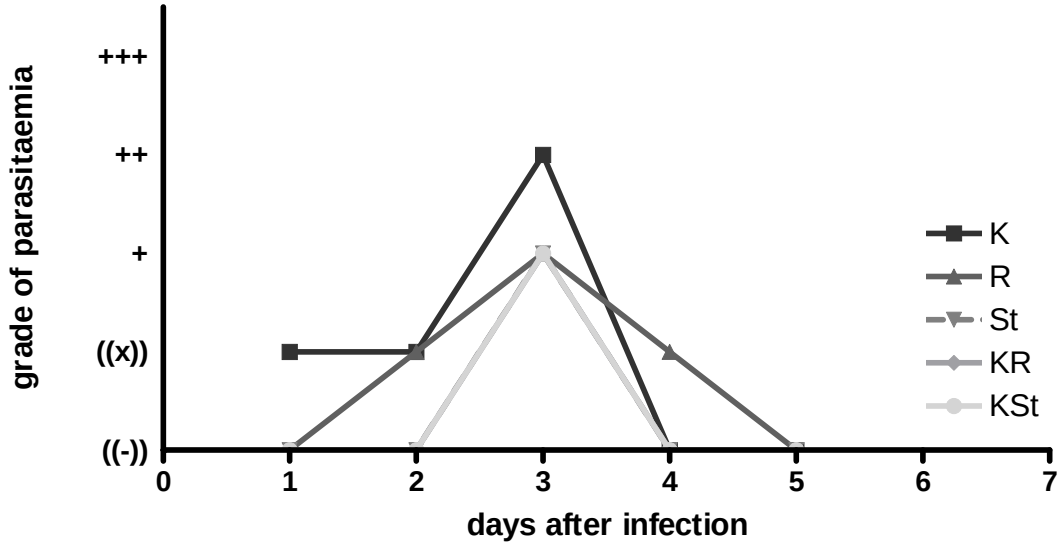






Group C:

Fig. 28: Course of parasitaemia:
STIB 795 infected NMRI mice with one treatment period



parasite free up to end of study at day 185

Fig. 29: Counts on Neubauer:
STIB 795 infected NMRI mice with one treatment period

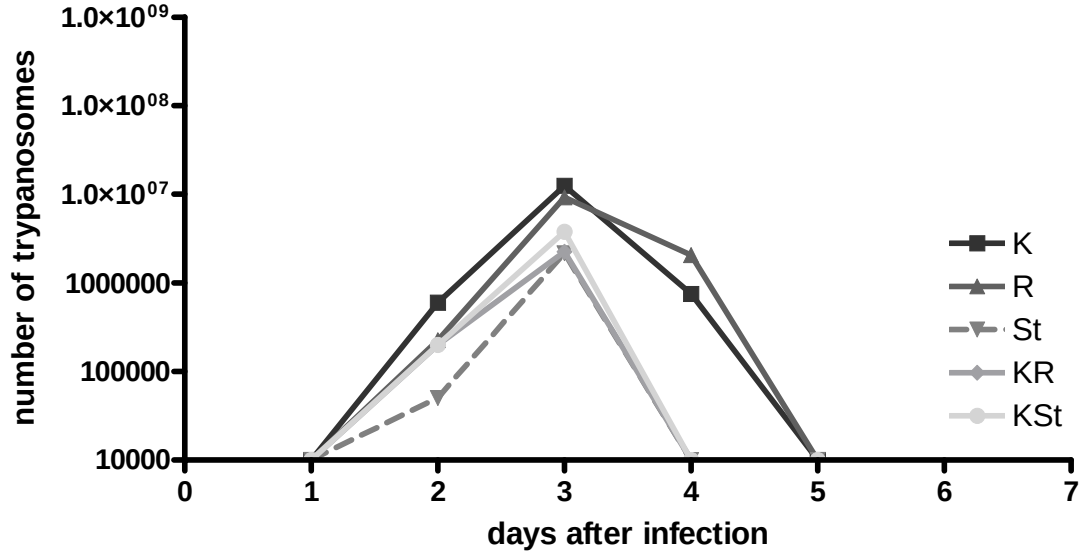
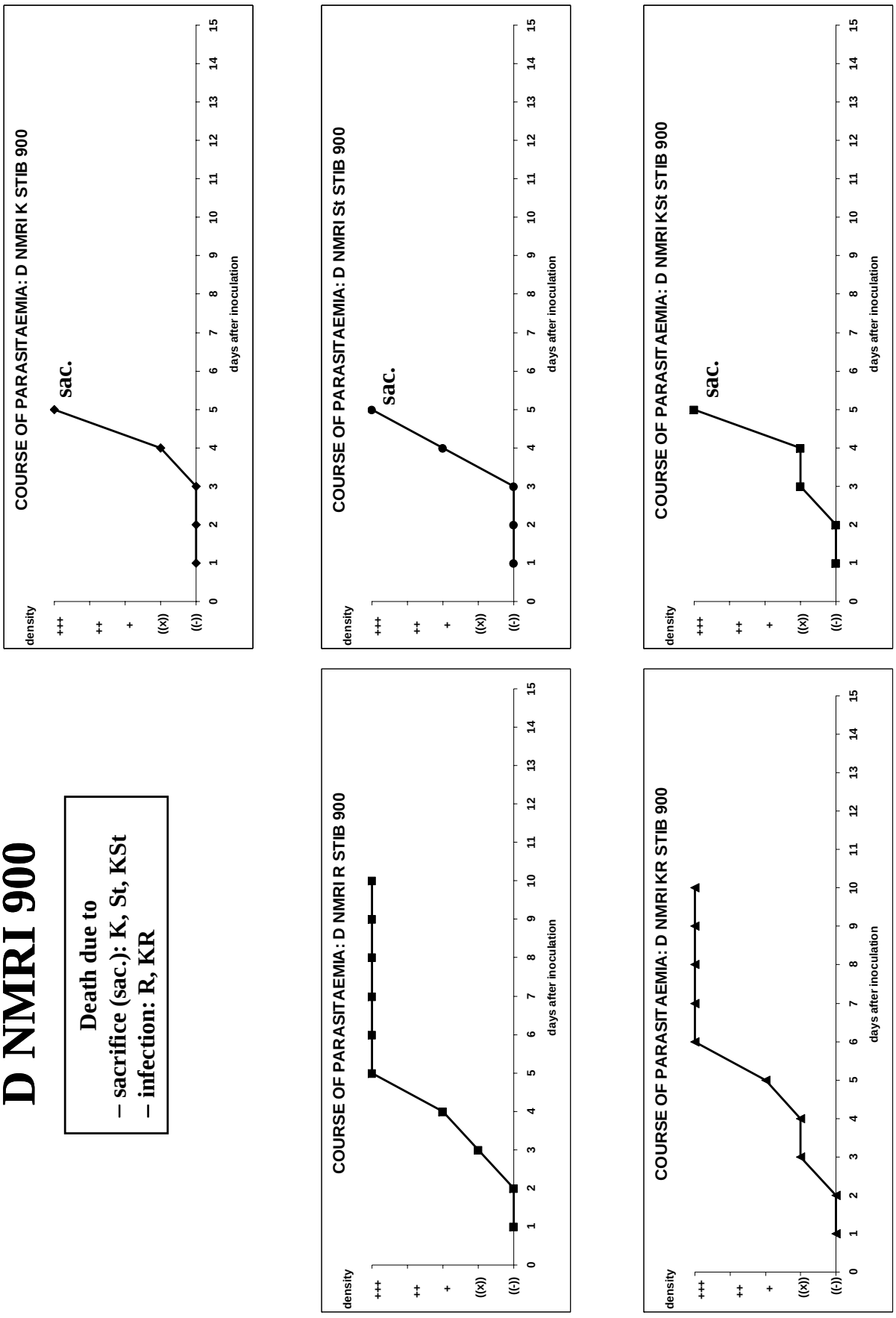
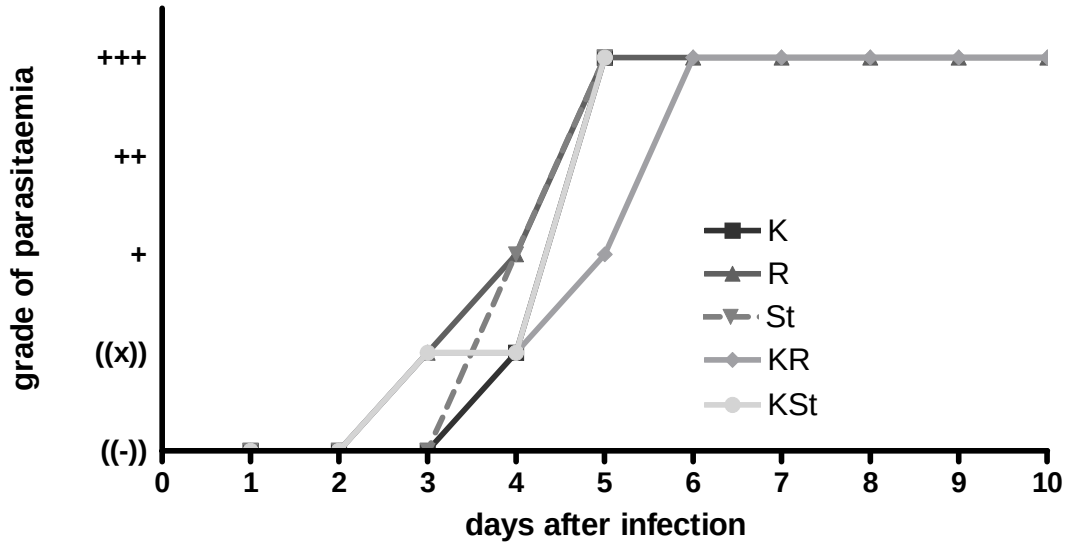


Fig. 30: Course of parasitaemia: untreated subpassaged STIB 900 infected NMRI mice



Group D:

Fig. 31: Course of parasitaemia: untreated subpassaged STIB 900 infected NMRI mice



death ·due to sacrifice: K, St, KSt ·due to infection: R, KR

Fig. 32: Counts on Neubauer: untreated subpassaged STIB 900 infected NMRI mice

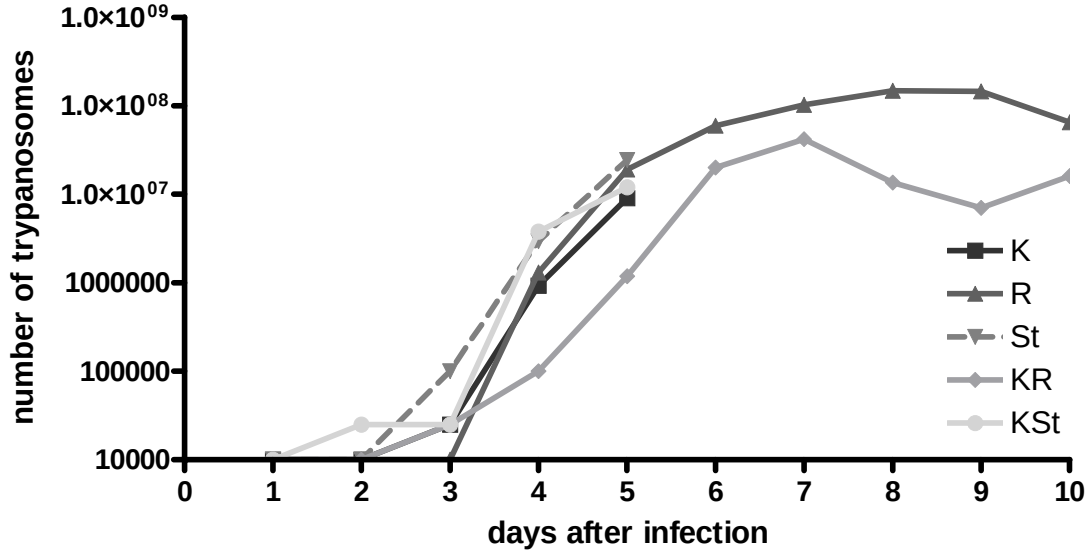
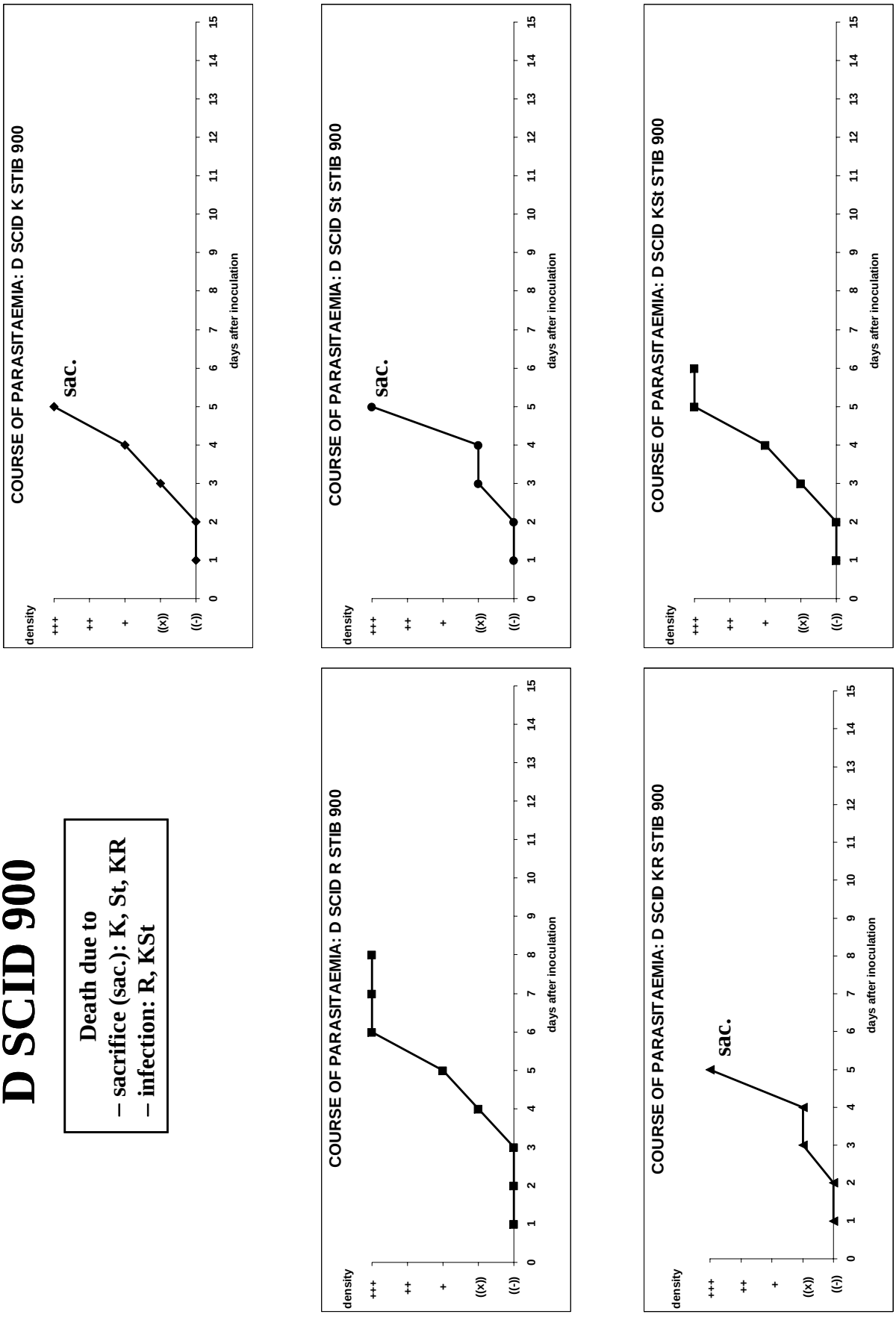
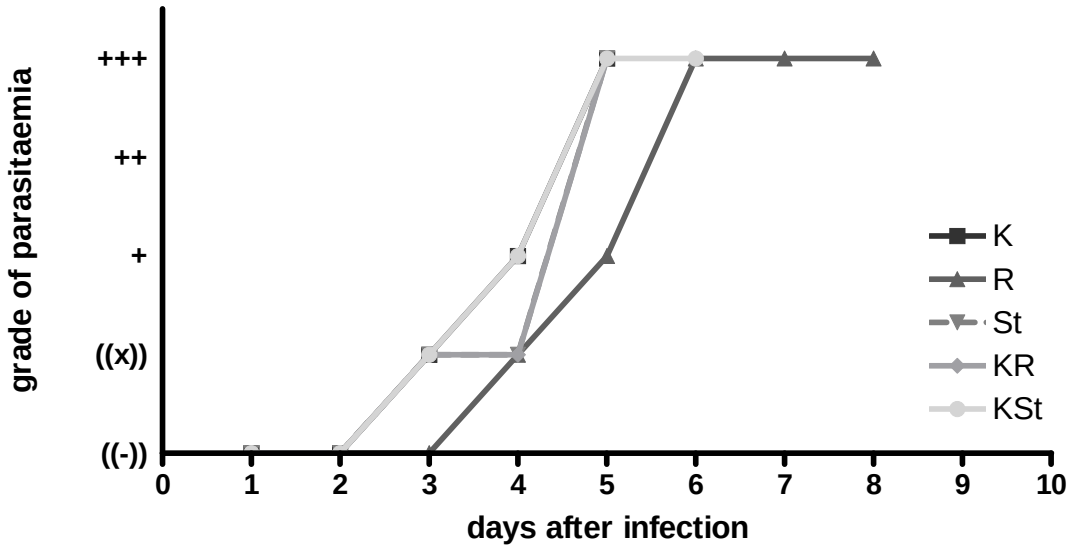


Fig. 33: Course of parasitaemia: untreated subpassaged STIB 900 infected SCID mice



Group D:

Fig. 34: Course of parasitaemia: untreated subpassaged STIB 900 infected SCID mice



death ·due to sacrifice: K, St, KR ·due to infection: R, KSt

Fig. 35: Counts on Neubauer: untreated subpassaged STIB 900 infected SCID mice

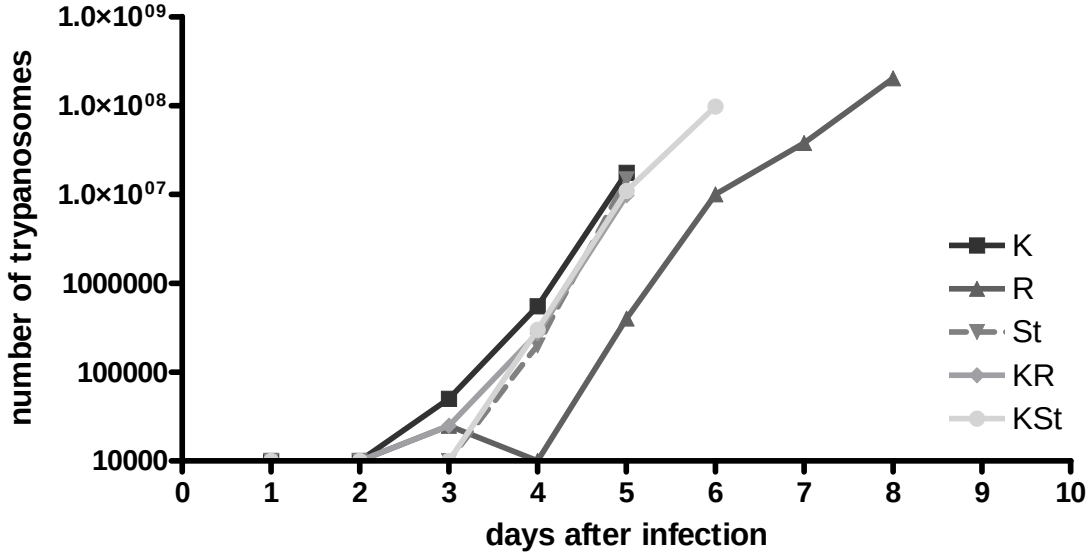
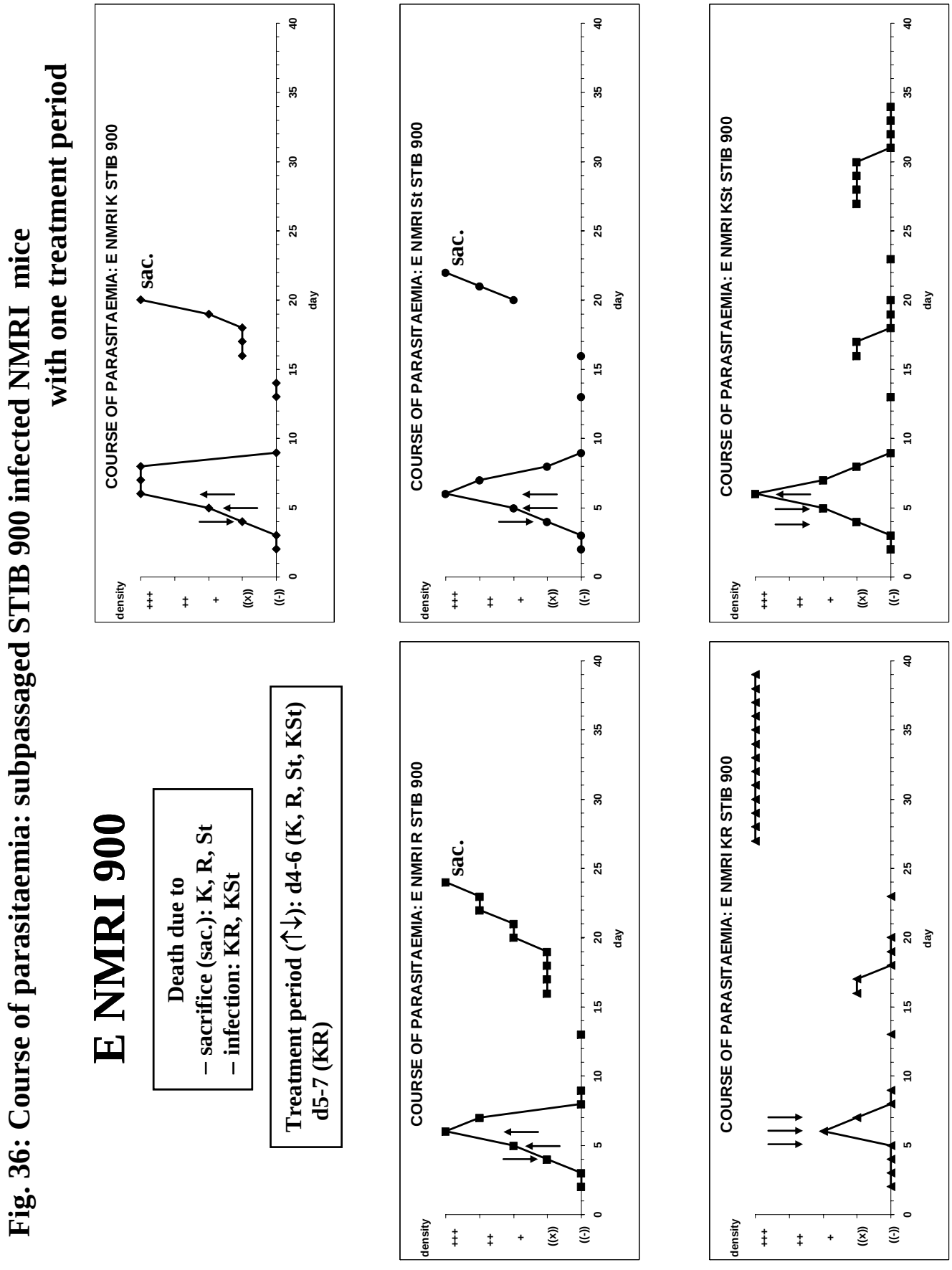
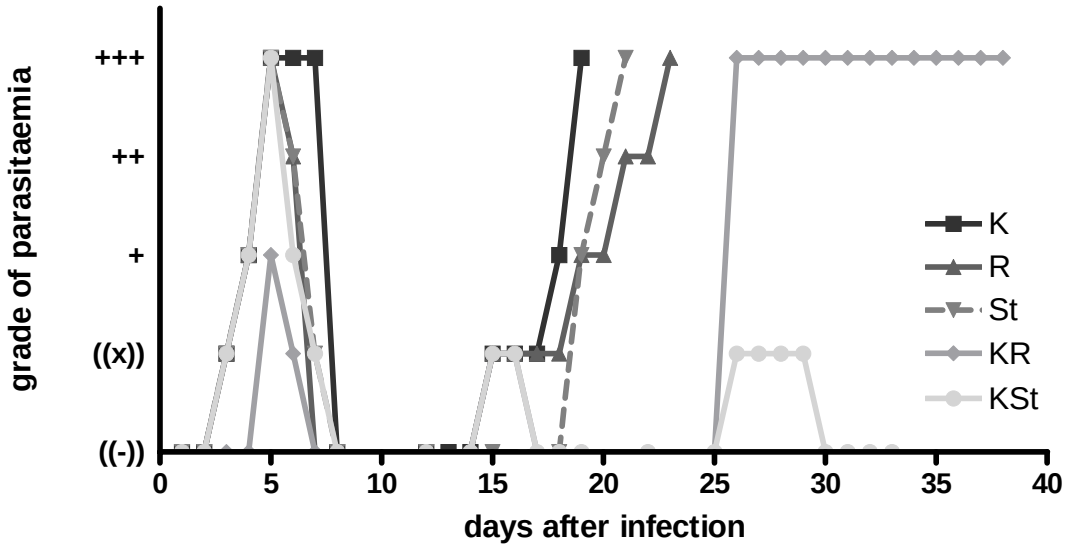


Fig. 36: Course of parasitaemia: subpassaged STIB 900 infected NMRI mice with one treatment period



Group E:

Fig. 37: Course of parasitaemia: subpassaged STIB 900 infected NMRI mice with one treatment period



death ·due to sacrifice: K, R, St ·due to infection: KR, KSt

Fig. 38: Counts on Neubauer: subpassaged STIB 900 infected NMRI mice with one treatment periods

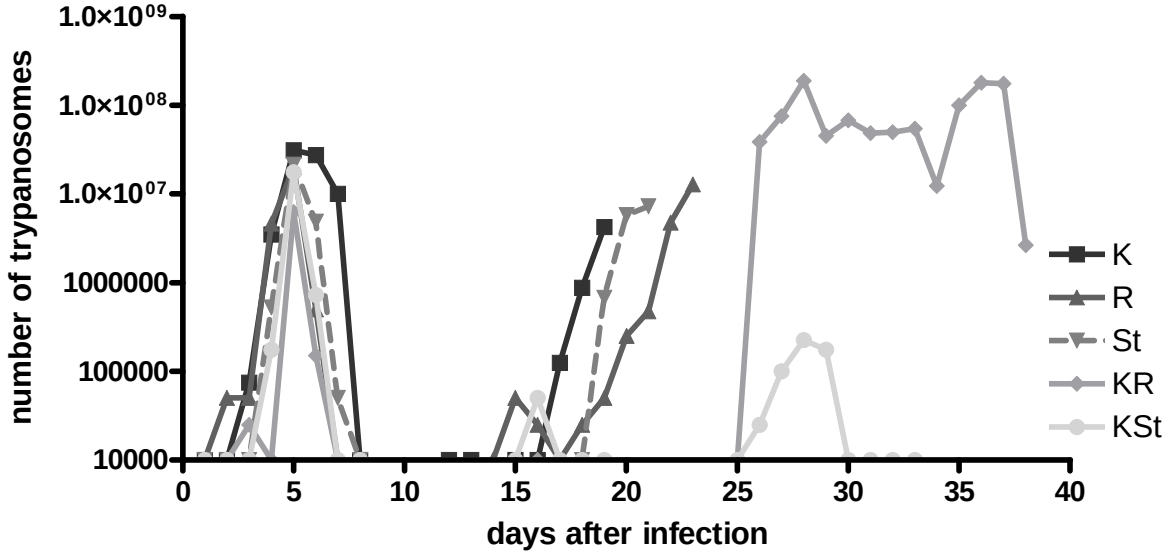
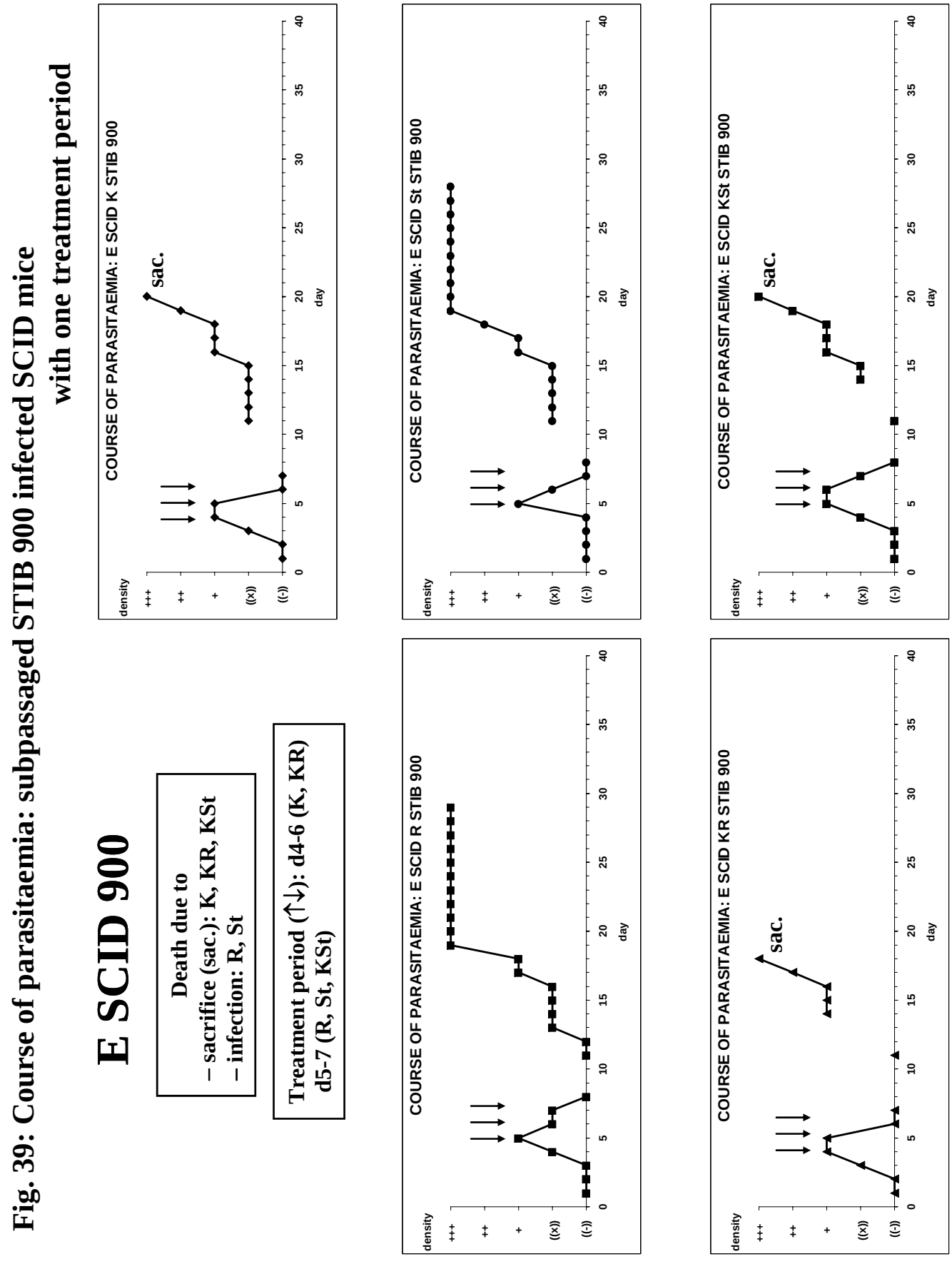
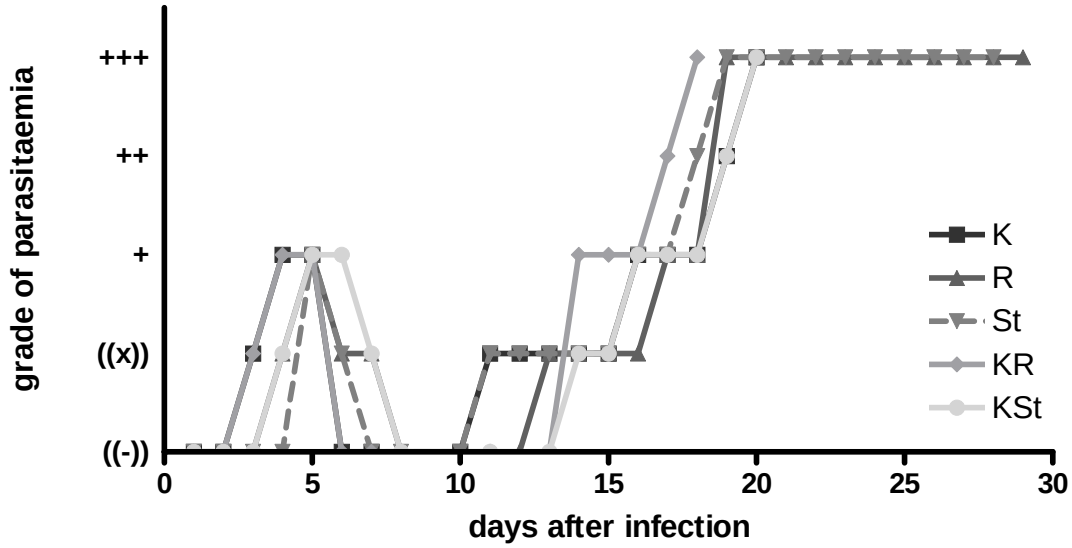


Fig. 39: Course of parasitaemia: subpassaged STIB 900 infected SCID mice with one treatment period



Group E:

**Fig. 40: Course of parasitaemia:
subpassaged STIB 900 infected SCID mice with one treatment period**



death ·due to sacrifice: R, KR, KSt ·due to infection: K, St

**Fig. 41: Counts on Neubauer:
subpassaged STIB 900 infected SCID mice with one treatment period**

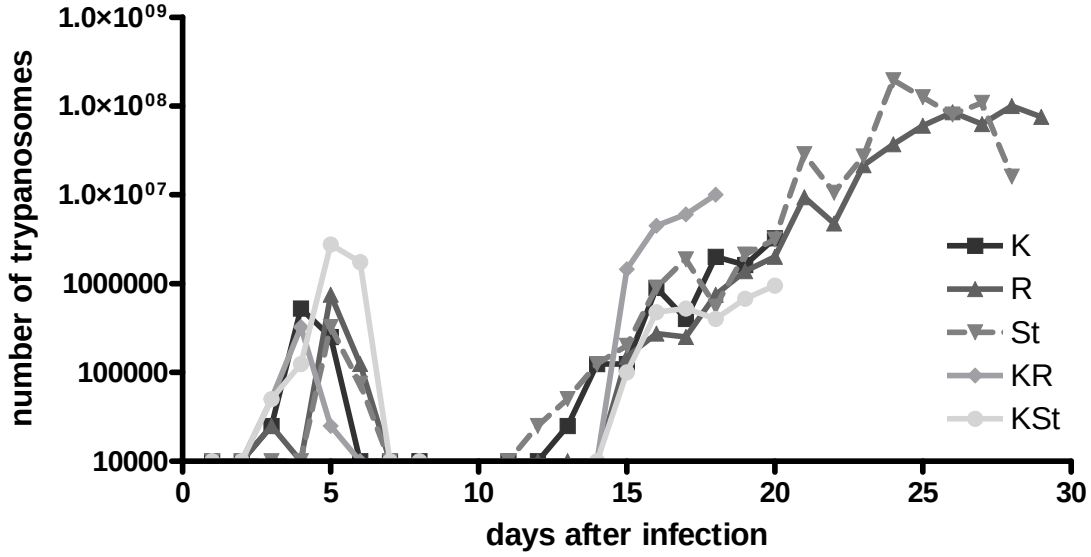
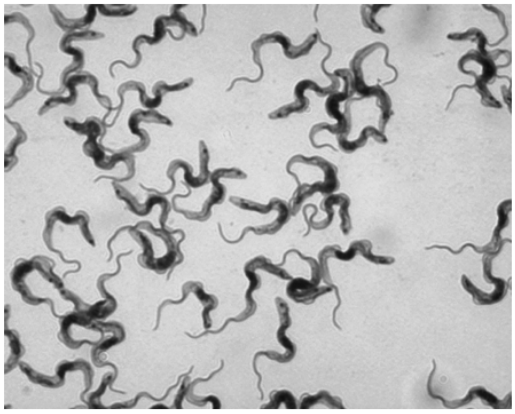
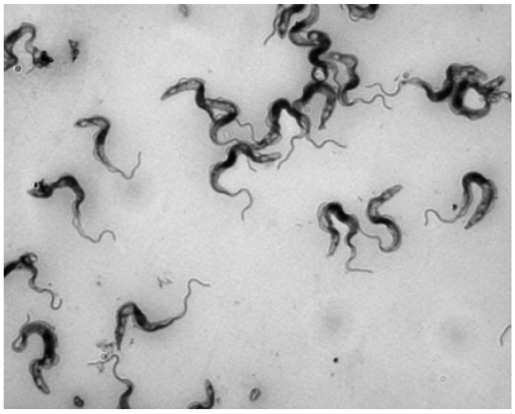


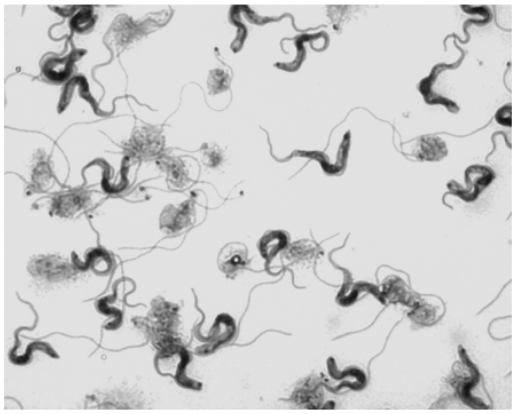
Fig. 42: Morphological appearance at magnification 1000x



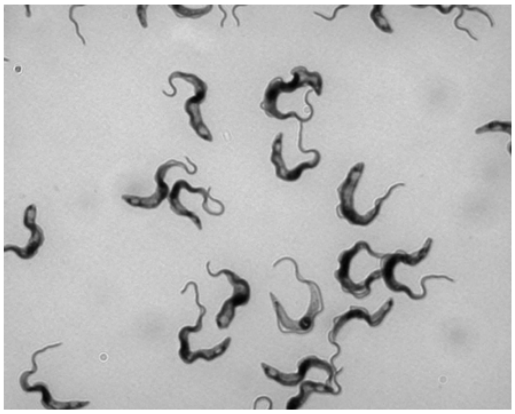
A NMRI K 100



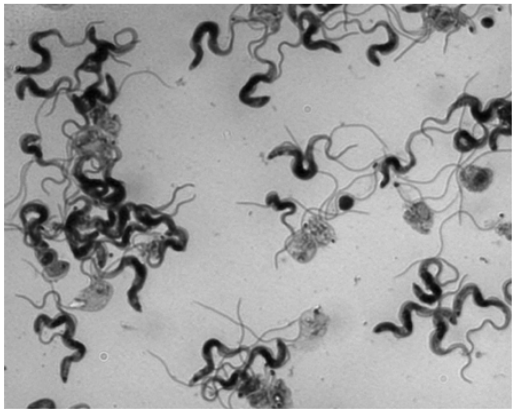
B NMRI R 100



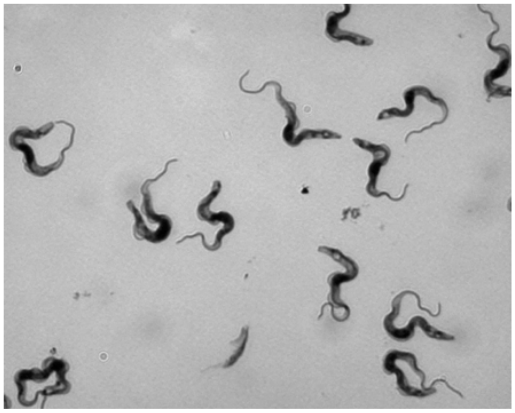
A NMRI KR 100



B NMRI St 100



A NMRI KSt 100



B NMRI KSt 100

Fig. 43: Example of CASY data

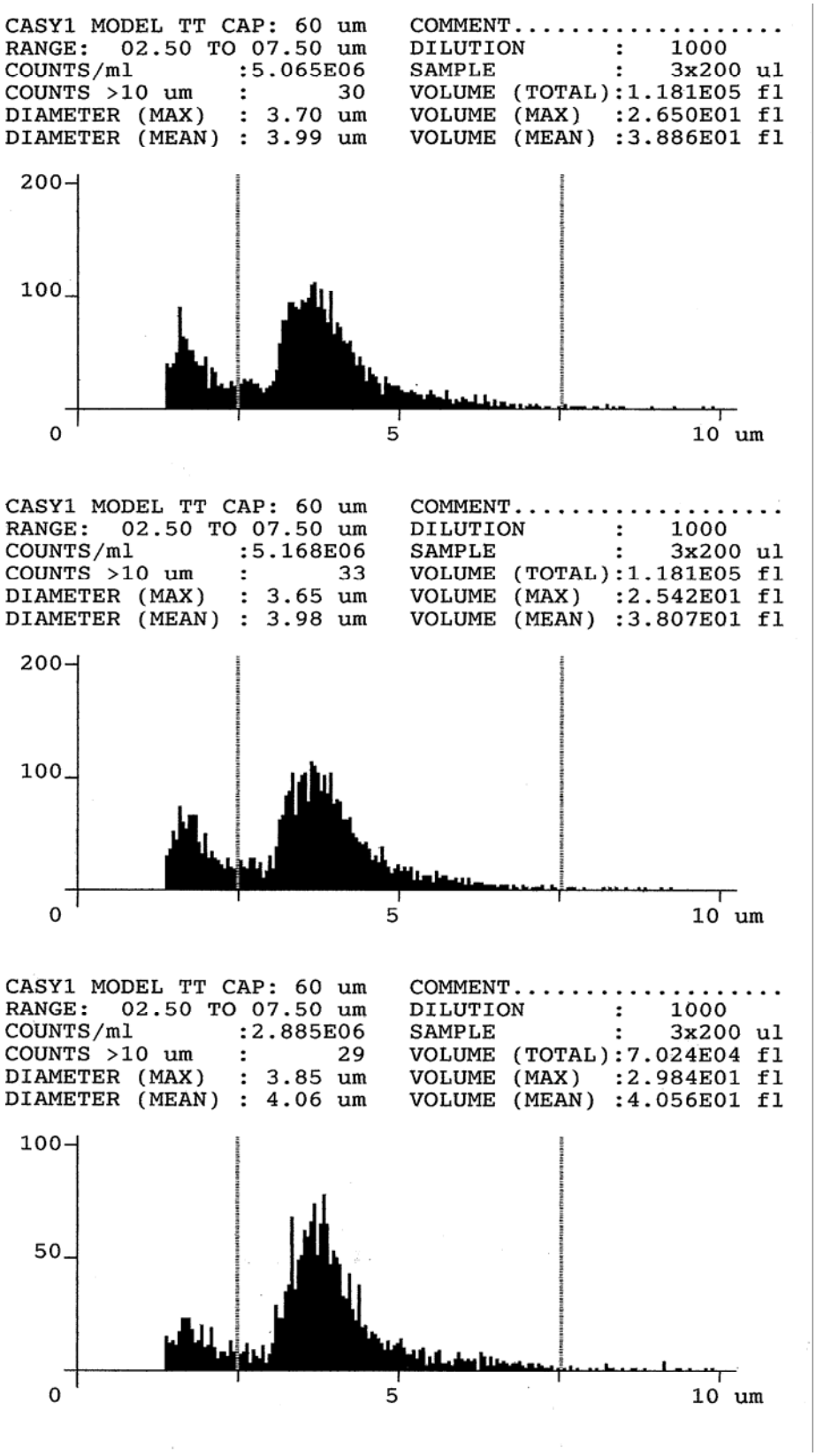
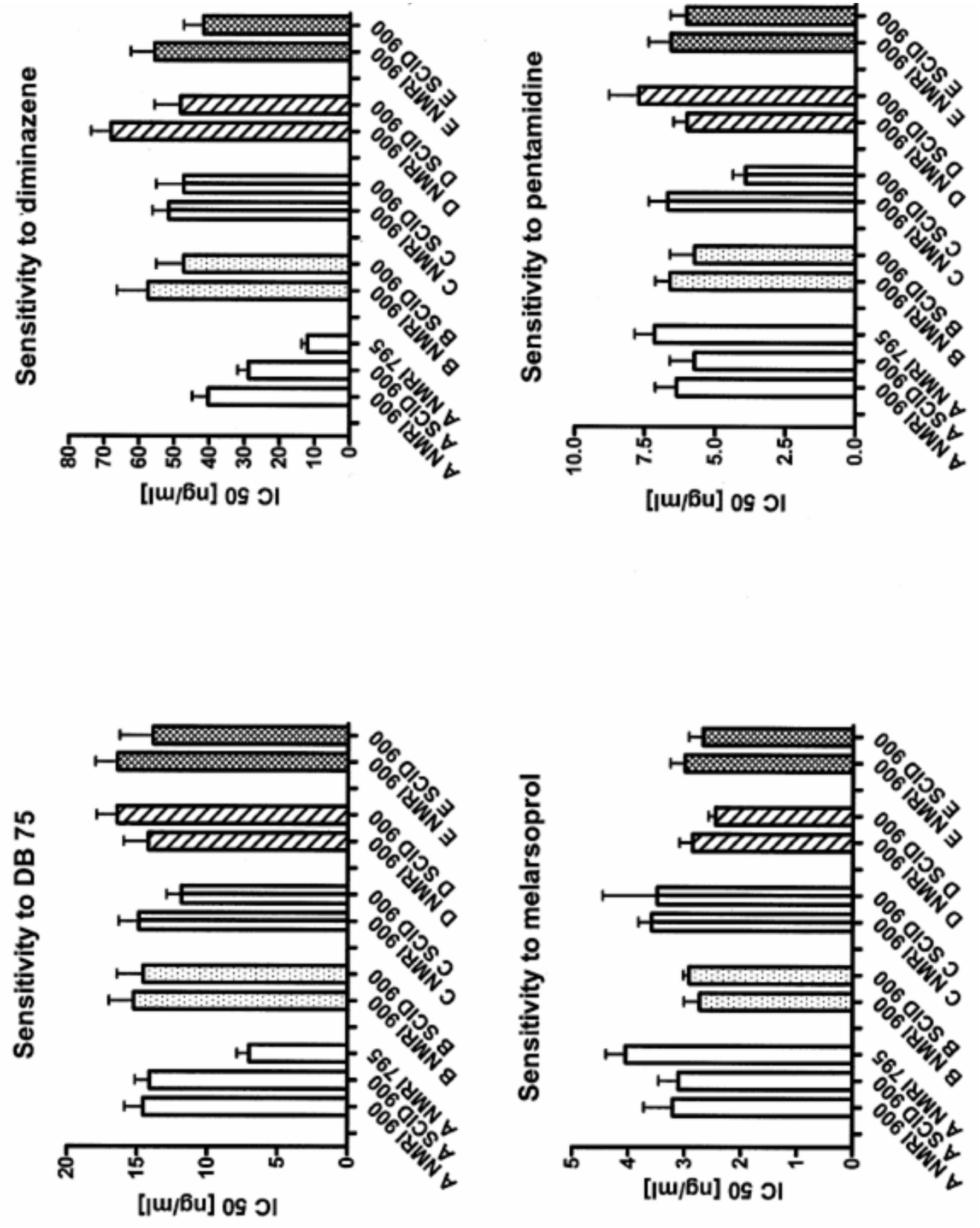


Fig. 44: Sensitivity of isolated trypanosomes to selected compounds



References:

1. 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 4:1273-7.

2. Bitonti AJ, McCann PP, Sjoerdsma A. Necessity of antibody response in the treatment of African trypanosomiasis with alpha-difluoromethylornithine. Biochem Pharmacol 1986; 35:331-4.

3. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. Nature 1983; 301:527-30.

4. Bosma MJ, Carroll AM. The SCID mouse mutant: definition, characterization, and potential uses. Annu Rev Immunol 1991; 9:323-50.

5. Brun R, Kunz C. In vitro drug sensitivity test for *Trypanosoma brucei* subgroup bloodstream trypomastigotes. Acta Trop 1989; 46:361-8.

6. Campbell GH, Esser KM, Weinbaum FI. *Trypanosoma rhodesiense* infection in B-cell-deficient mice. Infect Immun 1977; 18:434-8.

7. Czitrom AA, Edwards S, Phillips RA, Bosma MJ, Marrack P, Kappler JW. The function of antigen-presenting cells in mice with severe combined immunodeficiency. J Immunol 1985; 134:2276-80.

8. de Gee AL, McCann PP, Mansfield JM. Role of antibody in the elimination of trypanosomes after DL-alpha-difluoromethylornithine chemotherapy. J Parasitol 1983; 69:818-22.

9. Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol 1970; 28:521-34.

10. Osman AS, Jennings FW, Holmes PH. The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice. Acta Trop 1992; 50:249-57.

11. Peregrine AS, Mamman M. Pharmacology of diminazene: a review. Acta Trop 1993; 54:185-203.

12. Sacks DL, Askonas BA. Trypanosome-induced suppression of anti-parasite responses during experimental African trypanosomiasis. Eur J Immunol 1980; 10:971-4.

An attempt to identify organs in mice which may serve as niches for trypanosomes for relapse development

Abstract

In vivo screening of new diamidine analogues (DB compounds) in mice infected with the trypanosome strain STIB 900 revealed that treatment with some of these new compounds did not lead to total cure of the mice, whereas when infected with the trypanosome strain STIB 795 total cure was achieved. Blood was cleared from parasites in both cases, but STIB 900 trypanosomes reappeared after an aparasitic period again, proliferated and lead to death when no further treatment was performed. Taking the results from "Studies of kinetics of parasitaemia and trypanosome disappearance after drug treatment of first infection and relapses" into account, reappearance of parasites due to resistant trypanosomes was excluded. Therefore, the hypothesis was set up, that trypanosomes invade extravascular sites (niches) where they are not fully exposed to the drug and cause relapses by re-invading the blood again when no drug was present. In this study, a method to evaluate which organs may serve as niches was developed by tracing mRNA of trypanosomes using nested PCR. Organs of mice were homogenised and tested for trypanosomal presence. The method was shown to work, but unfortunately, the extraction of mRNA was not always successful. Additionally, it could be shown that improvements have to be made to increase the sensitivity of the detection when mouse mRNA is predominantly present. However, one organ, the spleen was detected to contain trypanosomal mRNA. This indicates that this organ may be the or one of the niches.

1. Introduction

The UNC lead consortium to discover new drugs for the treatment of parasitic diseases was founded in 1999. One of their projects is to improve treatment of human African trypanosomosis. Thereto, compounds were synthesised in analogy to pentamidine, the first line drug in treatment of first stage trypanosomosis. Promising results were obtained for some of the synthesised compounds, the so called DB compounds, when tested in vitro. While screening these compounds in vivo routinely, the observation was made, that no total cure could be achieved after treatment with DB 75 in the acute mouse model [Brun, R. (1999)],

when mice were infected with the trypanosome strain STIB 900 (*Trypanosoma brucei rhodesiense*). The compounds which were tested and did not achieve total cure belong chemically to the group of diamidines. They were synthesised as analogues to pentamidine with the aim to improve beside its efficacy its pharmacokinetic and pharmacodynamic properties. Although a rapid parasite clearance is achieved after treatment with DB compounds, relapses occur after a certain time with doses which are not curative. These relapses were thought to result from trypanosomes sequestering in extravascular sites inaccessible for the drug (niches) and from where re-invasion of the blood may take place once drug levels drop below therapeutic levels. The duration of the aparasitic periods between clearance of the parasites after drug treatment and re-appearance of trypanosomes in the blood depend on the drug, the dose, the dosage scheme and the line of the infected mouse. The protocol of the acute mouse model looks as follows: mice are infected intraperitoneally with $1*10^3$ trypanosomes. Three days post infection, when parasites can be detected microscopically in the tail blood, the 4 day treatment period starts with a high but tolerated dose using the intraperitoneal route.

To identify possible niches, some adaptations to this protocol were made: mice were infected with 10^5 trypanosomes. Additionally, the duration of the treatment period was reduced to shorten the aparasitic period between clearance and re-appearance of trypanosomes in the blood. In summary: NMRI mice infected with the trypanosome strain STIB 900 were treated 3 days post infection with 10 mg/kg DB 75 once per day for 3 days. Already after the first treatment, trypanosomes were cleared from blood in almost all mice, a day later 100% of the animals were parasite free. In the case of DB 75, the duration of the aparasitic period between clearance and re-appearance of trypanosomes in the blood is different in each mouse, lasting from 9 to 20 days (see “ Studies of kinetics of parasitaemia and trypanosome disappearance after drug treatment of first infection and relapses”). After this period, trypanosomes can be found again when tail blood is inspected microscopically. The number of trypanosomes increases and the mice die if untreated. Taking all the above mentioned facts into account, the hypothesis was set up, that trypanosomes sequestered in organs could circumvent the influence of the drug and initiate a parasitaemia when drug levels drop below therapeutic doses. From literature such relapses were already known. Jennings observed relapses occurring dependent on the beginning of the treatment period in mice infected with the trypanosome strain GVR 35 (*T. b. brucei*), when treated with diminazene. Mice which were treated within 7 days after trypanosome infection could be cured totally, whereas mice

undergoing treatment after day 21 post infection relapsed after a certain period. The source of the relapses was identified to be the CNS [Jennings, F.W. (1979)].
The blood-brain-barrier (BBB) is thought to be the reason for relapses. This barrier allows only small and lipophilic molecules to cross unless there is a specific transporter for a molecule. The existence of the BBB creates an unique milieu where trypanosomes may be protected from certain drugs circulating in the blood stream. Polar molecules like diamidines would be too charged to cross, and to date no specific transporter for diamidines is known to be located in the cells forming the BBB. For this reason, the CNS is thought to be the ideal niche for trypanosomes. One could speculate if invasion of the brain by trypanosomes may be facilitated when an infectious disease is present, because factors accompanying an infection were known to break down the integrity of the BBB. Then, passage of the BBB would be easier not only for the parasites but also for other molecules [Chaudhuri, J.D. (2000)]. One of these factors is nitric oxide (NO), which is synthesised by a NO oxidase which may be stimulated by stress, inflammation and infection [Thiel, V.E. (2001)].Whether a trypanosome infection is able to break down the integrity of the BBB within 3 days after infection is not known, nor is the mechanism of the trypanosomes to cross this barrier.
Furthermore, to date only late stage trypanosomosis is associated with CNS infection.
Reflecting this, one could draw the following conclusions:

1. All organs, not only the CNS, in which lower drug levels than in the blood are found could serve as niches.

And/ Or

2. CNS infection could take place at an earlier stage of the disease (at least in this model).

Various methods to identify these niches were discussed in advance. An approach to detect trypanosome presence with the aid of antibodies was rejected, since antibody-based assays cannot always distinguish between past and current infections [Nutman, T.B. (1994)]. Additionally, differentiation between trypanosomes which are alive or dead is not possible by this method, what is a pre-requisite for this experiment since only trypanosomes which are alive may cause relapses. Using polymerase chain reaction (PCR) was thought to be the most appropriate tool to detect these niches by amplifying a trypanosome-specific gene.
Evidence should have been supplied on the genomic DNA level, selecting primers amplifying a sequence occurring only in the genome of the trypanosome. Since the trypanosomal genome

is known to possess 200 copies of a 1390 bp long sequence, which is found mostly clustered as direct tandem repeats, and contains the 35 nucleotides known as spliced leader (SL or mini exon), primers within this sequence were chosen [de Lange, T. (1983)], [Dorfman, D.M. (1984)]. Nested PCR was performed to increase the sensitivity of this method. Thereto, primers were chosen which amplify a fragment within the sequence selected for the primary PCR (Fig.1).

The method of detection had to be changed from amplification and detection of a DNA sequence to a mRNA sequence (see results and discussion). Additionally, inoculation of brain pellets obtained from infected and melarsoprol, DB 75 respectively, treated mice into recipient mice was performed to determine whether brains may serve as niches. Thereto, blood of the recipient mice was checked for the presence of trypanosomes.

2. Material and Methods

2.1. Trypanosomes:

Trypanosoma brucei rhodesiense (STIB 900):

STIB 900 is a derivative of STIB 704. Pathogens were isolated from a male patient at St. Francis Hospital in Ifakara/Tanzania in 1982. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenical growth in vitro.

Trypanosoma brucei brucei (GVR 35):

Trypanosomes were isolated from a wilde-beast in the Serengeti in 1966 and were passaged in a group of irradicated mice before stabilates were made [Jennings, F.W. (1982)].

2.2. Mice

Female NMRI mice with a body weight of 20-25 g, obtained from RCC, Ittingen, Switzerland, were used.

2.3. Drugs and Primers

2.3.1. DB 75

DB 75 was synthesised by Dr. D. W. Boykin, Georgia State University, Atlanta, USA. An aqueous solution of 1 mg/ml DB 75 in 10% DMSO was prepared the day of treatment.

2.3.2. Melarsoprol

Melarsoprol was obtained from WHO (Geneva, Switzerland, donated by Sanofi-Aventis). A suspension 0.8 mg/ml melarsoprol in water was prepared the day of treatment.

2.3.3. Endoxan®, Cyclophosphamide

Cyclophosphamide was purchased from Baxter (Volketswil, Switzerland).

2.3.4. Diminazene

Diminazene was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.3.5. Primers

All primers designed were tested for self complementarity using "Oligonucleotide properties Calculator" software (www.basic.northwestern.edu/biotools/oligocalc.html). They were purchased from Qiagen (Qiagen, Hilden, Germany). A stock solution containing 100 μM primers in TE (Tris HCl 10 mM, EDTA 1 mM) was prepared. For PCR, primers were diluted further to 10 μM. The sequences of the primers were mentioned below.

2.4. Infection and treatment of mice

2.4.1. Infection of mice

Cryopreserved stabilates containing bloodstream forms of trypanosome strain STIB 900 were diluted with phosphate-buffered saline with glucose (PSG) buffer to a final concentration of $1*10^5$ trypanosomes/0.25 ml. Inoculation of parasites was performed intraperitoneally.

2.4.2. Monitoring the parasitaemia

The days treatment was performed, blood was checked by tail blood examination. Thereto, one drop of blood, obtained by tail snip, was diluted with one drop of sodium citrate (Merck, Darmstadt, Germany) 3.2% and inspected under the microscope at a magnification of 200x.

2.4.3. Treatment of mice

Treatment was performed 3 days after inoculation, once a day for 3 days with 10 mg/kg bodyweight DB 75 or 8 mg/kg bodyweight melarsoprol, respectively.

2.5. Preliminary experiment: check for absence of trypansomal DNA in blood after clearance of parasites

In a preliminary study, blood obtained from five mice which were infected with trypanosomes and treated three days post infection for 3 days with 10 mg/kg DB 75 was tested for the presence of trypanosomes. Thereto, blood obtained by cardiac puncture was DNA extracted using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) performing the spin protocol. Concentration of DNA was performed by extraction with 3 M sodium acetate (Merck, Darmstadt, Germany). Precipitate was washed with ethanol 70% (Merck, Darmstadt, Germany) and reconstituted in 20 µl TE/0.1% Tween 20 (Fluka Chemie GmbH, Buchs, Switzerland). PCR was performed as mentioned for DNA extracted from tissues.

2.6. Microscopical blood monitoring

One week after the last treatment was performed, blood was harvested by performing a cardiac puncture. As anticoagulant 0.1 ml of a solution containing 3.2 % sodium citrate was used. Trypanosomes were separated from blood cells using a DEAE column [Lanham, S.M. (1970)]. The column eluent was centrifuged at 2700 rpm (Jouan, Johnson Control, Basel, Switzerland). Supernatant was discarded except for 0.5 ml. Pellet was resuspended and transferred into a pasteur pipette with a tip which had been melted to seal. A second centrifugation step was performed (2700 rpm). The tip of the pasteur pipette was inspected for the presence of trypanosomes under the microscope at a magnification of 200x. If no trypanosomes could be detected, preparation of the isolated organs was performed, if trypanosomes were detected the whole infection and treatment procedure was performed with a new mouse. A similar centrifugation method was described by Lumsden and co-workers [Lumsden, W.H. (1977)].

2.7. Preparation of organs

Mice were sacrificed and dissected. Each organ was stored separately in PSG on ice until it was further proceeded. The following organs were isolated:

- pancreas
- liver
- brain
- genitalia
- lung
- heart
- spleen
- kidney
- fat tissue
- muscle tissue
- thymus
- spine
- eyes
- skin

The identification of the isolated organs was confirmed by an experienced technician and a mouse anatomy book [Iwaki, T. (2001)].

Homogenisation of the isolated organs was performed using a homogeniser (Ultra-Turax T 25, IKA Labortechnik, Janke & Kunkel GmbH & Co KG, Staufen, Germany) with disperser (S 25 N 10 G). After homogenisation of each organ, the Ultra-Turax was disassembled, cleaned with soap and rinsed with plenty of water to prevent contamination from one organ to another. After being homogenised, tissues underwent a slow centrifugation step (700 rpm). Supernatant was transferred and underwent a further centrifugation step (2700 rpm). With these two centrifugation steps, the amount of material could be reduced drastically without losing any trypanosomes. If trypanosomes would have been in any of these organs, they should be found in the pellet after these two centrifugation steps.

Positive control: trypanosomes from culture

For positive control, DNA/mRNA were extracted from a well growing trypanosome culture. Thereto, a solution containing $1*10^6$ trypanosomes was centrifuged at 2750 rpm. The supernatant was discarded and the pellet was extracted for DNA/mRNA as mentioned for the tissue samples.

Negative control: uninfected and untreated mouse

DNA/mRNA of a mouse, which had never been infected nor treated was isolated from organs (liver, brain, spleen) with the aid of the Qiagen® Kit/mRNA extraction method respectively, as mentioned below. These samples were used additionally to determine the limit of detection of the performed PCR.

2.8. Screening for trypanosomal DNA

2.8.1. Extraction of DNA with Qiagen® Kit

DNA of the pellet was extracted using the Qiagen DNeasy® Tissue Kit for DNA purification from animal tissues according to the DNeasy® protocol for animal tissues (DNeasy Tissue Kit Handbook 05/2002). After the extraction, the obtained volume was further reduced to concentrate the DNA by precipitation with 3 M sodium acetate (Merck, Darmstadt, Germany) and absolute EtOH (Merck, Darmstadt, Germany). After an additional washing step with EtOH 70% and subsequently drying, the pellet was reconstituted in 20 µl TE/0.1% Tween 20.

2.8.2. PCR

All PCR reactions were performed in an Eppendorf Mastercycle gradient (Dr. Vaudaux AG, Schönenbuch/Basel, Switzerland). Amplification by PCR was done with the following reaction mixtures (50 µl):

	DNA	cDNA	
buffer (10x)	5.0 µl	buffer for advantage taq (10x)	
dNTPs (2 mM)	5.0 µl		
primer fwd (10 µM)	2.5 µl		
primer rv (10 µM)	2.5 µl		
taq (5 units/µl)	0.2 µl	taq (advantage: 50 units/µl)	0.3 µl
water	31.8 µl		31.7 µl
template	3.0 µl		

Unless not stated otherwise the following protocol was used: initial denaturation was performed at 94° C for 3 min. Thereafter, a program performing 25 cycles for DNA samples, 30 cycles for cDNA samples respectively, of 30 s at 94° C, 40 s annealing at 60° C and 1 min extension at 72° C was run. The obtained products were separated by electrophoresis on a 0.5% agarose (QA-Agarose TM, Qbiogene, Basel, Switzerland) gel and stained with ethidium bromide.

Primary PCR of DNA extracts

Primary PCR was performed with additional $MgCl_2$ in the reaction mixture. Thereto, 3 µl of water were replaced by 3 µl a of solution containing 25 mM $MgCl_2$, leading to a total $MgCl_2$ concentration of 3 mM in the reaction mixture. As forward primer SLfa: 5`- GAG AAG CTC CCA GTA GCA GC and as reverse primer SLr3: 5`- TCG CCT GCT CAG TAG ACA TG was used. The expected length of the amplified fragment was 554 bp.

Secondary PCR of DNA extracts

In the nested PCR the following primers were used: forward: SLfb: 5`- GCC AAC ACA CGC ATT GTG C and reverse: SLr2: 5`- CAT GAA AGC GCC CAT CGA GG. The product is expected to be 342 bp.

Limit of detection

To find out the limit of detection of this method, mouse DNA was mixed with DNA obtained from cultured trypanosomes. Dilutions containing 100, 20, 4, 0.8, 0.16 and 0.0032 trypanosomes per µl were tested with nested PCR.

2.9. Screening for trypanosomal mRNA

All reagents used for mRNA extraction and transcription to cDNA were supposed to be RNase free.

2.9.1. mRNA Isolation

The pellet obtained after the centrifugation steps was transferred to an Eppendorf tube and five times the volume of this pellet Trizol (Invitrogen AG, Basel, Switzerland) was added. After vortexing, the tube was incubated at 37° C for 5 minutes and then stored at –80° C. After thawing, chloroform (Sigma Aldrich GmbH, Steinheim, Germany) was added (1/5 of the Trizol volume). The content of the tube was mixed well and incubated at room temperature for 3 min, then spun for 30 minutes at 4° C. The aqueous phase (supernatant) was transferred into a new Eppendorf tube and 0.8 volume of isopropanol was added. The mixture was vortexed and precipitated over night at –20° C.
The tube was spun 30 min at 4° C. Supernatant was discarded and the pellet was washed with Ethanol 75% (Merck, Darmstadt, Germany) and dried. Trizol was added and the whole procedure was performed again. The obtained pellet was processed as mentioned under 2.9.2..

2.9.2. DNA digestion of RNA

The pellet was dissolved in 38 µl 5 mM Tris/0.1 mM EDTA (Fluka Chemie GmbH, Buchs, Switzerland) and incubated for 5 min at 50° C with open lid. 2.5 µl 0.1 M DTT, 1 µl RNase inhibitor (10 units/µl), 5 µl RQ1 10x buffer and 3.5 µl (1 unit/µl) DNase RQ1 (all Promega, Catalys AG, Wallisellen, Switzerland) was added and the mixture was incubated at 37° C for 30 min. 180 µl Trizol was added, followed by another incubation period of 5 min at 37° C. Chloroform (40 µl) was added, the tube was vortexed and incubated at room temperature for 3 min. After having spun the tube for 30 min at 4° C, the aqueous phase was transferred to a new Eppendorf tube and isopropanol (0.8 volume) was added. The mixture was vortexed and incubated at –20° C overnight to precipitate the RNA. The pellet was washed with

ethanol 75% and dried. This procedure was repeated once, the obtained pellet was processed as mentioned in 2.9.3..

2.9.3. Reverse transcription

The pellet was dissolved in 50.2 µl 5 mM Tris/0.5 mM EDTA. 0.3 µl hexamers (random primer: 3 µg/µl, Invitrogen AG, Basel, Switzerland) was added. After incubation at 65° C for 5 min, the tube was placed on ice. 3.5 µl 0.1 mM DTT, 2 µl RNase inhibitor (10 units/µl), 7 µl reaction buffer 10x, 7 µl 5 mM dNTPs (Qiagen, Hilden, Germany) was added. 40 µl of this mixture were place into a new tube and served as positive control. The remaining 30 µl represented the negative control. To the positive control 1 µl of reverse transcriptase (Sensiscript, Qiagen, Hilden, Germany) was added. All tubes were incubated at 37° C for 1 h 15 min. Afterwards, the tubes were incubated for 3 min at 93° C and 1 µl RNase A (500 µg/ml) was added. An incubation period of 20 min at 37° C took place, before tubes were put on ice. A solution of 10% Tween 20 was added to the cDNA to achieve a final concentration of 0.01% Tween 20. Then cDNA was stored at –20° C.

2.9.4. Test for presence of cDNA

A fragment of 785 bp is expected to be amplified from genomic DNA when using the mouse primers poly_fwd_2: 5´- CTT CCT TGA CTG TTC TAG C and poly_rv_2: 5´- AAT GGC CAG GAC GGA AC as reverse primer. Fragments amplified from cDNA will show a length of 395 bp using the same primers. 55° C annealing temperature was evaluated to give the best results. To be more sensitive, advantage taq and corresponding advantage buffer (Becton Dickinson AG, Basel, Switzerland) was used.

2.9.5. Primary cDNA PCR for trypanosome presence

In the primary PCR spliced_l_for:5`- ATT ATT AGA ACA GTT TCT GTA CTA TAT TG and tub_b_rv: 5`- ACA CAT CGA GCA CAG AGT CG were used as primers. The amplified cDNA sequence is expected to have 436 bp. PCR was performed using advantage taq.

2.9.6. Nested cDNA PCR

Spliced_l_for: 5`- ATT ATT AGA ACA GTT TCT GTA CTA TAT TG and tub_b_nested_rv: 5`- CAC CCT CCG TGT AGT GGC C were used as primers for secondary PCR, resulting in a fragment of 406 bp on the cDNA level using advantage taq.

2.10. Inoculation of brain homogenates

2.10.1 Brain of a GVR 35 infected mouse

A mouse, infected with trypanosomes of the strain GVR 35, was treated at day 21 with diminazene 40 mg/kg intraperitoneally and sacrificed nine days later. Blood obtained by cardiac puncture was analysed for trypanosomal presence microscopically. Since no parasites were found in the blood, the brain was homogenised, centrifuged and injected i.p. into an immunosuppressed recipient mouse. Immunosuppression was performed with cyclophosphamide (200 mg/kg) applied 5 hours in advance of injection of the brain pellet. Tail blood was checked for trypanosomes a week after injection of the homogenate.

2.10.2. Brain of a STIB 900 infected mouse

Half of the organ homogenates of brains from melarsoprol and DB treated mice, were injected into immunosuppressed mice. Thereto, mice were treated with cyclophoshamide 200 mg/kg 5 hours prior to application of the homogenate intraperitoneally. Weekly, parasitaemia was checked in tail blood and immunosuppression was performed once a week for 4 weeks.

3. Results

3.1. Detection of trypanosomal DNA

3.1.1. Preliminary experiment (Fig. 2)

Blood of infected and subsequently treated mice, was collected in the aparasitic period and checked for presence of trypanosomal DNA by nested PCR. Only when blood samples were PCR negative, testing for trypanosomes hidden in tissue would be sensible. Otherwise, it could not be excluded that positive signals obtained for tissues may be caused by trypanosomal DNA from dead trypanosomes which was found in the circulating blood. Since no trypanosomal DNA could be detected in DNA extractions obtained from the blood of 5 mice one week after the last treatment when checked by PCR, the experimental procedure was modified. Instead of extracting DNA from the blood and testing for the presence of trypanosomal DNA, blood was checked visually for trypanosomes. Thereto, blood underwent a centrifugation procedure and was inspected microscopically. The advantage of this method was that re-appearance of trypanosomes could be detected in a very early stage already. Therefore, time and material was saved when organs were prepared, since the presence of any trypanosomes would lead to an abort of the experiment before organs were proceeded further after isolation from the mouse. With the help of this centrifugation procedure it is possible to

detect single trypanosomes, which is of importance when evaluating if these mice already relapsed or were still in the aparasitic period.

3.1.2. Limit of detection (Fig. 3)

It was possible to detect less than 1 trypanosome by PCR. Reaction containing 0.32 trypanosomes tested by PCR showed a clear band at the same position that was obtained for the positive control. For the solution containing 0.064 trypanosome no band could be detected.

3.1.3. Melarsoprol and DB treated mice

Mice having been infected and subsequently treated with melarsoprol or DB 75 were sacrificed. Blood was checked microscopically after double centrifugation to confirm that mice had been still in the aparasitic period. Organs were isolated, homogenised and amount of material was reduced further by centrifugation. The obtained pellets underwent DNA extraction and were tested for the presence of trypanosomal DNA.

The melarsoprol treated mouse was thought to serve as negative control, since in vivo observations showed that these mice were cured totally, developing no relapse anymore within 185 days after treatment (see “Studies of kinetics of parasitaemia and trypanosome disappearance after drug treatment of first infection and relapses”). Clearance of parasites from blood after drug treatment was even a bit faster than the one seen after DB 75 treatment, since all mice were trypanosome negative within 24 hours. However, when DNA extracted from organs isolated one week after the last treatment was checked for the presence of trypanosomal DNA, all organs gave a positive signal after nested PCR (Fig. 4).

Inspecting organs obtained from four DB treated mice, no clear pattern was obtained when organs were checked by PCR for the presence of trypanosomal DNA (Fig. 5).
This lead to the assumption that the DNA from dead trypanosomes had not been degraded yet, and was therefore still detectable.

The consequence of this finding was, that it is not possible to identify these niches using the genomic level. Therefore, the evidence for the existence of the invasion of niches should be

supplied by amplifying a mRNA sequence. mRNA is known to be very unstable in non-living cells and is therefore only traceable when the parasite is alive.

3.2. Detection of trypanosomal cDNA

To prove the existence of trypanosomes in any tissue, organs were isolated, homogenised and centrifuged as mentioned for DNA. The obtained pellet underwent a RNA extraction, processed by a subsequent transcription of the RNA into cDNA. The advantage of this reverse transcription (RT) was that introns were spliced out and that only the sequence of the trypanosomal mRNA, and not the one of DNA, could be amplified by choosing specific primers. For amplification, the forward primer was designed complementary to a sequence within the spliced leader gene (Fig. 1) and the reverse primer was designed reverse complementary to a sequence within the tubulin beta gene (Fig. 6). Approximately 15 α-tubulin and β- tubulin genes are arranged alternating in a tandem array within the genome of a trypanosome. In the intergenic region of those tubulins no spliced leader sequence was found, indicating that the SL sequence originates from small RNA transcript encoded at other sides and is trans-spliced subsequently (Fig. 7) [Sather, S. (1985)], [Ullu E. (1996)].
To increase the sensitivity of this procedure, nested PCR was performed. Thereto, additional primers had to be designed. Since the part of spliced leader sequence which is trans-spliced in front of the tubulin beta gene is very short (35 bp), the same forward primer was used also in the nested PCR. The nested reverse primer was designed a few base pairs upstream of the primer of the first PCR. Fragments which were amplified by these PCRs showed the size of 436 bp after primary and 406 bp after the nested PCR (Fig. 6).

Before performing a PCR to detect trypanosomal cDNA, it was checked whether mRNA extraction and transcription to cDNA was successful. Thereto, a sequence of the mouse genome was amplified, since mouse cDNA would be present in excess after the mRNA extraction and transcription procedure. Primers were chosen, which bind to the polymerase II sequence in the mouse genome only. Intron spanning primers were designed, so that two products may be obtained when performing PCR: one product from genomic (DNA) level and the other from mRNA level (Fig. 8). The advantage of this selection was that within one PCR both fragments may be obtained and therefore contaminating DNA which got co-extracted in the mRNA procedure may be monitored.

3.2.1. Detection of mouse cDNA

PCR detecting mouse cDNA showed that all organs of melarsoprol treated mice were extracted successfully (Fig. 9). Variations in the amount of cDNA were clearly visible, reflected by the intensity of the obtained bands.

In the first run, only pancreas, liver, brain, spleen and spine gave a positive signal when organs of DB 75 treated mice were tested by PCR (Fig. 10). In the second run, when organs of 3 DB 75 treated mice were isolated and mRNA extracted, 3 pancreas, 1 liver, 2 brains, 1 lung, 1 heart, 3 spleens, 2 thymus, 3 spines and 3 eyes showed presence of mouse cDNA (Fig. 11).

Brain homogenates (Fig. 12)

6 out of 8 brain pellet from melarsoprol and all brain pellets from DB 75 treated mice were extracted successfully as could be shown by the presence of mouse cDNA.

3.2.2. Detection of trypanosomal cDNA

Limit of detection (Fig. 13, Fig. 14)

When a dilution series of trypanosomal cDNA in TE buffer was tested by PCR, 0.0857 trypanosomes per reaction were still detectable. A dilution series of trypanosomal cDNA in mouse cDNA showed a remarkable reduction in sensitivity, since at least 40 trypanosomes had to be present in the PCR reaction mixture, otherwise no positive signal was obtained.

Melarsoprol and DB 75 treated mice (Fig. 15, Fig. 16)

The homogenate obtained from the brain of the melarsoprol treated mouse gave a positive signal for trypanosomal cDNA (Fig. 15). No cDNA of trypanosomes could be detected by PCR in organs obtained after treatment with DB 75 from the first run (Fig. 15). When analysing cDNA obtained from mice treated with DB 75 obtained from the second run, the spleen of one mouse gave a positive signal (Fig. 16).

Brain homogenates (Fig. 17)

The presence of trypanosomal cDNA could be detected in none of the brain homogenates, neither in the ones of mice treated with melarsoprol nor the ones treated with DB 75.

3.3. Injection of brain homogenates

In a parallel experiment it was checked whether trypanosomes get damaged, lose their infectivity or get lost when organs undergo the homogenisation and centrifugation procedure. Basis of this experiment was the protocol for the in vivo testing of compounds against late stage trypanosomosis [Gichuki, G. (1999)]. Within this model a relapse based on re-invasion of trypanosomes out of the brain is simulated. Thereto, a mouse infected with the trypanosome strain GVR 35 (*T. b. brucei*) was treated on day 21 after infection with diminazene. Since diminazene is not able to cross the BBB, only the blood is cleared from parasites. Some days after diminazene treatment, the pellet of the brain of the GVR infected mouse (obtained as described for tissue samples), was injected into an immunosuppressed recipient mouse, since no trypanosome could be detected in the blood of the donor mouse after double centrifugation microscopically. Establishment of an infection due to trypanosomes present in the brain pellet, was checked by tail blood inspection one week after injection.

Additionally, half of the pellet volume obtained after centrifugation of homogenised brains obtained from melarsoprol and DB 75 treated mice, was injected into immunosuppressed mice. This was performed with 8 mice, treated with either melarsoprol or DB 75. Tail blood was checked for trypanosomes within a 4 week period to detect if a parasitaemia results from those pellets. Immunosuppression was performed once a week.

One week after injection of the brain homogenate obtained from the GVR 35 infected mouse parasites were found in the blood of the recipient mouse when tail blood was inspected. Whereas, brain pellets obtained from STIB 900 infected mice treated with melarsoprol or DB 75 did not lead to a parasitaemia in immunosuppressed recipient mice.

4. Discussion

General aspects

When blood was collected, sodium citrate was used as anticoagulant, since heparin the anticoagulant usually used, would inhibit the polymerase within the PCR. Heparin, a negatively charged polysaccharide (glucosaminoglycan), is known to bind positive ions like Mg^{2+}, Ca^{2+} and others. Especially the presence of Mg^{2+} is important for PCR, since it forms

complexes with dNTPs, primers and DNA templates which were needed by the polymerase to anneal and to synthesise the anti-strand.

Blood check microscopically vs. PCR

Testing mouse blood by PCR revealed that trypanosomal DNA was cleared within 7 days after the last DB 75 treatment. DNA extracted from blood of 5 mice clearly showed no presence of trypanosomal DNA. Therefore, it was possible to change the protocol and to introduce the microscopical control after double centrifugation of blood obtained by cardiac puncture in the aparasitic period. With the shift to the microscopical control instead of the check by PCR, the detection of any parasites could be performed within a shorter time and with less effort. Additionally, a lot of material and labour could be saved, since parasites were detected in an early stage of the experiment, before organs were DNA extracted.

To reduce the amount of material which was obtained by homogenising the isolated organs, centrifugation steps played an important role. With the aid of a first slow centrifugation step at 700 rpm, dense particles could be eliminated. Carrying out a fast centrifugation at 2700 rpm with the supernatant obtained after the first centrifugation step, trypanosomes were found in the pellet. This centrifugation procedure leads to a reduced pellet size of a volume from approximately 100 to 250 µl.
The homogenisation and centrifugation procedure performed did not lead to damage, reduction in infectivity nor total loss of trypanosomes, since pellets of brains of GVR 35 infected mice were shown to trigger a parasitaemia in immunosuppressed recipient mice.

Genomic level

After having optimised parameters like annealing temperature and the Mg^{2+} concentration for the PCR to detect trypanosomal DNA, a dilution series was generated to test the sensitivity of this method. It could be shown, that it is possible to detect less than 1 trypanosome with this method despite the predominant presence of mouse DNA. Since DNA of one organ was extracted in 20 µl, it is possible to detected 3-4 trypanosomes per organ.

Organs of melarsoprol treated mice were tested for the presence of trypanosomal DNA. These mice should have served as negative control, showing that total clearance of trypanosomal DNA had taken place within one week after treatment. Microscopical blood check after

double centrifugation technique revealed no trypanosomes, indicating that the mouse was in the aparasitic period when sacrificed. Since melarsoprol treated mice were known to be cured after treatment and not to relapse within the observation period of 185 days after the last treatment, presence of trypanosomal DNA was not expected. Surprisingly, DNA extracts of all organs showed a positive band identical with the band obtained for the positive control. This result lead to the assumption, that DNA persisted in organs longer than 7 days after the last treatment without being degraded.
The problem of persistent free circulating DNA which may be detected during a certain time is already known from the treatment of visceral leishmaniasis, were kinetoplast DNA could be detected within 37 days after initiation of the drug treatment. One of the patients did not even turn PCR negative within 145 days after treatment [Disch, J. (2004)].
Another example is the free circulating DNA that is exploited in the diagnosis of occult infection with microfilariae (loa loa) by PCR [Toure, F.S. (1998)]. In occult loiasis, no microfilariae can be found in the peripheral blood and patients do not show any clinical symptoms.

DNA of organs from DB 75 treated animals did not show a clear pattern in invading organs when checked by PCR. The reason could be that no specific niche is preferred and therefore depending on the individual mouse different organs may be invaded and serve as niche. More likely, it could be that these organs were positive because DNA of trypanosomes had not been degraded totally and could therefore be detected. DNA in tissues may be degraded slower than DNA circulating in the blood, as PCR of blood samples indicated.

Taking the PCR results for melarsoprol and DB treated mice into account, it is not sure whether these positive signals obtained were due to free circulating DNA or if the DNA detected belonged to trypanosomes hidden in these organs. Results of melarsoprol treated mice indicate that the detected trypanosomal DNA is more likely to be DNA of dead trypanosomes, whose DNA was not degraded completely within the week after the last treatment. Since relapses after DB 75 treatment occur between day 9 and day 20, it was not possible to postpone the day of sacrifice any longer, since then relapses would have to be encountered. A mouse being in the early stage of the relapse would also deliver positive signals from any organ as soon as trypanosomal DNA within the blood could be detected by

PCR. In this case, trypanosomes would be found also when inspected microscopically after double centrifugation.

mRNA level

The presence of mouse cDNA increased the limit of detection over 400-fold from 0.0857 trypanosomes per reaction in TE to 40 trypanosomes per reaction in mouse cDNA. Therefore, an organ has to be populated by at least 270 trypanosomes, in order to be identified as positive.

Melarsoprol treated mice

Extraction and transcription of mRNA from organs of melarsoprol treated mice seemed to be successful, since mouse cDNA could be detected for all samples. When these cDNAs were tested for the presence of trypanosomal DNA, one positive signal was obtained. The sample represented a homogenate of the brain. A possible explanation for this may be that trypanosomes managed to cross the BBB before drug treatment was performed. Treatment with melarsoprol, which is able to cross the BBB to a certain extent, did clear the blood from parasites and had also a certain toxic effect on parasites in the brain. The parasites in the brain may not be killed immediately and could have survived for some time. Since control mice did not relapse anymore, it is assumed that this population of parasites in the brain died without triggering a relapse.

After having shown a trypanosome positive brain homogenate, 8 additional brains of melarsoprol treated mice were tested for the presence of trypanosomes. This time, half of the pellet obtained after the centrifugation steps was injected i.p. into immunosuppressed mice. Results will be discussed below. The remaining half of the pellet underwent mRNA extraction and transcription to cDNA. None of these samples could confirm the colonisation of the brain, when brain pellets were analysed by PCR.

DB treated mice

Unfortunately, extraction and transcription of organs from DB treated mice was unsuccessful for most of the samples in the first run. Why only 5 organs were extracted successfully and the others not, is not known. Since this extraction was performed parallel to the extraction of the organs obtained from the melarsoprol treated mouse, it could be excluded that one of the

substrates used for extraction and transcription did not work properly or was contaminated with RNases. Therefore, the whole experiment was performed again with 3 mice. Here again, the extraction of mRNA and transcription to cDNA was only partly successful. Organs which delivered cDNA were tested for presence of trypanosomes. Interestingly, one positive organ could be detected, showing a band at the same position as the positive control (Fig. 16). This organ was identified to be the spleen. The spleen serves not only as efficient blood-filtering system, but also as important element of the immune system. Based on its structure, a distinction between red and white pulp is possible. The red pulp consists of large numbers of sinuses and sinusoids. Removal of old and damaged erythrocytes and platelets is performed within this zone. In addition, the present macrophages are discussed to be involved in iron recycling and removal of bacteria from the blood. The white pulp represents the lymphoid region of the spleen. There, T and B cell compartments can be distinguished. In the T cell zone, T cells interact with dendritic cells and passing B cells, whereas in the B cell follicles (B cell zones) clonal expansion of activated B cells takes place. Both zones are of importance when an immunological response to antigens in the blood has to be generated [Mebius, R.E. (2005)]. An intact immune system is of importance not only in the control of a developing parasitaemia, but also in clearance of parasites after treatment (immune-dependence of chemotherapy) [Berger, B.J. (1992)]. Therefore, this organ was suspected to serve as niche already before the experiment was performed.

Since the detection of trypanosomal cDNA in mouse cDNA is not very sensitive, other organs also may serve as niche, but detection may just not be possible since less than 270 trypanosomes could be present.
As already mentioned for melarsoprol, brains of DB treated mice were checked both by PCR and by inoculation into naive, immunosuppressed mice. The inoculation of half of the pellet obtained after centrifugation was performed to improve the detection limit. As seen, detection limit for trypanosomal cDNA in mouse cDNA was not very sensitive. Inoculation of parasites is very sensitive, since one trypanosome may be sufficient to initiate a parasitaemia. No mice, neither injected with brain pellets of melarsoprol nor DB 75 treated mice turned parasitic within 4 weeks although immunosuppression took place once per week.

Since the centrifugation method was shown to work without having a negative effect on the trypanosomes, the result of this inoculation experiments indicated that the CNS had not been invaded within 3 days after injection of the parasites.

The performed experiment clearly showed the mRNA extraction process and transcription to cDNA to be the bottleneck. Maybe, yield of the extraction may be increased and degradation of mRNA may be reduced within the preparation when a commercial RNA kit like the RNeasy® Kit from Qiagen would be used.
This experiment was stopped at this stage due to limitations in time. To get better results, RNA extraction has to be improved, as well as the sensitivity of the PCR in presence of predominant mouse cDNA.

5. Conclusion

This experiment indicated that the brain is unlikely to be the source of relapse when treatment is performed already three days after infection as it is the case for the in vivo screening of compounds tested for activity in the acute mouse model. Based on the outline of this experiment and the results obtained from this study, other organs, like the spleen for example, have to be considered to serve as niche. It also showed, that the approach to detect the presence of trypanosomes by PCR would be possible on the level of mRNA. Thereto, sensitivity of the PCR performed with transcribed cDNA has to be increased. Using Southern blot technique would be one possibility to enhance sensitivity: after having transferred the amplified sequences to a membrane, denaturation to single strand cDNA is performed. Hybridising with a specific, radioactive labelled probe complementary to the sequence to detect, would lead to a signal indicating the presence of the amplified sequence. Another possibility to increase the sensitivity would be the use of different primers and/or a different gene, where inhibition of mouse cDNA may not be so strong. Additionally, improvements in the extraction of mRNA and its transcription to cDNA are of major importance.

Fig. 1: Nucleotide sequence of the 1390 bp fragment containing the spliced leader sequence:

NCBI Sequence Viewer v. 2.0
www. ncbi.nlm.nih.gov
1390 BP

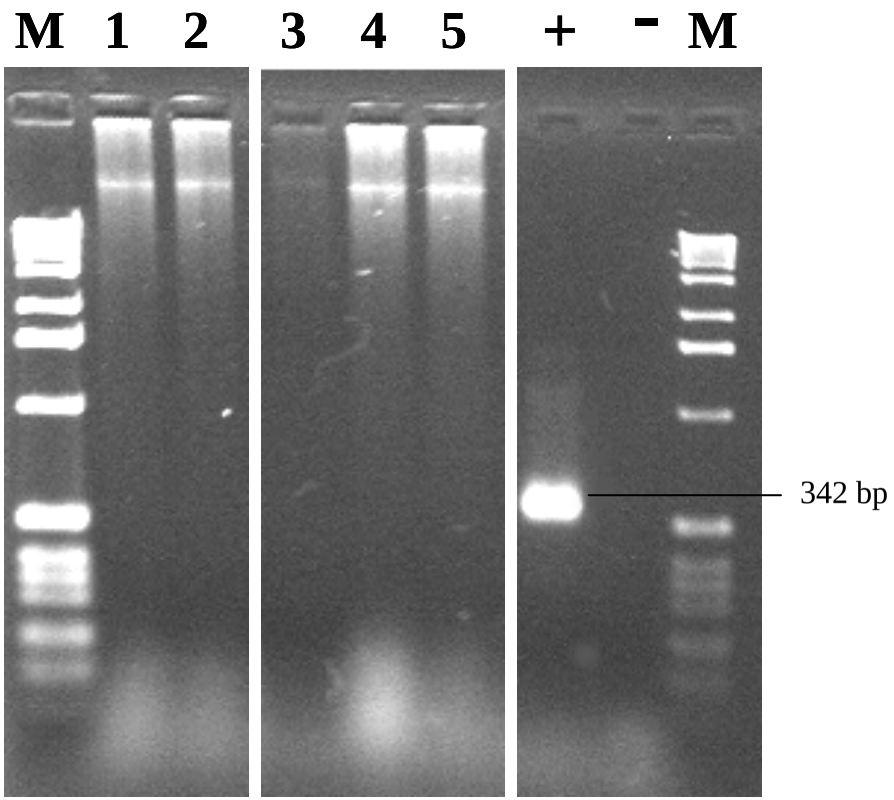
```
aacgctatta ttagaacagt ttctgtacta tattggtatg agaagctccc agtagcagct    60   SLfa
gggccaacac acgcattgtg ctgttggttc ctgccgcata ctgcggaatc tggaaggtgg   120   SLfb
ggtcggatga cctttatctc tttttatttt ttttatttt ttttatttat ttttttttg     180
atctttattt gctacgctga cacacacacg caaacacgca cacgcacact cacactcaca   240
ctcactcata tatatatata tatattttct ttatttattt atttatttat ttatttattt   300
gtttgtttat ttatttattt atatgcaaat ataattatac tatagcttat ggttttcttg   360
catatctgta taagcgcgtt ggggtcctcg atgggcgctt tcatggctta tacgtgctcg   420   SLr2
tttctcccgt tcatttttac gcagtcggac aatttcatgt cgctcttacc attgcaatta   480
ctcattttca ctttacacat cactttctta cacatatagg cgctttaaag tctgctgccc   540
gccgttttca atggcggtcg gcatgccccc ctacatgtct actgagcagg cgaacggccc   600   SLr3
cggcatggca acaccaaata tccccttca gggttttgcc tcattttgcc gatgttctta   660
acctggttat acccgcaata tgccagctgc accctcagtt cgtgatgtta tatactttcc   720
caattttggg gccggccccc gcccaacacc acccgccgct aataaacggc ggagaataac   780
agcgagcata ccgccggcca gccacagagc cgaaagaagc cggcctgcgc gccctattca   840
tgttattagc cgccattaag cattatatta cactcagttc aaccgtcctt cttctttgcg   900
ttgttgttgt tgccgttgtg ttctatataa agtttatcgg cagaggcgcc ctggctcctc   960
cccatcaccc cctgcccacc ctccaaaatc tggcgccgcc ggctggtcct gcacgcccca  1020
gaaacgcgtt tcttttattg ttggttgatt gccttaatgt tctgttgcag atggcgattc  1080
accattaagc atttaatatt ttagaaataa gagagcgctg gtaaaagacg gcgggtcgcg  1140
ccacacggtg gttgccgtta tgcacgatac cccatgtagt atatatgcgg tcccgcttat  1200
tccgccaccc tcccccataa cggcttaagc acaagacccc tttgtttccc ataggtctac  1260
cgacacattt ctggcacgac agtaaaatat ggcaagtgtc tcaaaactgc ctgtacagct  1320
tatttttggg acacacccat gctttcaact aacgctatta ttagaacagt ttctgtacta  1380   spliced_
tattggtatg                                                         1390   l_for
```

PCR products DNA level:

Primary PCR: SLfa – SLr3 : 554 bp

Nested PCR: SLfb – SLr2 : 342 bp

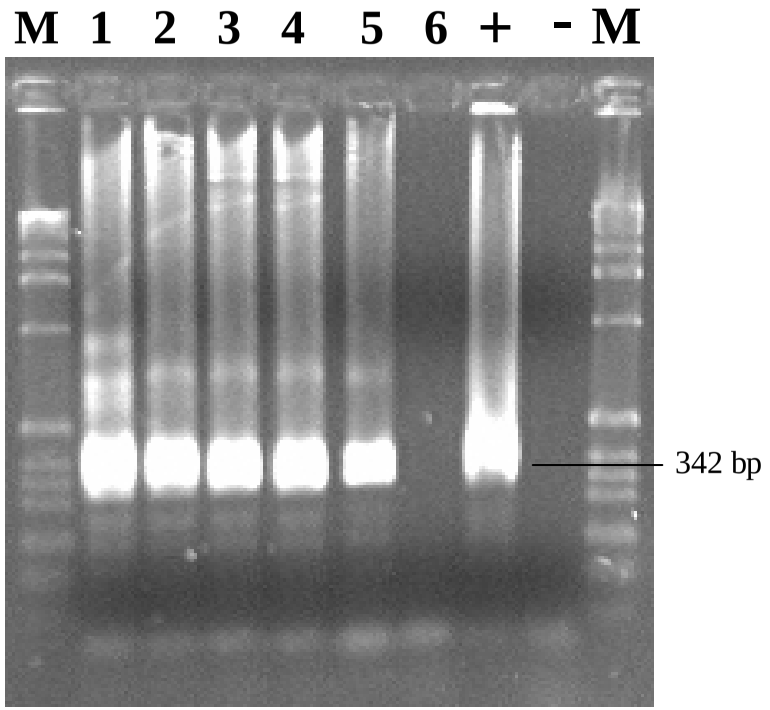
Fig. 2: Clearance of blood from trypanosomal DNA after treatment with DB 75



M: 1 kb DNA ladder
1-5: mice
+: DNA from cultured trypanosomes
-: DNA from mice

fragment length: 342 bp

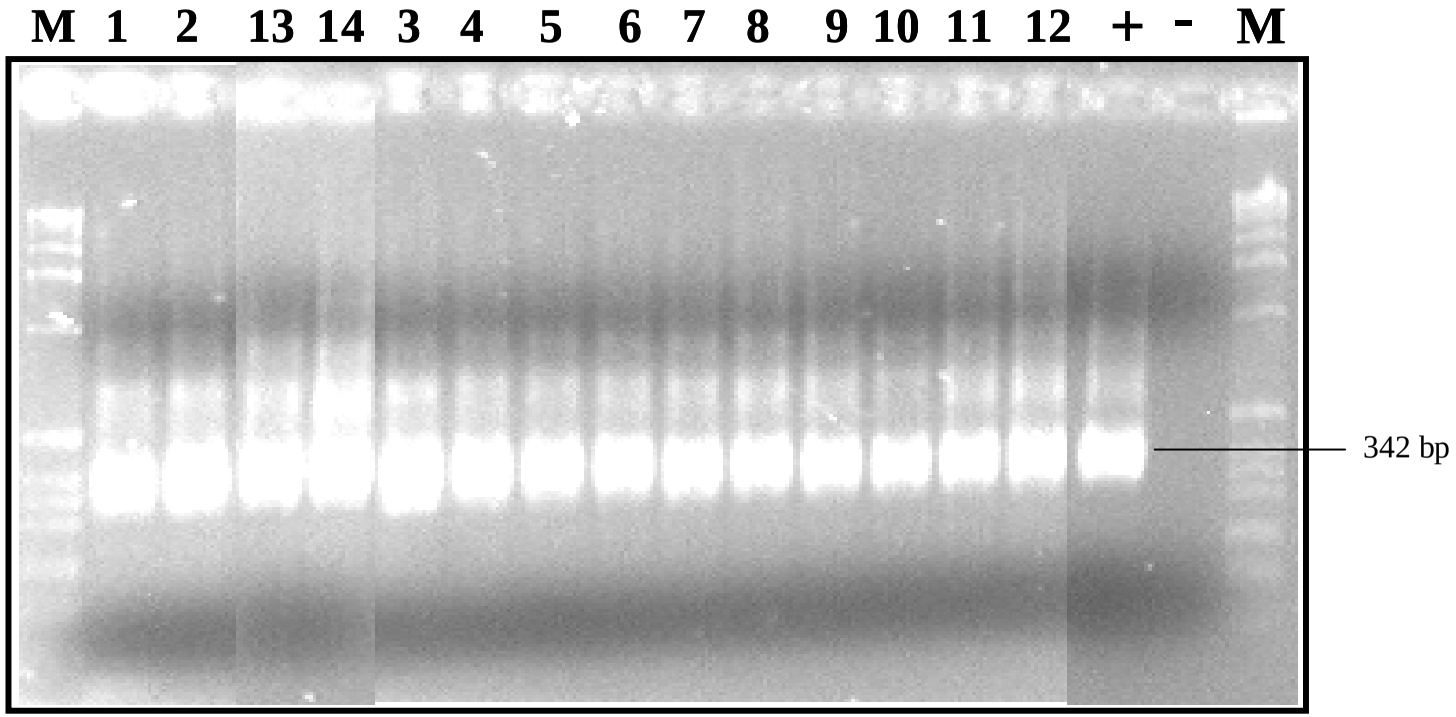
Fig. 3: Limit of detection: trypanosomal DNA in mouse DNA



M: 1 kb DNA ladder
1: 200 trypanosomes per reaction
2: 40 trypanosomes per reaction
3: 8 trypanosomes per reaction
4: 1.6 trypanosomes per reaction
5: 0.32 trypanosomes per reaction
6: 0.064 trypanosomes per reaction
+: DNA from cultured trypanosomes
-: DNA from mice

fragment length: 342 bp

Fig. 4: Trypanosomal DNA in organs of melarsoprol treated mice



M:	1 kb DNA ladder		
1:	pancreas	8:	kidney
2:	liver	9:	fat tissue
3:	brain	10:	muscle tissue
4:	genitalia	11:	thymus
5:	lung	12:	spine
6:	heart	13:	eyes
7:	spleen	14:	skin
+:	DNA from culture trypanosomes	-:	DNA from mice

fragment length: 342 bp

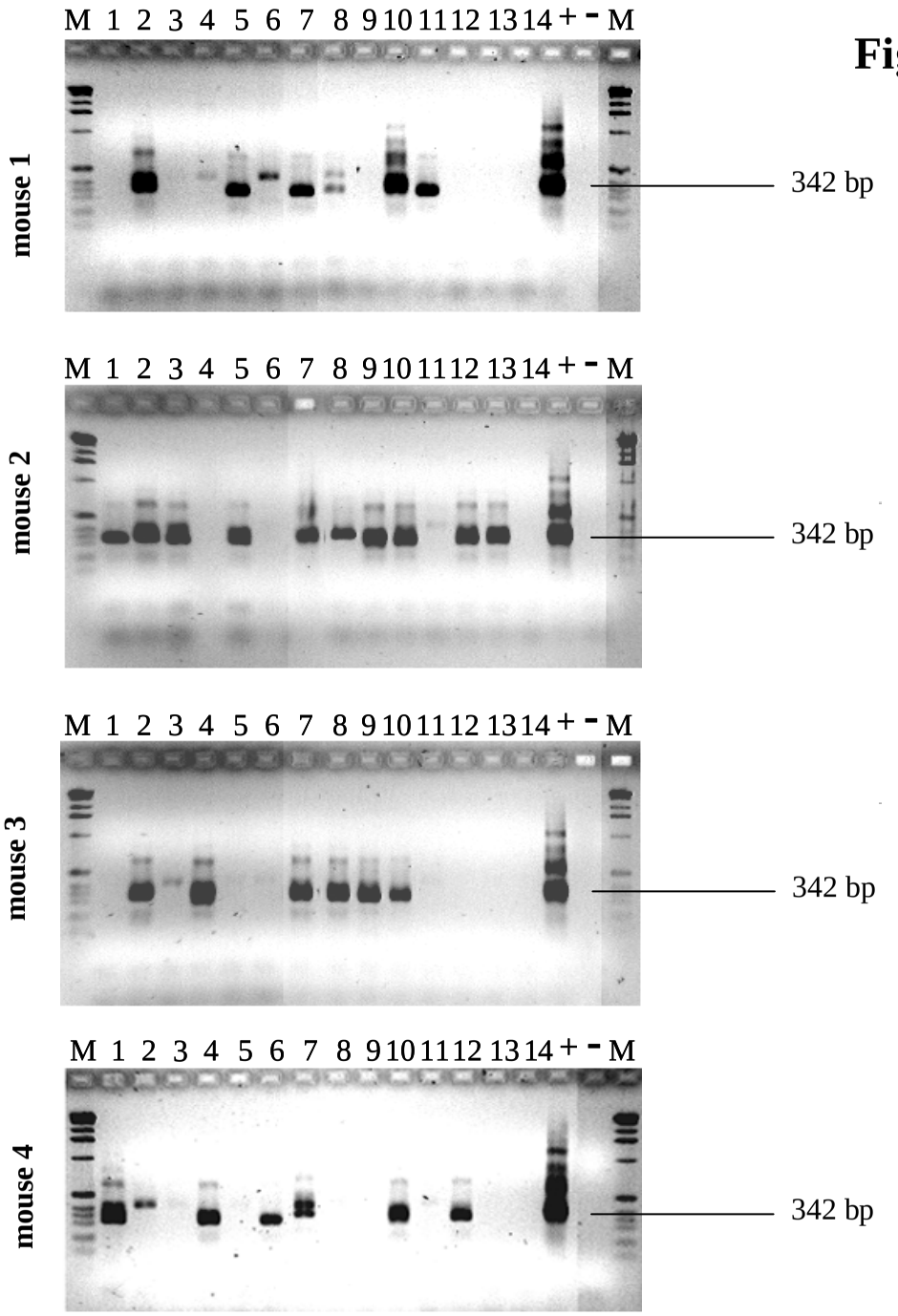


Fig. 5: Trypanosomal DNA in organs of DB 75 treated mice

M: 1 kb DNA ladder
1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin
+: DNA of trypanosomes out of culture
-: DNA from mice

fragment length: 342 bp

Fig. 6: Nucleotide sequence of Beta Tubulin

NCBI Sequence Viewer v. 2.0

www. ncbi.nlm.nih.gov

1329 bp

```
   1  atgcgcgaaa tcgtctgcgt tcaggctggc caatgcggta accagatcgg ctcaaagttc
  61  tgggaggtga tcagtgacga gcacggtgtg gaccccacag gtacctacca gggtgactct
 121  gacctgcagc tggagcgcat caatgtgtac tttgatgagg caacgggagg tcgctatgtg
 181  ccccgctccg tgctgattga tctggagcca ggtacaatgg actccgtacg tgctggcccc
 241  tatggtcaga tcttccgccc cgacaacttc atctttggac agtctggcgc cggcaacaac
 301  tgggcaaagg gccactacac ggagggtgcg gaactgatcg actctgtgct cgatgtgtgc   tub_b_nested_
 361  tgcaaggagg cggagagctg tgactgcctc caaggcttcc agatctgcca ctcccttggt   rv/tub_b_rv
 421  ggtggtactg gctccggcat gggtacgctg ctcatctcga agcttcgcga gcagtaccct
 481  gaccgtatca tgatgacttt ctccatcatc ccatccccca aggtgtccga cactgtcgtc
 541  gagccgtaca atacgactct ctccgtgcac caacttgtgg aaaactccga tgagtcgatg
 601  tgcattgaca acgaggcact gtacgatatt tgcttccgca ccctgaaact gacaacacca
 661  acgttcggtg acctgaacca cttggtgtct gctgttgtgt ccggcgtcac ctgctgcctg
 721  cgcttccctg gtcagttgaa ctctgacctc cgtaagttgg ctgtgaacct tgtcccattc
 781  ccgcgtctgc acttcttcat gatgggcttc gccccgctga ccagccgcgg ctcgcagcag
 841  taccgcggtc tctccgtgcc cgagctaacg cagcagatgt tcgatgcgaa aaacatgatg
 901  caagctgcag atcctcgtca cggccgctac ctgacagcgt ctgcactctt ccgcggccgc
 961  atgtcgacga aggaggttga tgagcagatg ctgaacgtgc agaacaagaa ctcgtcctac
1021  ttcattgagt ggatcccgaa caacatcaag tcctctgttt gcgatatccc acccaaggga
1081  ctcaagatgg ctgtcacctt cattggcaac aacacctgca tccaggagat gttccgccgt
1141  gtgggagagc agttcaccct catgttccgt cgcaaggcgt tcttgcactg gtacactggc
1201  gagggtatgg acgagatgga attcacggag gcagagtcca acatgaacga tctcgtgtct
1261  gagtaccagc agtaccagga tgccacgatt gaggaggagg gcgagttcga cgaggaggag
1321  caatactag
```

PCR product:

Spliced leader sequence	5`UTR	β-tubulin	3`UTR	Poly A
35 bp	49 bp	1329 bp		
in PCR: 29 bp	49 bp	358 bp (1°), 328 bp (2°)		
primary PCR/secondary PCR:		436 bp / 406 bp		

Fig. 7: Processing of β-tubulin mRNA

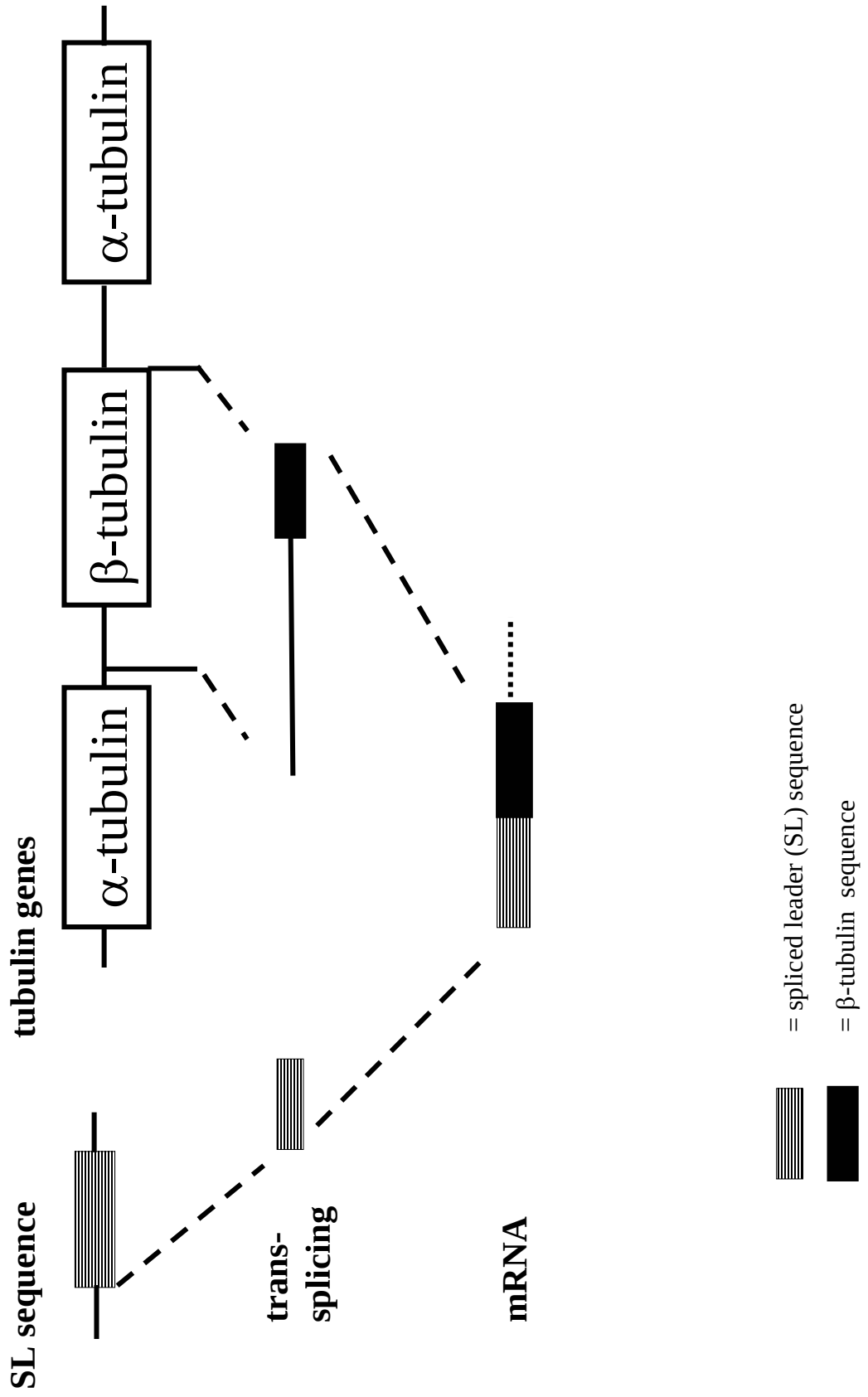


Fig. 8: CLUSTAL W (1.82) multiple sequence alignment: Mouse polymerase II and its mRNA sequence

```
polyII          GCGTCCGGCGCAGGCTCCGTGCTGGCAGCTCCACGAGCGCGCGTGCACAGTTGTTTGGGA 60
transcript      ------------------------------------------------------------

polyII          AGCGCGACAGCATGGACGACGAGGAGGAGACCTACCGGCTGTGGAAGATCCGCAAGACGA 120
transcript      ------------------------------------------------------------

polyII          TCATGCAGGTGATCAAGCGTGAGGAGCTTTATGCGAGCGAGCGTTCAGTTGCGGACTAAC 180
transcript      ------------------------------------------------------------

polyII          TGCGGGTTGTCCGCCGAGGCCCGCCTCCCGCAGGGGCCAATCCTCCTGGATTGGAATGCA 240
transcript      ------------------------------------------------------------

polyII          TCCCCGTTTAAAGTGTGGAGCCCACCCACGCTGGCTTCAACTCTTAGAAATCCTACTGCT 300
transcript      ------------------------------------------------------------

polyII          TCCACCTCCTAACTCTGCCTCAGGGGAAAAGGCCTGGGTGGGCTCCGTTTCGGGAGAGGG 360
transcript      ------------------------------------------------------------

polyII          GGCTGCTGCGTGGTCACACTTGGTTTTATGCGAGCTAGGTAGCCGCCCACACGAAAGCTA 420
transcript      ------------------------------------------------------------

polyII          TCAACCATCTGTAACCCCAGTTCCAGGGGATCTGACGCCCTGTCCTGATCTCTGGGGACT 480
transcript      ------------------------------------------------------------

polyII          CCAGACACACATAGGATGAAAAAACAGTATTTTTAAAAACAGAACAAGCCGGTATGATGG 540
transcript      ------------------------------------------------------------

polyII          TACACGCCTTTAATCCCAGCACTCTGGAGGCAGAAGCAAGCGAATCTCTTTAAATTAGAA 600
transcript      ------------------------------------------------------------

polyII          GTCAGCCTCATCTACATAGTGATTTCCAGAATAGACAAAGTCACGCAGAGAAACCCTGTC 660
transcript      ------------------------------------------------------------

polyII          TTGAAAAAGAAAAAGGGAATAAAAGAAAAAATTCTTGGTTGTTTTGAGTTGAACACATGA 720
transcript      ------------------------------------------------------------

polyII          GACACCATTATTAGATAAAGAGGTATGCATAGTTTTGACTTATGCTGTGTTGGTGGAGCC 780
transcript      ------------------------------------------------------------

polyII          CAGGGCCACCTCATGCCTGTTCCTCAGCCCTGATCCTGGATGATGCTCTAGTCTCCCTCA 840
transcript      ------------------------------------------------------------

polyII          TCAGGAACACTCAAGACTGGGGACAGGAAGGTGCCTGTATTCAGAAGGTTGCCTACAGTT 900
transcript      ------------------------------------------------------------

polyII          GCCAGGGGATTAATCCCAGCCTGCTCCACTGAGGTTGGGGGGAGGAGAGGCTTGGCCGGG 960
transcript      ----------------------------------------CCACGCGTCCGGCGCAG 17
                                                         *   *   ***   *
```

```
polyII          CCGCTGTGGTCTGAGCCTATACAGAGTTTGGGTACTGCTGGATCAGTTCAGTGTTAGTCC 1020
transcript      GCTCCGTGCTGGCAGCTCCACGAGCGCGCGTGCACAGTTG--TTTGGGAAGCGCGA---- 71
                 * * *** *   ***      ** *   * * ** * **  *  *   ** *  *

polyII          CCAGCCTCGGCCATTCAGGGTCAGGTCTGCGAGCTAAGTGCTCAGGCTGACTGCCTCTTT 1080
transcript      -CAGCATGGACGACG-AGGAGGAGACCTACCGGCTGTGGA---AGA----------TCC 115
                 **** * * * *   ***   **  ** *  ***  *     **           *

polyII          GCCTCTTGTCTCTGCAGCTGTGTCATGACCGTGGCTACCTGGTGACCCAGGATGAACTGG 1140
transcript      GCAAGACGATCATGCAGCTGTGTCATGACCGTGGCTACCTGGTGACCCAGGATGAACTGG 175
                **     *    **********************************************

polyII          ACCAGACACTTGAGGAATTCAAGGCGCAGTTTGGGGACAAGCCCAGCGAAGGACGGCCAA 1200
transcript      ACCAGACACTTGAGGAATTCAAGGCGCAGTTTGGGGACAAGCCCAGCGAAGGACGGCCAA 235
                ************************************************************

polyII          GGCGCACAGACCTCACGGTGCTGGTGGCCCACAACGATGACCCCACAGACCAGATGTTTG 1260
transcript      GGCGCACAGACCTCACGGTGCTGGTGGCCCACAACGATGACCCCACAGACCAGATGTTTG 295


polyII          TGTTCTTCCCAGGTGAGGCCCTTGGGCTGGCAGGAGTGGTCTCCTCCAGGCCCTGGCTCA 1320
transcript      TGTTCTTCCCAGGTGAGGCCCTTGGGCTGGCAGGAGTGGTCTCCTCCAGGCCCTGGCTCA 355
                ************************************************************

polyII          GGTGCCAGTGAATGCATGTCCCACTCTGGCCTAACGCTGCCTGTAAGATTGTGGCAGCTG 1380
transcript      GGTGCCAGTGAATGCATGTCCCACTCTGGCCTAACGCTGCCTGTAAGATTGTGGCAGCTG 415
                ************************************************************

polyII          CCTGCTTTTGTCCCTGTCTCCCCTGGGAAGCCTCCCTTTTGCTGGTGTCGTTTTCATCCC 1440
transcript      CCTGCTTTTGTCCCTGTCTCCCCTGGGAAGCCTCCCTTTTGCTGGTGTCGTTTTCATCCC 475
                ************************************************************

polyII          ATAAGCCCCAGAACCAGCTCTCAGCCCTCCACCCCCTGGCTCCTATATGCTGTATTCTAT 1500
transcript      ATAAGCCCCAGAACCAGCTCTCAGCCCTCCACCCCCTGGCTCCTATATGCTGTATTCTAT 535
                ************************************************************

polyII          AGCTTCTGCCTGGGAGTCCCAGGGCCAGCTATGCCACCCCCACACCCCCTGTGTGCTGTA 1560
transcript      AGCTTCTGCCTGGGAGTCCCAGGGCCAGCTATGCCACCCCCACACCCCCTGTGTGCTGTA 595
                ************************************************************

polyII          TTCTACAGCTTCTGCCAGCAGCGCAGGTGTCTTGTGGGAGTGAAGGCTATTGGGGAGCCC 1620
transcript      TTCTACAGCTTCTGCCAGCAGCGCAGGTGTCTTGTGGGAGTGAAGGCTATTGGGGAGCCC 655
                ************************************************************

polyII          AGGACCTCCCTGTGCAGCTACATTCAGTCTCCCCAGACAGCTTGCCTCTTTTAATACCTA 1680
transcript      AGGACCTCCCTGTGCAGCTACATTCAGTCTCCCCAGACAGCTTGCCTCTTTTAATACCTA 715
                ************************************************************

polyII          ATGCTTTGTGGCCCCGGGACCTGGTTTTTAGTGTGAGAATTGGGGGGTGCGGGGATGGGC 1740
transcript      ATGCTTTGTGGCCCCGGGACCTGGTTTTTAGTGTGAGAATTGGGGGGTGCGGGGATGGGC 775
                ************************************************************

polyII          ATGGTAGCATATGCCTGTAAGCCCAGTGTTTGAGAGGCAGAGGCAGGCAGTCGAGGCCAG 1800
transcript      ATGGTAGCATATGCCTGTAAGCCCAGTGTTTGAGAGGCAGAGGCAGGCAGTCGAGGCCAG 835
                ************************************************************

polyII          CCTGGTCTATACAATGACTTCCGGGACAACTATGGAGGGCATTGGTTCTCAGCCTTCCTG 1860
transcript      CCTGGTCTATACAATGACTTCCGGGACAACTATGGAGGGCATTGGTTCTCAGCCTTCCTG 895
                ************************************************************

polyII          ATGCTGGGACCCCTTAGTACACTTCACATTGTGACCTCCAACCATAAAATCTTCATTGCT 1920
transcript      ATGCTGGGACCCCTTAGTACACTTCACATTGTGACCTCCAACCATAAAATCTTCATTGCT 955


polyII          AATTCATAACTAATTTTGCTACTATTATGAGTCATAATGTAAATATACCTGTGTTTATCC 1980
transcript      AATTCATAACTAATTTTGCTACTATTATGAGTCATAATGTAAATATACCTGTGTTTATCC 1015
                ************************************************************
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polyII          AGCAATTTGCAAACTAGGTGGCCTGGGTCTGCTCCTGCGCTCTGCCTGAGGAGACAAGGC 2040
transcript      AGCAATTTGCAAACTAGGTGGCCTGGGTCTGCTCCTGCGCTCTGCCTGAGGAGACAAGGC 1075
                ************************************************************

polyII          TAGCCTCATGTAGACACAGGGTCCATCTCTTGGTCAGTCAGTTTCTCCTTGAGGCTGTCT 2100
transcript      TAGCCTCATGTAGACACAGGGTCCATCTCTTGGTCAGTCAGTTTCTCCTTGAGGCTGTCT 1135
                ************************************************************

polyII          AAGCTGACAGTACCATATCCCCCAAACCCCAGAGCTGCTGAGAGCCCCCACCCCCGTTCC 2160
transcript      AAGCTGACAGTACCATATCCCCCAAACCCCAGAGCTGCTGAGAGCCCCCACCCCCGTTCC 1195
                ************************************************************

polyII          AGGGGGGCCGCAGTTCCTGCATACCCTGCAGCTTCTGCAGTGATAGCTGAGCCCCCTGGC 2220
transcript      AGGGGGGCCGCAGTTCCTGCATACCCTGCAGCTTCTGCAGTGATAGCTGAGCCCCCTGGC 1255
                ************************************************************

polyII          AGCCCCATCATGGCTGCCTTGATGTTCTAGTCTTCCCTTGCAAGCAGAGAAACTAAGACC 2280
transcript      AGCCCCATCATGGCTGCCTTGATGTTCTAGTCTTCCCTTGCAAGCAGAGAAACTAAGACC 1315
                ************************************************************

polyII          ACAGGCTCCAGTAGTGAGTGTGAGGGTGGTGCTTGTGTGCCCGCAGAGGAGCCCAAGGTG 2340
transcript      ACAGGCTCCAGTAGTGAGTGTGAGGGTGGTGCTTGTGTGCCCGCAGAGGAGCCCAAGGTG 1375
                ************************************************************

polyII          GGCATCAAGACCATCAAGGTATACTGCCAGCGCATGCAGGAGGAAAACATCACACGGGCG 2400
transcript      GGCATCAAGACCATCAAGGTATACTGCCAGCGCATGCAGGAGGAAAACATCACACGGGCG 1435
                ************************************************************

polyII          CTGATTGTGGTGCAGCAGGGCATGACGCCCTCCGCCAAGCAGTCCCTGGTGGACATGGCC 2460
transcript      CTGATTGTGGTGCAGCAGGGCATGACGCCCTCCGCCAAGCAGTCCCTGGTGGACATGGCC 1495
                ************************************************************

polyII          CCGAAGTATGTGCTGGAACAGTTTCTACAGCAGGAGCTGCTCATCAATATCACAGAGCAC 2520
transcript      CCGAAGTATGTGCTGGAACAGTTTCTACAGCAGGAGCTGCTCATCAATATCACAGAGCAC 1555
                ************************************************************

polyII          GAGGTGTGTGTGGTGGGGTGGGGCGTAGTGCTGCCTGCATCCAGGACACGGGCCGGGGCG 2580
transcript      GAGGTGTGTGTGGTGGGGTGGGGCGTAGTGCTGCCTGCATCCAGGACACGGGCCGGGGCG 1615


polyII          GGGTGCTTCCCCGGTTTTGGCCCTGAGCATCCAACTTGGTGATATTAATAGCCTGGTTTT 2640
transcript      GGGTGCTTCCCCGGTTTTGGCCCTGAGCATCCAACTTGGTGATATTAATAGCCTGGTTTT 1675
                ************************************************************

polyII          GTCCTCTGTCCCAGCCCCATGACAATACCCATTAGTCTGTTGGTCTGTTGAAGTTCCTGA 2700
transcript      GTCCTCTGTCCCAGCCCCATGACAATACCCATTAGTCTGTTGGTCTGTTGAAGTTCCTGA 1735
                ************************************************************

polyII          TGGCCGCGTGCCTGTGGGTGGGTCTCTGTCTCCACAGCTAGTCCCTGAGCACGTGGTCAT 2760
transcript      TGGCCGCGTGCCTGTGGGTGGGTCTCTGTCTCCACAGCTAGTCCCTGAGCACGTGGTCAT 1795
                ************************************************************

polyII          GACAAAGGAGGAGGTGACTGAACTGCTGGCTCGATAGTATCCTTTACTCTGTCCCCACAC 2820
transcript      GACAAAGGAGGAGGTGACTGAACTGCTGGCTCGATAGTATCCTTTACTCTGTCCCCACAC 1855
                ************************************************************

                                                poly_fwd_2
                                                ----------->
polyII          CCCCCTCACCCCAGCGCTGCCCGCCAGCTGCTTCCTTGACTGTTCTAGCAAGCTTCGTGA 2880
transcript      CCCCCTCACCCCAGCGCTGCCCGCCAGCTGCTTCCTTGACTGTTCTAGCAAGCTTCGTGA 1915
                ************************************************************

polyII          GAGCCAGCTACCCAGAATCCAGGCCGGGGACCCAGTGGCACGGTACTTTGGGATCAAGCG 2940
transcript      GAGCCAGCTACCCAGAATCCAGGCCGGGGACCCAGTGGCACGGTACTTTGGGATCAAGCG 1975
                ************************************************************

polyII          AGGGCAGGTGAGAAGCCACAGTGGTTGTTCCCCTCCTAGGCCCTTTGGGAATTATTGGAT 3000
transcript      AGGGCAGGT------------------------------------------------------ 1984
                *********
```

```
polyII          CTCCCTGGTTCCTCCATCTCCTCCCTGTCTCTGAGGCCAAGACCACCATTGCCATAGGCC 3060
transcript      ------------------------------------------------------------

polyII          AGTGGTCCCAAGGCCCTGCCACCACCACTCCCTCGCCTGCCTGCTCCCACAGGTCGTGAA 3120
transcript      ----------------------------------------------------CGTGAA 1990
                                                                   ******

polyII          GATCATCCGCCCCAGTGAGACGGCTGGCCGCTACATCACCTACCGCCTGGTGCAGTAGCC 3180
transcript      GATCATCCGCCCCAGTGAGACGGCTGGCCGCTACATCACCTACCGCCTGGTGCAGTAGCC 2050
                ************************************************************

polyII          GCAGGTGAGCCCAGCACTGCCCAGATGAGCCTGCTACTTGTCCTTGACCCGGTAGAGGGT 3240
transcript      GCAGG------------------------------------------------------- 2055
                *****

polyII          CAGAGCTGCACCCACAAAAATAAAATGTAACGGGAGGGTAGGCTTGAATCCTAATGCCCA 3300
transcript      ------------------------------------------------------------

polyII          GGGGCAAAGGCAGGCAGATGTCTGTGAGTTCCAGACCAGCCAGGGCTACATAGTAAGCCT 3360
transcript      ------------------------------------------------------------

polyII          CAGAAAGCAAGCAAACAAACAAACCCAAAACAACCCATACTCTTGCCAGGGGAAACCTAG 3420
transcript      ----------------------------------------------GGAAACCTAG 2065
                                                              **********

polyII          TGCTCAGAGACTGAAGCAGGAGGATTGCTGCACATCTGTGGACTCCTTCTCGGTGTGAGT 3480
transcript      TGCTCAGAGACTGAAGCAGGAGGATTGCTGCACATCTGTGGACTCCTTCTCGGTGTGAGT 2125
                ************************************************************

polyII          GTCTCCAGGAAAACAATGAAGAGGAACCCACAGCTGCACCGCATGCAGGGAAACCCAGAG 3540
transcript      GTCTCCAGGAAAACAATGAAGAGGAACCCACAGCTGCACCGCATGCAGGGAAACCCAGAG 2185
                ************************************************************

polyII          CCCACTGCTCTGCACAGCACCCTGACAGCTGTGGGCCACAGCCTGTGGGTCATAACTGGA 3600
transcript      CCCACTGCTCTGCACAGCACCCTGACAGCTGTGGGCCACAGCCTGTGGGTCATAACTGGA 2245
                ************************************************************

                                          poly_rv_2
                                          <-------
polyII          TTGCCTTTTGACTGACGGGTTCCGTCCTGGCCATTGTCGCAGGATGAGTGACTCCAGAGC 3660
transcript      TTGCCTTTTGACTGACGGGTTCCGTCCTGGCCATTGTCGCAGGATGAGTGACTCCAGAGC 2305
                ************************************************************

polyII          TGCAGCCACACCCCCACTCCTTACTCCAGGGTCACTGTTCTGTTCAGAGTTCTCCATTCT 3720
transcript      TGCAGCCACACCCCCACTCCTTACTCCAGGGTCACTGTTCTGTTCAGAGTTCTCCATTCT 2365
                ************************************************************

polyII          CGAAGGACTCTCCTGAGAATTAAAGGATACTTTGATAAGTT------------------- 3761
transcript      CGAAGGACTCTCCTGAGAATTAAAGGATACTTTGATAAGTTAAAAAAAAAAAAAAAAAAA 2425
                ****************************************

polyII          ----
transcript      AAAA 2429
```

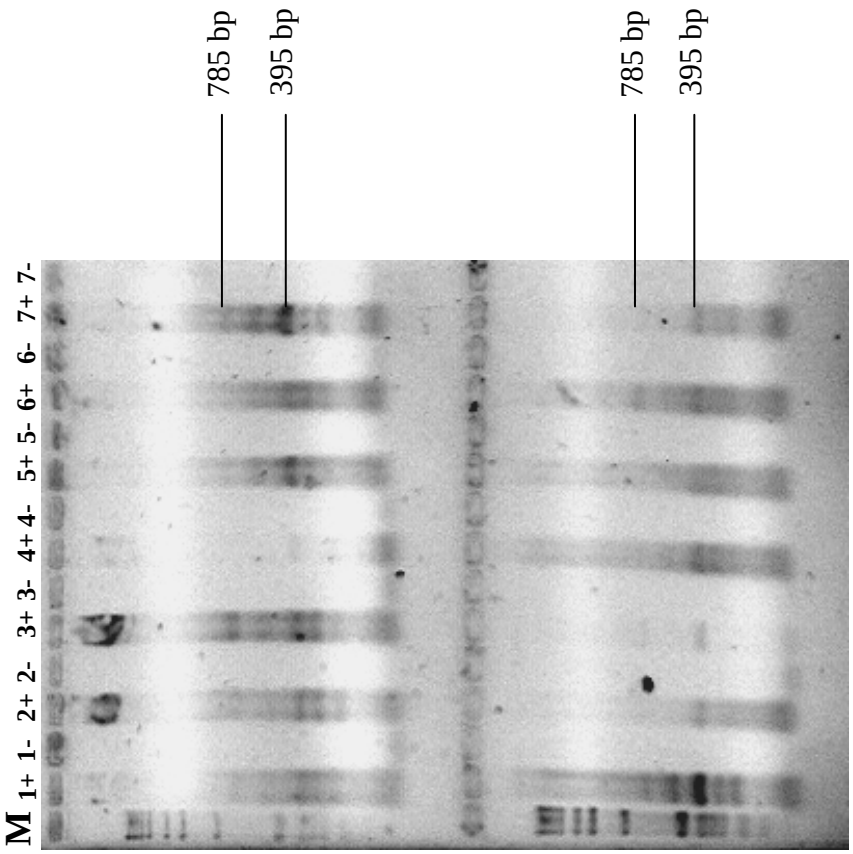
PCR products:

poly_fwd_2 – poly_rv_2:

DNA level: 785 bp

mRNA level 395 bp

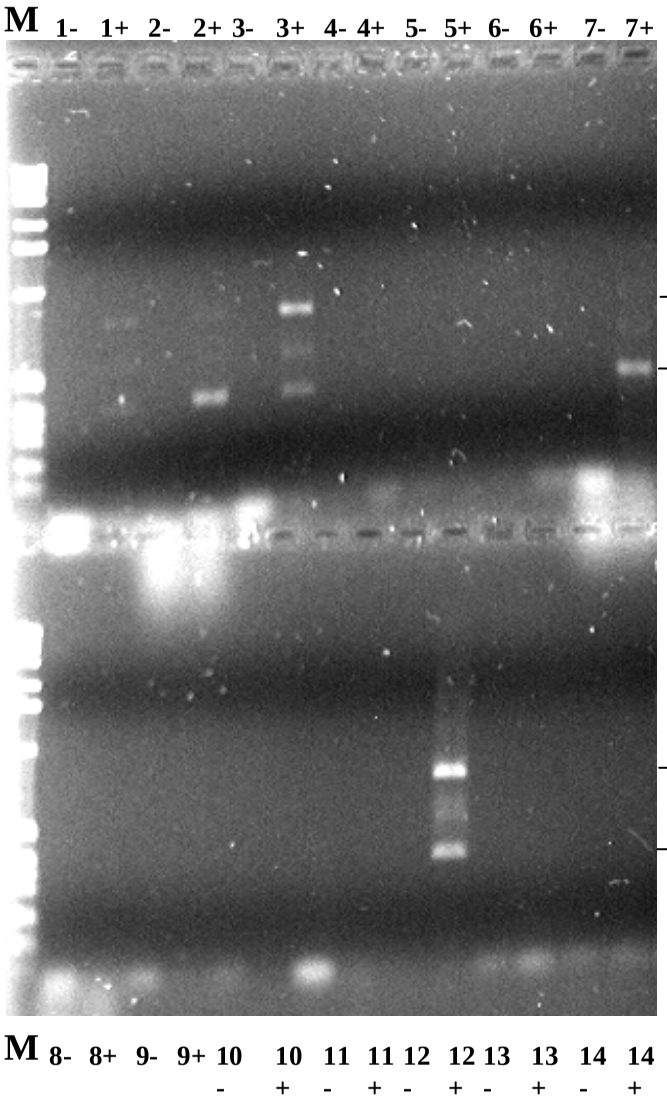
Fig. 9: Detection of mouse cDNA in organs of a melarsoprol treated mouse



M: 1 kb DNA ladder
\+ with - without sensicript
1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin

fragment length: DNA: 785 bp
cDNA: 395 bp

Fig. 10: Detection of mouse cDNA in organs of a DB 75 treated mouse, first run

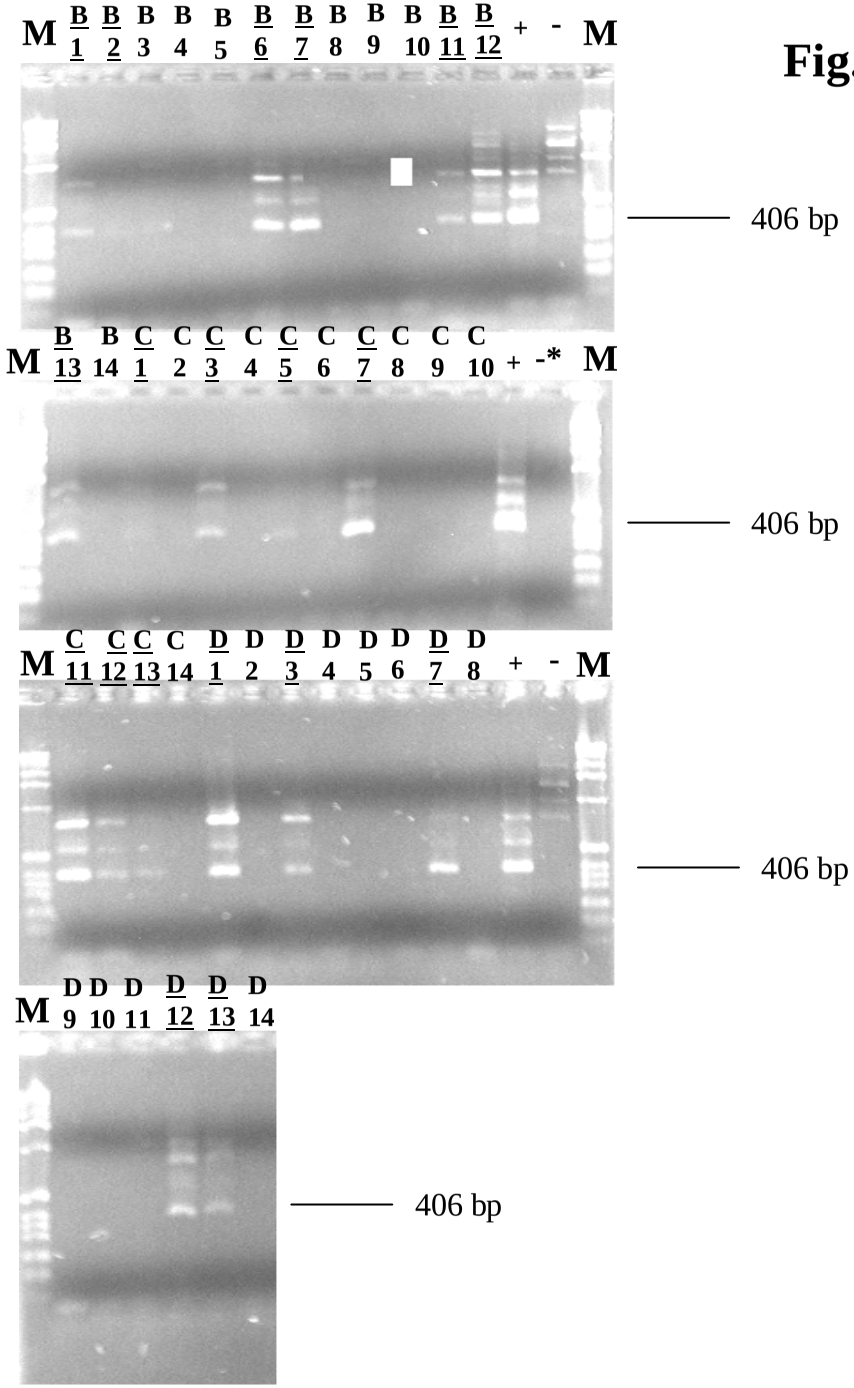


M: 1 kb DNA ladder
\+ with - without sensicript

1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin

fragment length: DNA: 785 bp
cDNA: 395 bp

Fig. 11: Detection of mouse cDNA in organs of DB 75 treated mice, second run



M: 1 kb DNA ladder
organs of DB 75 treated mice (mouse B, C, D)

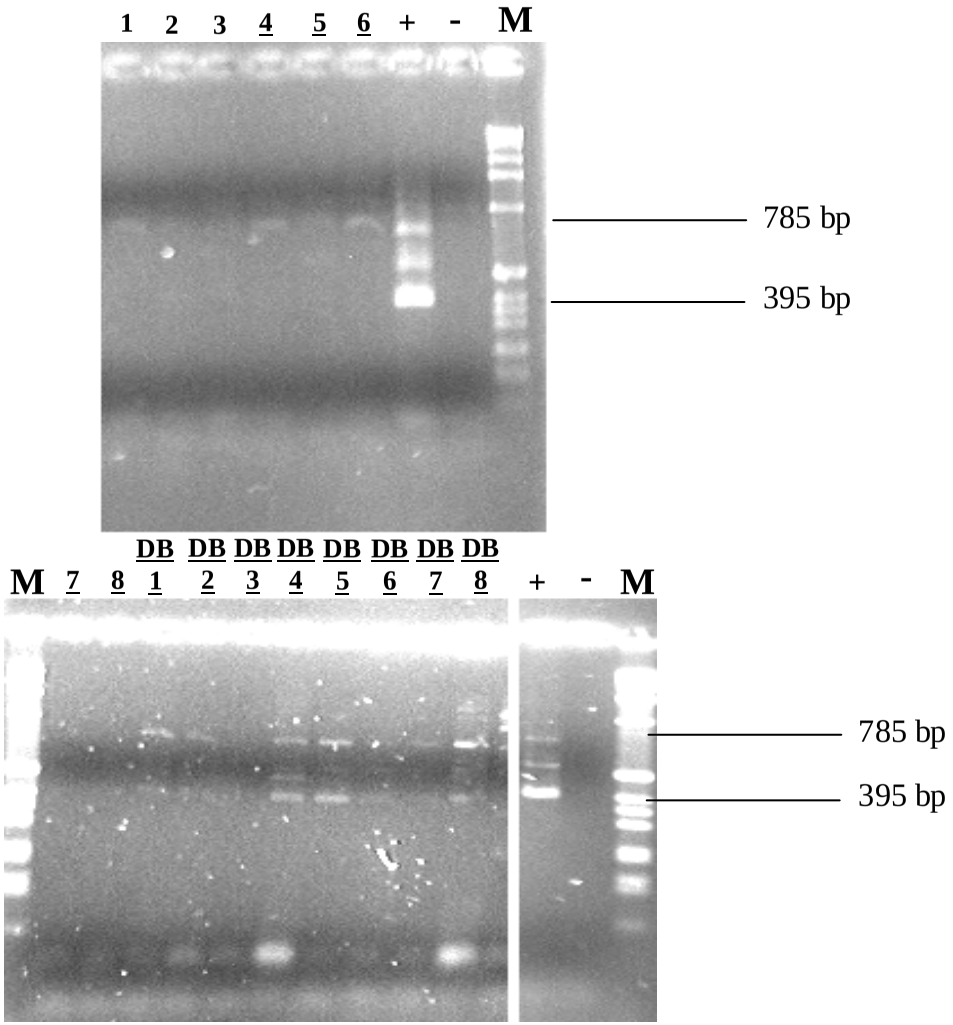
1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin

+: cDNA of trypanosomes
-: DNA mouse
-*: DNA trypanosomes

fragment length: cDNA: 406 bp

positive organs are underlined

Fig. 12: Detection of mouse cDNA in brain homogenates



M: 1 kb DNA ladder

brain homogenates of
- melarsoprol (1 - 8)
- DB 75 (DB 1 - DB 8)

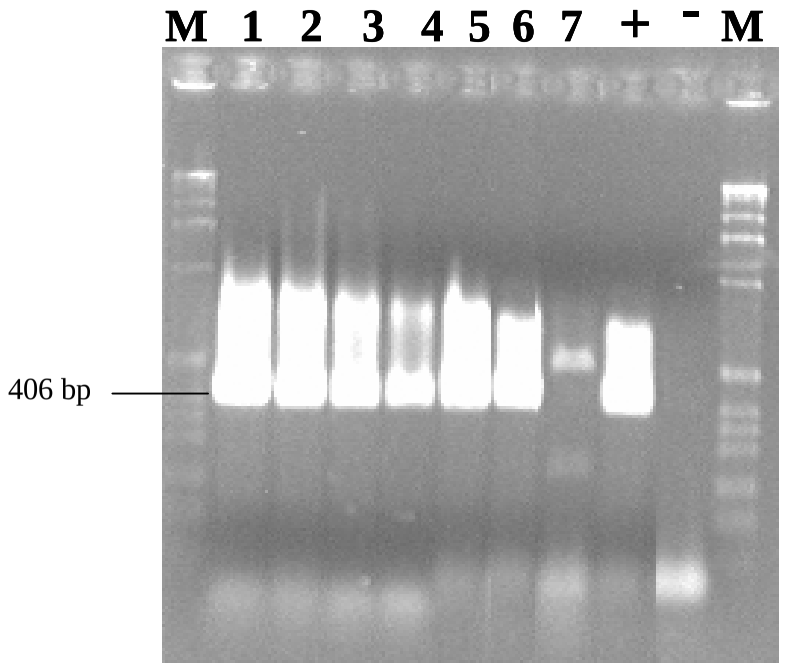
treated mice

+: cDNA of mouse
-: DNA of trypanosomes

fragment length: DNA: 785 bp
cDNA: 395 bp

positive organs are underlined

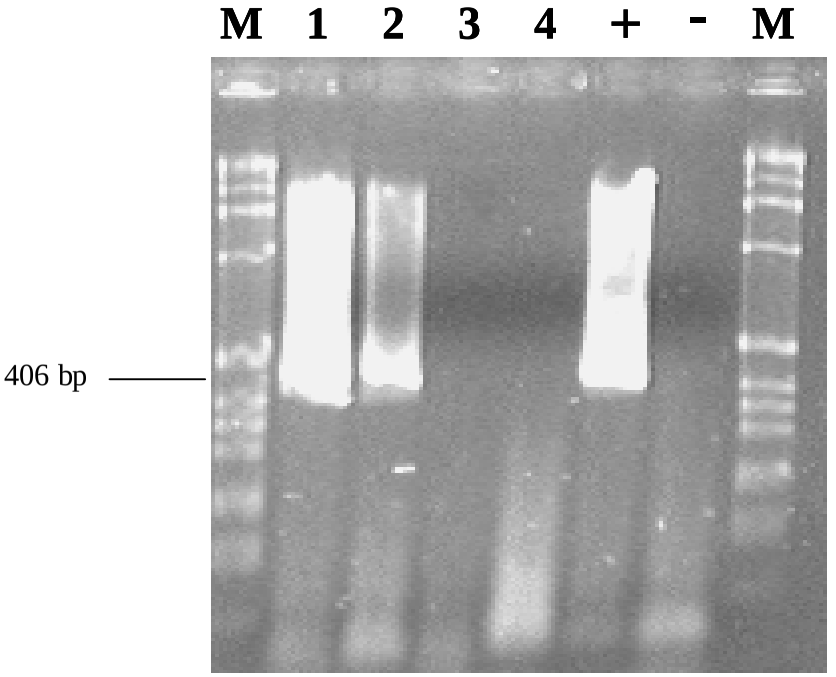
Fig. 13: Limit of detection: trypanosomal cDNA in TE



M: 1 kb DNA ladder
1: 8570 trypanosomes per reaction
2: 857 trypanosomes per reaction
3: 85.7 trypanosomes per reaction
4: 8.57 trypanosomes per reaction
5: 0.857 trypanosomes per reaction
6: 0.0857 trypanosomes per reaction
7: 0.00857 trypanosomes per reaction
+: cDNA from cultured trypanosomes
-: cDNA from mice

fragment length: cDNA: 406 bp

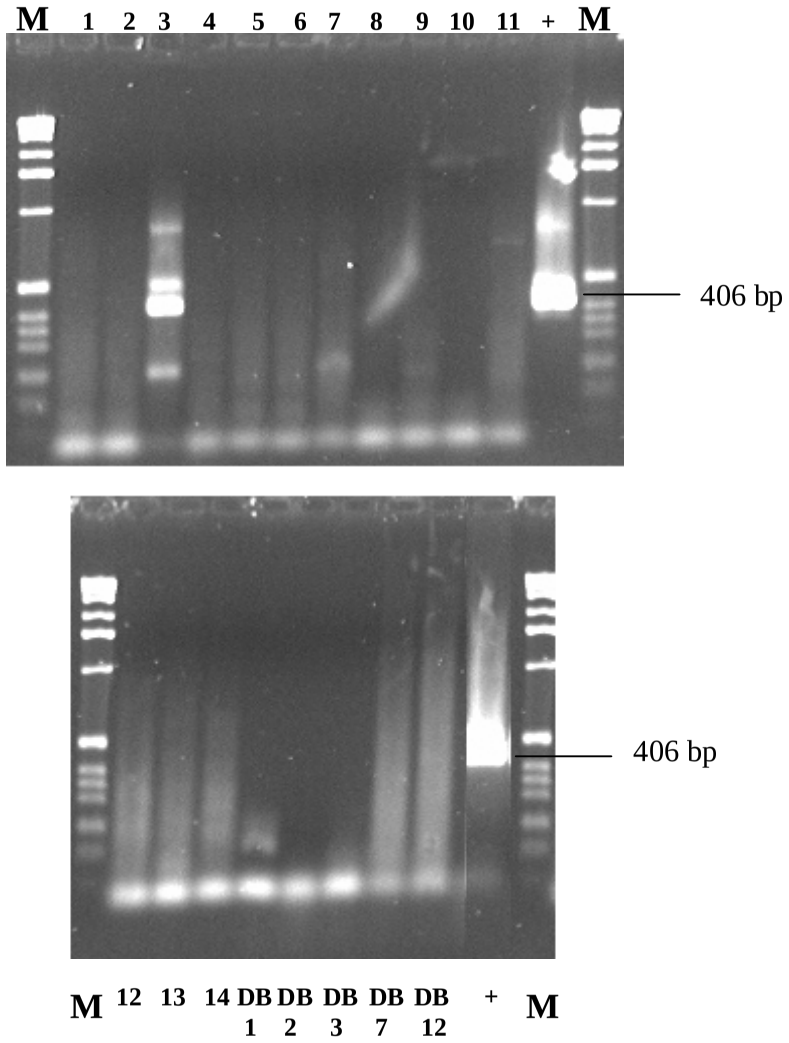
Fig. 14: Limit of detection: trypanosomal cDNA in mouse cDNA



M: 1 kb DNA ladder
1: 200 trypanosomes per reaction
2: 40 trypanosomes per reaction
3: 8 trypanosomes per reaction
4: 1.6 trypanosomes per reaction
+: cDNA from cultured trypanosomes
-: cDNA from mice

fragment length: cDNA: 406 bp

Fig. 15: Detection of trypanosomal cDNA in mouse cDNA positive organs



M: 1 kb DNA ladder

organs of melarsoprol treated mice

1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin

organs of DB 75 treated mice

DB 1: pancreas
DB 2: liver
DB 3: brain
DB 7: spleen
DB 12: spine

+: cDNA of trypanosomes

fragment length: cDNA: 406 bp

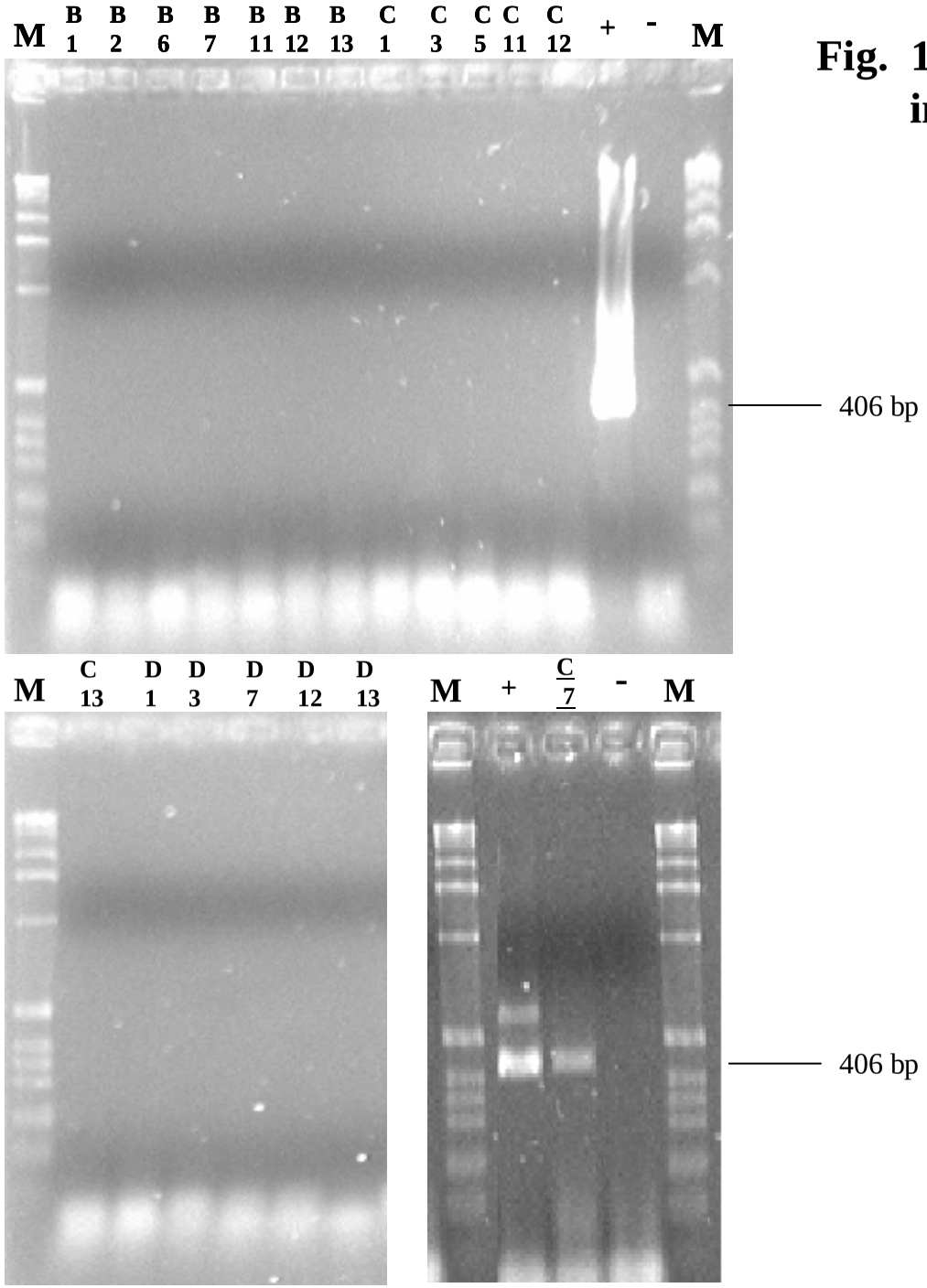


Fig. 16: Detection of trypanosomal cDNA in mouse cDNA positive organs, second run

M: 1 kb DNA ladder

organs of DB 75 treated mice (mouse B, C, D), which were mouse cDNA positive

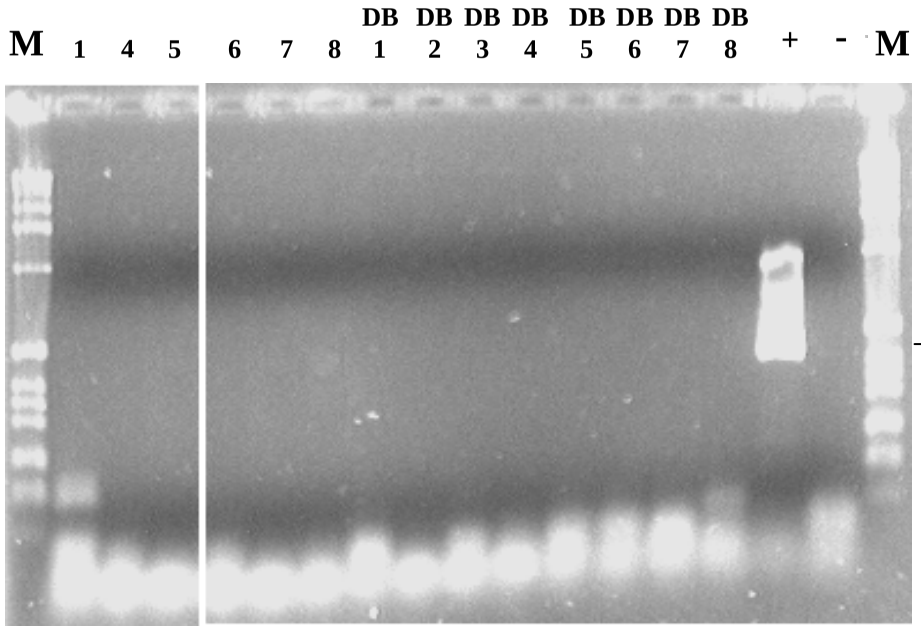
1: pancreas
2: liver
3: brain
4: genitalia
5: lung
6: heart
7: spleen
8: kidney
9: fat tissue
10: muscle tissue
11: thymus
12: spine
13: eyes
14: skin

+: cDNA of trypanosomes
-: DNA of trypanosomes

fragment length: cDNA: 406 bp

positive organ is underlined

Fig. 17: Detection of trypanosomal cDNA in mouse cDNA positive brain homogenates



M: 1 kb DNA ladder

brain homogenates of
- melarsoprol (1 - 8)
- DB 75 (DB 1 - DB 8)

treated mice

+: cDNA of trypanosomes
-: DNA of trypanosomes

fragment length: cDNA: 406 bp

References:

1. Berger BJ, Fairlamb AH. Interactions between immunity and chemotherapy in the treatment of the trypanosomiases and leishmaniases. Parasitology 1992; 105 Suppl:S71-S78.

2. Brun R, Kaminsky R. Animal Models of Acute (first-stage) Sleeping Sickness. 1999. p. 789-93.

3. Chaudhuri JD. Blood brain barrier and infection. Med Sci Monit 2000; 6:1213-22.

4. de Lange T, Liu AY, Van der Ploeg LH, Borst P, Tromp MC, Van Boom JH. Tandem repetition of the 5' mini-exon of variant surface glycoprotein genes: a multiple promoter for VSG gene transcription? Cell 1983; 34:891-900.

5. Disch J, Oliveira MC, Orsini M, Rabello A. Rapid clearance of circulating Leishmania kinetoplast DNA after treatment of visceral leishmaniasis. Acta Trop 2004; 92:279-83.

6. Dorfman DM, Donelson JE. Characterization of the 1.35 kilobase DNA repeat unit containing the conserved 35 nucleotides at the 5'-termini of variable surface glycoprotein mRNAs in *Trypanosoma brucei*. Nucleic Acids Res 1984; 12:4907-20.

7. Gichuki G., Brun R. Animal Models of CNS (second-stage) Sleeping Sickness. Handbook of Animal Models of infection. 1999. p. 794-8.

8. Iwaki T, Yamashita H, Hayakawa T. A Color Atlas of Sectional Anatomy of the Mouse. Adthree Publishing Co.,Ltd.; 2001.

9. Jennings FW, Gray GD, Urquhart GM. The use of Erlangen diamidine 98/202 in relapsing *Trypanosoma brucei* infections in mice. Trans R Soc Trop Med Hyg 1982; 76:204-7.

10. Jennings FW, Whitelaw DD, Holmes PH, Chizyuka HG, Urquhart GM. The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. Int J Parasitol 1979; 9:381-4.

11. Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol 1970; 28:521-34.

12. Lumsden WH, Kimber CD, Strange M. *Trypanosoma brucei*: detection of low parasitaemias in mice by a miniature anion-exchanger/centrifugation technique. Trans R Soc Trop Med Hyg 1977; 71:421-4.

13. Mebius RE, Kraal G. Structure and function of the spleen. Nat Rev Immunol 2005; 5:606-16.

14. Nutman TB, Zimmerman PA, Kubofcik J, Kostyu DD. A universally applicable diagnostic approach to filarial and other infections. Parasitol Today 1994; 10:239-43.

15. Sather S, Agabian N. A 5' spliced leader is added in trans to both alpha- and beta-tubulin transcripts in *Trypanosoma brucei*. Proc Natl Acad Sci U S A 1985; 82:5695-9.

16. Thiel VE, Audus KL. Nitric oxide and blood-brain barrier integrity. Antioxid Redox Signal 2001; 3:273-8.

17. Toure FS, Kassambara L, Williams T, Millet P, Bain O, Georges AJ et al. Human occult loiasis: improvement in diagnostic sensitivity by the use of a nested polymerase chain reaction. Am J Trop Med Hyg 1998; 59:144-9.

18. Ullu E., Tschudi C., Gunzl A. Trans-splicing in trypanosomatid protozoa. In: Smith D.F, Parsons M., editors. Molecular Biology of Parasitic Protozoa. IRL press; 1996. p. 115-29.

Melarsoprol and pentamidine resistant strains and their cross resistance

Abstract

Resistance to melarsoprol and pentamidine, respectively, was induced in trypanosomes of the strain STIB 900 in vitro. The obtained resistant trypanosome populations were tested for their sensitivity to DB 75, melarsoprol and pentamidine.

Additionally, resistant populations were inoculated in immunosuppressed mice to check whether they were still infective and to monitor if a selection takes place. After having been proliferated in the mouse, trypanosomes were isolated and their IC_{50} to the above mentioned drugs were determined. To assess the stability of this drug induced resistance, drug pressure was ceased for 2 months. Then again, sensitivity to the drugs mentioned above was tested. Resistance was shown to be stable. IC_{50} of the drugs showed that cross resistance occurred between all drugs, but to a different extent. Melarsoprol resistant trypanosomes showed the same resistance to pentamidine as the pentamidine resistant strain did. This relationship was not the same vice versa, since melarsoprol resistance was lower in pentamidine resistant trypanosomes compared to the one in melarsoprol resistant trypanosomes. Interestingly, the same sensitivity to DB 75 was determined for both resistant strains. Proliferation of resistant trypanosomes in mice did not have any effect on the sensitivity to the tested drugs.

1. Introduction

Resistance to drugs is a major problem in treatment of diseases. Basically, a distinction between an innate and an acquired resistance can be made. Innate resistance is found, when an organism per se is not sensitive to treatment without having had a previous contact to the drug, whereas an acquired resistance may be the result of drug exposure, drug pressure, cross resistance or mutagenesis. Today, mechanisms of resistance of bacteria against most antibiotics are well investigated and documented.

Trypanosomal resistance to treatment had been reported very early: Ehrlich postulated resistance of trypanosomes to fuchsin already in 1907 [Ehrlich, P. (1907)]. However, little is known about the mechanism of resistance in trypanosomes. The identification of the P2 transporter and its recognition motif was a big breakthrough in the investigation of a developing resistance of trypanosomes to melarsoprol.

Problems of anti-parasitic drugs are, that they are acting specifically against a target within the parasites, and therefore resistance may occur very easily when minor changes within this target happen. These minor changes of the target are mostly based on spontaneous mutation in the genome and emerge particularly in organisms which show a short generation time and a high frequency of combinatorial events. High intergenetic exchange is occurring mainly in bacteria and viruses. In trypanosomes, combinatorial events were known to exist only in the procyclic forms in the tsetse fly and not in bloodstream forms [Jenni, L. (1986)]. Generally, mutations of the genome leading to drug resistance have to deliver a selective advantage of the mutant over the wild type. Additionally, this mutation has to be passed on to the descendants and should not get lost when parasites enter the next stage of their life-cycle. Acquiring resistance always imposes a certain cost for the parasites, possibly a reduction in infectivity or in the transmission rate. The cost for melarsoprol resistance is not exactly known, but mutations in the P2 transporter could lead to reduced uptake of nucleobases like adenosine, which are essential for the parasites.

Resistance to a drug is defined by the ability to tolerate certain concentrations without having any negative effects on the parasite. Many mechanism of resistance are known to date:

One strategy is to produce more targets (overexpression), so that some of the targets are still free available. Binding of morphine at the opioid receptor is known to stimulate the synthesis of this receptor. Therefore, an increase of drug is necessary to reach the same effect: so called pharmacodynamic tolerance in pain therapy.

Another mechanism is that the target itself undergoes mutations. Differences at the drug binding site may lead to a decrease in affinity. An example for this type of resistance is the reduced affinity of streptomycin which can not bind anymore to the modified 30 s subunit of the bacterial ribosome. The modification of the RNA-polymerase beta subunit is responsible for resistance to rifampicin.

Mutations of drug transporters are also a strategy in development of resistance. Transporters responsible for drug uptake show mutations as the example of the chloroquine transporter in plasmodia does. Resistance to chloroquine is linked to multiple mutations in a protein (PfCRT: *P. falciparum* chloroquine resistance transporter) that is assumed to function as transporter in the parasite´s digestive vacuole. Chloroquine is assumed to interact in the detoxification of hematin by binding to it and forming toxic complexes. [Wellems, T.E. (2001)], [Wellems, T.E. (2004)].

Induction of export pumps like P-Glycoprotein (P-gp), a protein encoded by the multiple drug resistance gene (MDR), is also one strategy to decrease drug concentration within a cell. Protease inhibitors used in therapy of HIV were shown to be exported massively by P-gp. Combination with P-gp inhibitors, like other protease inhibitors (ritonavir, saquinavir, nelfinavir) or a calcium channel blocker like verapamil, is used to reach higher levels of protease inhibitors [Fardel, O. (1996)].

Resistance may be transient, as the example of chloroquine resistance in Malawi showed [Kublin, J.G. (2003)]. After having had high treatment failures due to resistance of malaria parasites to chloroquine, first line treatment was changed to the combination sulfadoxine/pyrimethamine. Several years later, re-introduction of chloroquine was possible since resistance got lost. A possible explanation for the increase of the sensitivity to chloroquine may be caused by re-emergence of chloroquine sensitive plasmodia due to withdrawal of drug pressure. It seemed that resistance burdens in vivo fitness and therefore wild type plasmodia spread again after drug pressure had gone.

Today, one strategy to delay or prevent development of resistances is to combine drugs acting on different targets and therefore exhibiting different modes of action. Combination chemotherapy decreases the probability of resistance, since it is less likely that one parasite is resistant both to drug A and drug B. Another advantage, which is of importance especially in respect to antibiotics, is that when two different acting drugs were combined the spectrum may be extended (extended-spectrum antibiotics). Additionally, synergistic effects may result from a combination of two different acting drugs. A positive effect often observed, is that a combination allows to decrease the concentration of the constituents compared to monotherapy and therefore therapy may be tolerated better in patients. Whiteside suggested the combination of so called “sanative” pairs of drug, which do not induce cross resistance to each other [Whiteside, E.F. (1958)]. Also, resistances are less likely to occur for drugs with a short half life than for drugs with a long half life. Another factor which has to be mentioned in this context is that not only the concentration of drug the parasite is exposed to, but also the duration of exposure is of importance in developing resistance.

Within this study, in vitro resistance to drug was induced by growing trypanosomes of the strain STIB 900 (*Trypanosoma brucei rhodesiense*) in rising concentration of melarsoprol or

pentamidine, respectively. Sensitivity of these resistant strains was tested. Thereto, the concentration at which 50% of the parasites were killed compared to the control (IC_{50}) of trypanosomes resistant to melarsoprol and pentamidine were tested in the Alamar Blue assay [Raz, B. (1997)]. Additionally, cross resistance to DB 75, pentamidine resistance in melarsoprol resistant trypanosomes and melarsoprol resistance in pentamidine resistant trypanosomes was determined. By dividing the IC_{50} of a compound obtained for the resistant strain by the IC_{50} of this compound obtained for the wild type, the resistance factor is obtained [Carter, N.S. (1995)].
Since resistant trypanosomes were kept in culture conditions for several months, infectivity in a host was checked by inoculating trypanosomes in immunosuppressed mice. After proliferation in the mouse, stabilates were made and IC_{50} of trypanosomes were determined and compared to the ones obtained for trypanosomes from culture.

Comprehension of the mechanism underlying drug resistance is of importance since gained knowledge may help to evaluate new drug targets. The presence of cross resistance would indicate that the drugs tested do have the same target or the same transporter.

2. Material and Methods

2.1. Trypanosomes

STIB 900 is a derivative of STIB 704 which was isolated from a male patient at St. Francis Hospital in Ifakara/Tanzania in 1982. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenical growth in vitro.

2.2. Medium

Trypanosomes were cultivated in Minimum Essential Medium (MEM) with Earle`s salts (powder, GIBCO, Invitrogen, Basel, Switzerland), supplemented with 25 mM HEPES (GIBCO, Invitrogen, Basel, Switzerland), 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM nonessential amino acids (100x, GIBCO, Invitrogen, Basel, Switzerland). The medium was further supplemented with 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine and 15% heat inactivated horse serum [Baltz, T. (1985)].

2.3. Drugs

DB 75 was synthesised by Dr. D. W. Boykin, Georgia State University, Atlanta, USA [Das, B.P. (1977)]. Melarsoprol was obtained from WHO, Geneva, Switzerland donated by Sanofi-Aventis, and pentamidine as Pentacarinat® from Rhone-Poulenc, Thalwil, Switzerland. Endoxan® (cyclophosphamide) was purchased from Baxter, Volketswil, Switzerland and Resazurin from Fluka, Buchs, Switzerland.

2.4. Generation of resistant strains

Trypanosomes were cultured under a subcurative dose of either melarsoprol or pentamidine. After several subpassages, when proliferated trypanosomes showed no difference in size, morphology and fission rate compared to trypanosomes cultured without drug, the amount of drug applied was increased stepwise. After several of these adaptation steps, resistant strains were obtained.

2.5. Alamar blue assay

Trypanosomes were tested for their in vitro sensitivity to melarsoprol, pentamidine and DB 75. Thereto, trypanosomes were incubated 72 hours in serial drug dilutions of these drugs in a 96-well plate [Raz, B. (1997)]. After addition of the viability indicator resazurin (12.5 mg in 100 ml phosphate buffered saline), plates were read at excitation wavelength 536 nm and at emission wavelength 588 nm using the fluorescence reader SpectraMAX Gemini XS (Bucher Biotech AG, Basel, Switzerland). To determine the $IC_{50,}$ data analysis was performed with SoftmaxPRO 3.1.2..

2.6. Proliferation of resistant trypanosomes in immunosuppressed mice

Female NMRI mice with a body weight of 18-22 g were purchased from RCC, Ittingen, Switzerland. Mice were immunosuppressed applying 200 mg/kg Endoxan® intraperitoneally. Resistant trypanosomes were concentrated to $5*10^6$ trypanosomes and were inoculated i.p. into mice. Tail blood was checked for trypanosomes. When parasitaemia was high, stabilates were made and stored in liquid nitrogen. Trypanosomes proliferated in mice were tested for their sensitivity using the Alamar Blue assay.

Animal use adhered to the guidelines issued by the Swiss Federal Veterinary Department (BVET) for laboratory animals.

3. Results

A summary of the obtained results is presented in Table 1.

3.1. Melarsoprol and pentamidine resistant STIB 900

Trypanosomes grown in melarsoprol showed an IC_{50} of 74.5 ng/ml to melarsoprol compared to the wild type STIB 900 strain with an IC_{50} of 3 ng/ml (resistance factor: 24.8). In the case of pentamidine, IC_{50} of the resistant strain was 165.8 ng/ml compared to an IC_{50} of 1 ng/ml for its non resistant counterpart (resistance factor: 165.8).

3.2. Cross resistances

IC_{50} to pentamidine determined in the melarsoprol resistant strain was 175.6 ng/ml (resistance factor: 175.6), and IC_{50} to melarsoprol in pentamidine resistant strains was determined to be 38 ng/ml (resistance factor: 12.7).

DB 75 was determined to have an IC_{50} of 17.1 ng/ml (resistance factor: 11.4) in the melarsoprol resistant strain and of 20.4 ng/ml (resistance factor: 13.6) in the pentamidine resistant strain.

3.3. Transience of resistance

Trypanosomes showing resistance were kept in culture without drug pressure. After approximately 120 generation times (2 months = 60 days = 60*2, assuming a generation time of 12 h), sensitivity to melarsoprol, pentamidine and DB 75 was assessed. Melarsoprol resistant strains showed an IC_{50} of 77.9 ng/ml to melarsoprol (resistance factor: 26), 161.3 ng/ml to pentamidine (resistance factor: 161.3) and 19.1 ng/ml to DB 75 (resistance factor: 12.7). Pentamidine resistant strains had an IC_{50} of 121.2 ng/ml to pentamidine (resistance factor: 121.2), 17.8 ng/ml to melarsoprol (resistance factor: 5.9) and 18.5 ng/ml to DB 75 (resistance factor: 12.3). Results showed that a low cross resistance to both resistant strains was obtained for DB 75. A high cross resistance was found to pentamidine in the melarsoprol resistant strain, whereas in the pentamidine resistant strain a low cross resistance to melarsoprol was detected.

3.4. Infectivity

Inoculated trypanosomes lead to a moderate to high parasitaemia within a few days.

3.5. Selection in mice

Resistant trypanosomes proliferated in mice underwent several passages in culture medium before they were tested in the Alamar Blue assay.

Melarsoprol resistant trypanosomes isolated from mice showed an IC_{50} of 64.7 ng/ml to melarsoprol (resistance factor: 21.6), an IC_{50} of 118.5 ng/ml to pentamidine (resistance factor: 118.5) and an IC_{50} of 23.7 ng/ml to DB 75 (resistance factor: 15.8).

IC_{50}s for pentamidine resistant trypanosomes isolated from a mouse were shown to be on average 140.3 ng/ml to pentamidine (resistance factor: 140.3), 30.4 ng/ml to melarsoprol (resistance factor: 10.2) and 19.1 ng/ml to DB 75 (resistance factor: 12.7).

Cross resistance to DB 75 was low in both melarsoprol and pentamidine resistant strains. The melarsoprol resistant strain showed a high cross resistance to pentamidine, whereas low cross resistance to melarsoprol was found in the pentamidine resistant strain.

4. Discussion

Cross resistances between melaminophenyl arsenicals and diamidines in laboratory strains had been reported several times [Barrett, M.P. (2003)]. The reason for this cross resistance had been attributed to the loss of the P2 transporter, which transports both classes of drugs inside the parasite, since the mode of action of the two classes of drugs is assumed to be different [Barrett, M.P. (1999)], [de Koning, H.P. (2001)]. The proposed mode of action of arsenicals is an interaction on energy metabolism and on membrane integrity, whereas diamidines were thought to interact with negatively charged molecules like nucleic acids, and/or membrane phospholipids [Barrett, M.P. (1999)].

However, to draw a conclusion referring to a possible cross resistance between an arsenical and a diamidine is not easy, as observations showed [Barrett, M.P. (1999)], [Matovu, E. (2001)]. One reason may be that drug uptake into the trypanosome may be additionally mediated by several transporters other than the P2 transporter.

Theoretically, loss or mutation of P2 transporter would induce high levels of resistance to melaminophenyl arsenicals and very low levels of resistance to pentamidine due to the amount of transporters the drug relies on.

In this experiment melarsoprol and pentamidine resistant strains both showed only a slight cross resistance to DB 75 (resistance factor 12.5). This indicates that the P2 transporter plays a certain but not exclusive role in uptake of DB 75. Recent studies on transport of DB 75

suggested also the P1 transporter to be involved [Brun R., personal communication]. A possible interplay between P1 and P2 transporters was suggested to have a key function in uptake of adenosine in a way that overexpression of the P1 compensated for lack of the P2 transporter [Geiser, F. (2005)]. A similar behaviour may occur when mutation of the P2 transporter decreases the uptake of adenosine, which may then be taken up via P1 transporter. The ribose moiety of adenosine, to be more precise two hydroxylgroups of the ribose molecule, serve as recognition motif for the P1 transporter [de Koning, H.P. (1999)]. The possible recognition motif for DB 75, if at all transported by P1, is not elucidated to date.

Interestingly, cross resistance of melarsoprol resistant strains to pentamidine was very high (average resistance factor 150), whereas pentamidine resistant strains showed low cross resistance to melarsoprol (average resistance factor 9.6). These results were in a way surprising as cross resistance of pentamidine resistant strains were thought to result also in a high resistance to melarsoprol. It was hypothesised that mutations occurring in the three transporters (P2, High affinity pentamidine transporter: HAPT, low affinity transporter: LAPT) involved in pentamidine uptake, had a higher impact on cross resistance to melarsoprol which is transported only by two transporters (P2 and a yet unidentified transporter [de Koning, H.P. (2004)]).
These results may indicate that the mutation of the P2 transporter plays a minor role when resistance is induced by pentamidine, and/or that overexpression of another drug transporter compensates for drug uptake of melarsoprol in the pentamidine induced resistant trypanosome strain as described for P1/P2 transporters in uptake of adenosine.

Stability of resistance

Resistance to drug did not decrease within approximately 120 generation times in culture when drug pressure was absent, neither for melarsoprol nor pentamidine resistant strains. Compared to the regained chloroquine resistance in Malawi, sensitive trypanosomes are not assumed to survive 120 generation times under drug pressure and therefore are not likely to re-spread again after cessation of the drug. Regaining sensitivity to a drug to which trypanosomes were resistant to may be caused by mutation reversals. Since spontaneous mutations are likely to occur every 1 of 10^9 bases when DNA is replicated [Alberts, B. (1994)], a lot of time and trypanosomes were needed to receive the mutation reversal exactly at the position were the mutation had been taken place before. The P2 transporter in

melarsoprol resistant trypanosomes was shown to have 6 mutated nucleosides in the TbAT1 gene, which referred to a change in the amino acid sequence [Maser, P. (1999)]. Mutations at other sites leading to a decrease in sensitivity are also possible. When these trypanosomes additionally show a shorter generation time, IC_{50} of the trypanosome population will decline as the amount of these trypanosomes increases.

Mouse passage

Since trypanosomes isolated from mice did not show any differences in their sensitivity profile to the corresponding resistant strain obtained in vitro, mouse passage did not have a selective effect for or against resistant trypanosomes.

Generally, IC_{50} of melarsoprol, pentamidine and DB 75 did not change substantially in resistant strains after cessation of drug nor mouse passage compared to the original resistant strain. Also, both resistant strains showed the same sensitivity to DB 75. Interestingly, the same IC_{50} for pentamidine was determined in melarsoprol and pentamidine resistant trypanosomes. Differences in sensitivity to melarsoprol seemed to be little lower in the pentamidine induced resistant strain. Since the obtained IC_{50}s represent average values, these differences monitor a certain trend to a lower sensitivity to melarsoprol in the pentamidine resistant strain.

5. Conclusion

In vitro resistance was induced successfully in trypanosomes of the strain STIB 900 and was stable after mouse passage. Resistance seems not to be transient since no decrease in sensitivity was obtained when drug pressure was absent. Sooner or longer mutations reversals are expected to occur leading to more sensitive trypanosomes.
Good news were obtained referring to the cross resistance of these mutated trypanosomes to DB 75, since only a low cross resistance resulted.
With the aid of these resistant strains a system was created to test upcoming new drugs for their cross resistance to melarsoprol and pentamidine. This system may be an important tool when decisions regarding further development of certain drugs have to be made. A drug making its way to market, preferably shows no or only little cross resistance to drugs already on the market.

Table 1: IC_{50} and resistance factors of tested trypanosome strains

origin of strain	IC_{50} in ng/ml melarsoprol	IC_{50} in ng/ml pentamidine	IC_{50} in ng/ml DB 75	origin of strain	IC_{50} in ng/ml melarsoprol	IC_{50} in ng/ml pentamidine	IC_{50} in ng/ml DB 75
wildtype STIB 900	**3**	**1**	**1.5**		**3**	**1**	**1.5**
melarsoprol resistant strain	71.68	173.45	11.57	pentamidine resistant strain	27.81	153.67	12.94
	59.25	183.19	16.56		52.87	183.96	16.80
	92.44	169.77	23.17		33.38	159.77	31.36
average:	**74.46**	**175.57**	**17.1**	**average:**	**38.02**	**165.8**	**20.37**
resistance factor:	**24.8**	**175.5**	**11.4**	**resistance factor:**	**12.7**	**165.8**	**13.6**
melarsoprol resistant strain	81.00	138.06	10.54	pentamidine resistant strain	13.27	104.53	15.60
after cessation of	74.78	160.39	22.52	after cessation of	17.60	145.43	12.4
melarsoprol	77.98	185.46	24.22	pentamidine	22.54	113.5	27.38
average:	**77.92**	**161.3**	**19.09**	**average:**	**17.8**	**121.15**	**18.46**
resistance factor:	**26.0**	**161.3**	**12.7**	**resistance factor:**	**5.9**	**121.2**	**12.3**
melarsoprol resistant strain	56.83	115.38	17.61	pentamidine resistant strain	20.68	148.43	13.18
after mouse passage	77.42	86.21	18.65	after mouse passage	33.38	149.11	17.16
	59.86	153.89	34.79		37.25	123.21	26.95
average:	**64.7**	**118.49**	**23.68**	**average:**	**30.44**	**140.25**	**19.1**
resistance factor:	**21.6**	**118.5**	**15.8**	**resistance factor:**	**10.2**	**140.3**	**12.7**
overall average	**72.36**	**151.75**	**19.96**	**overall average**	**28.75**	**142.4**	**19.31**
overall resistance factor	**24.2**	**151.8**	**13.3**	**overall resistance factor**	**9.6**	**142.4**	**12.9**

References:

1. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Basic Genetic Mechanisms - DNA Repair. Molecular Biology of the Cell. Garland Publishing, Inc.; 1994. p. 242-50.

2. 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; 4:1273-7.

3. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ et al. The trypanosomiases. Lancet 2003; 362:1469-80.

4. Barrett MP, Fairlamb AH. The biochemical basis of arsenical-diamidine crossresistance in African trypanosomes. Parasitol Today 1999; 15:136-40.

5. Carter NS, Berger BJ, Fairlamb AH. Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. J Biol Chem 1995; 270:28153-7.

6. Das BP, Boykin DW. Synthesis and antiprotozoal activity of 2,5-bis(4-guanylphenyl)furans. J Med Chem 1977; 20:531-6.

7. de Koning HP. Transporters in African trypanosomes: role in drug action and resistance. Int J Parasitol 2001; 31:512-22.

8. de Koning HP, Anderson LF, Stewart M, Burchmore RJ, Wallace LJ, Barrett MP. The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in African trypanosomes. Antimicrob Agents Chemother 2004; 48:1515-9.

9. de Koning HP, Jarvis SM. Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. Mol Pharmacol 1999; 56:1162-70.

10. Ehrlich P. Chemotherapeutische Trypanosomen-Studien. Berliner Klinische Wochenzeitschrift 1907; 44.

11. Fardel O, Lecureur V, Guillouzo A. The P-glycoprotein multidrug transporter. Gen Pharmacol 1996; 27:1283-91.

12. Geiser F, Luscher A, de Koning HP, Seebeck T, Maser P. Molecular pharmacology of adenosine transport in *Trypanosoma brucei*: P1/P2 revisited. Mol Pharmacol 2005; 68:589-95.

13. Jenni L, Marti S, Schweizer J, Betschart B, Le Page RW, Wells JM et al. Hybrid formation between African trypanosomes during cyclical transmission. Nature 1986; 322:173-5.

14. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN et al. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J Infect Dis 2003; 187:1870-5.

15. Maser P, Sutterlin C, Kralli A, Kaminsky R. A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. Science 1999; 285:242-4.

16. Matovu E, Seebeck T, Enyaru JC, Kaminsky R. Drug resistance in *Trypanosoma brucei* spp., the causative agents of sleeping sickness in man and nagana in cattle. Microbes Infect 2001; 3:763-70.

17. Raz 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.

18. Wellems TE. Transporter of a malaria catastrophe. Nat Med 2004; 10:1169-71.

19. Wellems TE, Plowe CV. Chloroquine-resistant malaria. J Infect Dis 2001; 184:770-6.

20. Whiteside EF. The maintenance of cattle in tsetse-infested country. A summary of four years` experience in Kenya. Proceedings of the Seventh Meeting on the International Scientific Council for Trypanosomiasis Research. Bruxelles: CCTA publication; 1958. p. 83-90.

Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice

Abstract

A drug discovery and development project of a consortium funded by the Bill and Melinda Gates Foundation revealed highly active pentamidine analogues against *Trypanosoma brucei ssp*. Corresponding orally active prodrugs of these compounds were synthesised. To assess the influence of a present acute infection on the pharmacokinetics of a drug, trypanosome infected and uninfected mice were treated with those active drugs (intraperitoneally) and prodrugs (orally). Additionally, the effect of intraperitoneal vs. subcutaneous application was determined for DB 75 and DB 820.

Blood from mice (infected and uninfected) was harvested at selected times and plasma prepared. After precipitation of the plasma proteins, drug levels were determined using HPLC/MS/MS techniques. Evaluation of the pharmacokinetic profile was performed with two drugs (DB 75, DB 820) and their corresponding prodrugs (DB 289, DB 844), which differ structurally only within the spacer region, where a phenyl ring is replaced by a pyridyl moiety in case of DB 820/DB 844.

Plasma levels of infected animals were shown to be influenced by two different mechanisms: accumulation of active drug in trypanosomes and inhibition of metabolism due to infection or drug itself. The impact of those mechanisms was different for each of the analysed structures. Generally, inhibition of metabolism seemed to be more important for DB 820/DB 844, whereas accumulation of active compound had a bigger influence on the plasma levels of DB 75/DB 289. When comparing i.p. vs. s.c. administration, higher bioavailability was obtained after s.c. compared to i.p. application.

1. Introduction

Sleeping sickness still is one of the most life-threatening diseases for humans in Sub-Saharan Africa. It is estimated that each year 350´000 people get infected, and another 60 millions in 36 countries are at risk of infection. In some regions, where the prevalence of HAT is 20% to 50%, sleeping sickness is the first or second cause of mortality ahead of HIV/AIDS [www.who.int/mediacentre/factsheet/fs259/en/]. The causative agents of the disease are protozoan parasites of the genus *Trypanosoma*, namely *Trypanosoma brucei rhodesiense*

(*T. b. rhodesiense*) and *Trypanosoma brucei gambiense* (*T. b. gambiense*). The area in which human African trypanosomosis (HAT) occurs is restricted to the natural habitat of the tsetse fly, since parasites are transmitted by the bite of this fly.

Untreated trypanosomosis is always fatal. Different drugs are used for treatment depending on the stage of infection. During first stage, when parasites are present in the blood and lymphatic system, pentamidine and suramin are administered. Later, when trypanosomes have invaded the central nervous system (stage 2) the highly toxic arsenical compound melarsoprol is the most frequently used drug. Unfortunately, melarsoprol may cause encephalopathic syndromes, occurring in around 10% of all treated patients and leading to death in 10-50% of those cases [Blum, J. (2001)]. Eflornithine (DMFO), the sole alternative to treat second stage trypanosomosis, is effective against *T. b. gambiense* only. Since currently used drugs show severe adverse drug reactions and resistances to the applied drugs are not unusual, new drugs for the treatment of HAT are needed urgently.

An international consortium, the "UNC lead consortium to discover new drugs for the treatment of parasitic diseases", was founded in 1999 with the aim to develop a new drug to treat first stage HAT. New compounds were modelled based on their analogy to pentamidine. Screening activities in vitro revealed highly active pentamidine analogues: two of them, DB 75 and DB 820, showed promising results in vitro and in vivo in mice. Since these compounds are not orally active, their corresponding methoxyamidines (DB 289, DB 844) were synthesised by D. W. Boykin [Boykin, D.W. (1996)], [Anbazhagan, M. (2003)], [Ismail, M.A. (2003)]. By masking the diamidine groups with methoxy moieties, orally applicable molecules, which can be converted by enzymatic activation to the original compound, are obtained. Excellent activity and efficacy after oral administration was demonstrated for these methoxyamidine prodrugs.

The compounds were tested in vivo to estimate their toxicity and efficacy in mice. DB 75 was not toxic in mice when 50 mg/kg was applied i.p., as was DB 289 after oral administration of 100 mg/kg. However, after application of 100 mg/kg DB 75 i.p. all mice died. In the case of DB 820 i.p., DB 844 p.o. respectively, 100 mg/kg was tolerated well without showing any signs of intoxication.

Efficacy in the acute mouse model was shown after application of active compound at a dosage of 4 x 20 mg/kg. Treatment with 4 x 20 mg/kg DB 820 i.p. cured 4 out of 4 mice. Results for DB 75 were not as promising, since no mouse could be cured. However, a positive effect on the infection clearly was noticeable, resulting in a prolongation of the mean survival

days. Control mice died within 6 days after infection, whereas DB 75 treated mice (4 x 20 mg/kg i.p.) survived at least up to day 52.
In the acute mouse model oral application at 4 x 50 mg/kg DB 289 resulted in a cure of 3 out of 4 mice. After oral DB 844 application at 4 x 5 mg/kg all mice were cured.
When tested in the chronic mouse model, application of DB 844 at 6 x 100 mg/kg p.o. led to 100% cure, whereas only 3 out of 5 mice could be cured with the same treatment schedule when DB 289 was applied. These findings indicated that both prodrugs were able to cross the blood brain barrier (BBB) to a certain extent, otherwise no cure of mice would be observed. We anticipate DB 844 as new lead compound for second stage trypanosomosis.

Recently, DB 289 has completed phases IIa and IIb clinical trials quite successfully and is undergoing phase III now. Further derivatives of the diverse diamidines, like DB 844, are under investigation for activity in the late stage HAT at the moment.

In this study, pharmacokinetic profiles of active compounds (DB 75, DB 820) and prodrugs (DB 289, DB 844) were determined in infected and uninfected mice. We will report on differences in the pharmacokinetics due to the presence of parasites. Additionally, a possible influence of the parasites on the conversion of the prodrug to the active compound will be demonstrated. Furthermore, the reason for the superiority of DB 844 versus DB 289 in the chronic model will be explainable by the collected data.

2. Material and Methods

2. 1. Compounds and chemicals

	DB 75	**DB 289**	**DB 820**	**DB 844**
	active compound	prodrug	active compound	prodrug
salt	dihydrochloride	monomaleate	diacetate	trihydrochloride
formula	$C_{18}H_{16}N_4O$/ 2 HCl	$C_{20}H_{20}N_4O_3$/ $C_4H_4O_4$	$C_{17}H_{15}N_5O$/ 2 CH_3COOH/ 2.2 H_2O	$C_{19}H_{19}N_5O_3$/ 3 HCl / 1H_2O
MW (salt)	304.35 (377.267)	364.41 (480.47)	305.34 (490.705)	365.33 (492.783)
pK_a: *	11.8 10.41	4.09 2.53	11.91 10.45	3.71 2.49
log P * (neutral form)	2.681	3.78	1.20 ± 0.4⊗	3.2
log D ⊗				
pH 1	-2.88	-0.67	-3.99	-1.36
pH 4	-2.88	+3.37	-3.80	+2.85
pH 7	**-2.80**	**+4.28**	**-3.37**	**+3.35**
pH 8	-1.93	+4.28	-1.87	+3.35
pH 10	+1.43	+4.28	+0.81	+3.35
*** pH 7.4**	**> -2.0**	**+3.78**.	**n.d.**	**3.2**

* data determined by Saulter J. [Saulter, J.Y. (2005)]

⊗ calculated with ACD software

n.d.: not determined

2.1.1. Compounds

DB 75 and DB 289 were synthesised using methods previously described [Das, B.P. (1977)], [Boykin, D.W. (1996)]; DB 820 and DB 844, which show an alteration of the 2,5-phenyl group replacing the phenyl group by a pyridyl group according to Ismail [Ismail, M.A. (2003)].

2.1.2. Internal standards

d_8-labelled DB 75 and d_8-DB 289 were synthesised by D. W. Boykin as described previously [Stephens, C.E. (2001)]. Deuterium labelled d_4-820 and d_4-844 were synthesised according to Ismail [Ismail, M.A. (2004)].

2.1.3. Chemicals

DMSO and NaCl were purchased from Merck (Dietikon, Switzerland), potassium fluoride from Sigma Aldrich (Basel, Switzerland), heparin from Fresenius (Stans, Switzerland). HPLC- grade acetonitrile, methanol, water and formic acid were obtained from Fisher Scientific (Chicago, IL, USA), ammonium formate and trifluoroacetic acid (TFA) from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Drug solutions

2.2.1. Drug solutions for treatment of mice

The calculated amount of drug was weighed (either 2 mg/ml for active compounds or 10 mg/ml for prodrugs), dissolved in 10% DMSO, then water was added to the final volume.

2.2.2. Standard curves

20 mM stock solutions of prodrugs in methanol and 10 mM stock solutions of active compounds in methanol/water (1:1) were prepared and stored at –20° C. Dilutions were prepared the day the samples were analysed containing 5, 10, 25, 50, 100, 250, 500, 1000 and 10000 nM of the corresponding drug.

2.2.3. Internal standards

Stock solutions containing 10 mM deuterium labelled drug in water (active compounds) and methanol (prodrugs), respectively, were prepared and stored at –20° C. Further dilutions to concentrations of 25 nM of labelled prodrug (d_8-DB 289, d_4-DB 844) in acetonitrile and 125 nM of deuterium labelled active drug (d_8-DB 75, d_4-DB 820) in methanol/HCl 0.1N (8:1 v/v) were freshly prepared.

2.3. Animal experiment

2.3.1. Mice

In all experiments female NMRI mice with a bodyweight from 22 to 31 g were used. Mice were divided into 10 groups (as described in the table below), consisting of 24 mice each (3 mice per time point, 7 time points, 1 control group).

2.3.2. Administration of active compounds

20 mg/kg of DB 75, DB 820 respectively were administered intraperitoneally to infected and uninfected mice (groups B, G and A, F) and additionally subcutaneously to uninfected mice (groups C, H).

2.3.3. Administration of prodrugs

The prodrugs DB 289 and DB 844 were administered per os at 100 mg/kg to infected and uninfected mice (groups E, J and groups D, I).

2.3.4. Infection and animal care

Mice were infected with $5*10^3$ trypanosomes of the strain STIB 900 (*T. b. rhodesiense*) 3 days prior treatment. The animals showed a parasitaemia of 10^5 to 10^6 trypanosomes per ml the day the drug was applied.

The animal use adhered to the guidelines issued by the Swiss Federal Veterinary Department (BVET) for laboratory animals.

DB 75 / DB 289				**DB 820 / DB 844**			
group	compound	route of administration	status of infection	group	compound	route of administration	status of infection
A	DB 75	i.p.	uninf.	F	DB 820	i.p.	uninf.
B	DB 75	i.p.	inf.	G	DB 820	i.p.	inf.
C	DB 75	s.c.	uninf.	H	DB 820	s.c.	uninf.
D	DB 289	p.o.	uninf.	I	DB 844	p.o.	uninf.
E	DB 289	p.o.	inf.	J	DB 844	p.o.	inf.

2.3.5. Harvesting of blood

Blood was harvested after decapitation at seven different timepoints. For the active compounds the following timepoints were chosen: 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and

24 hr. Blood from prodrug-treated mice was collected 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr and 24 hr after drug application. Each timepoint is represented by the average obtained from data of three mice (± standard deviation).
As anticoagulant a concentrated heparin solution with potassium fluoride (2.5%) as stabiliser in NaCl solution (0.85%) was used to obtain a final concentration of 400 U/ml heparin.

2.3.6. Plasma preparation

Plasma was obtained after centrifugation at 5000 rpm for 5 min (Eppendorf centrifuge 5415, Vaudaux-Eppendorf AG, Schönenbuch/Basel, Switzerland) and frozen at –80° C.

2.4. Sample preparation

2.4.1. Standard curves

Calibration curves were prepared by spiking blank mouse plasma with varying amounts of drug. The range of the calibration curve was 5 nM to 10000 nM. 25 µl of the spiked plasma samples were transferred into Q-glass vials and treated as described below (sample preparation).

2.4.2. Sample preparation

2.4.2.1. Determination of prodrugs by triple quadrupole MS

After thawing, 25 µl aliquots of each sample were transferred to Q-glass vials loaded in a 96 well LC/MS plate. 100 µl of a 25 nM IS (d_8-289, d_4-844) solution in acetonitrile was added to each sample to precipitate the proteins. Vials in the plate were covered tightly with a polypropylene mat cap, and vortexed briefly at moderate speed on a microplate vortexer. The plate was centrifuged using a swinging bucket rotor equipped with microplate adaptors at 2600 g for 5 min at 4° C and loaded for MS analysis.

2.4.2.2. Determination of active compounds by iontrap MS

100 µl of 125 µM IS (d_8-75, d_4-820) in 8:1 methanol: HCl 0.1 N (v/v) was added to a 25 µl plasma aliquot. Samples were vortexed gently and spun using a swinging bucket rotor equipped with microplate adaptors at 2600 g for 5 min at 4° C. The supernatants were transferred to 1.5 ml eppendorf tubes and evaporated under 7 psi nitrogen at 45° C for 20 min in a Zymark TurboVap LV equipped with a 1.5-ml tube rack. Samples were reconstituted using 100 µL of a solvent consisting of 15 mM ammonium formate and 30 mM formic acid in water of HPLC quality.

Reconstituted samples were vortexed at high speed for 15 s and then centrifuged at 4000 rpm for 5 min at room temperature. The supernatants were transferred to a 200 µl polypropylene vial insert, and placed inside a 2.0-ml glass HPLC vial. Vials were capped and loaded onto the MS sample tray.

2.5. Instruments

2.5.1. Triple Quadrupole HPLC/MS/MS

Plasma concentrations of the prodrugs DB 289 and DB 844 were determined using reversed-phase HPLC with tandem triple quadrupole mass spectrometry (HPLC/MS/MS).

The analytical system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA), a thermostatic CTC PAL Leap autosampler (Hamilton Co., Reno, NV, USA), Valco solvent divert valve (Houston, TX, USA), and an Applied Biosystems API 4000 triple quadrupole mass spectrometer equipped with a TurboIon Spray® interface (MDS Sciex, San Fransisco, CA, USA) and PEAK nitrogen generator (Punta Gorda, FL, USA).

The equipment was controlled using Analyst 1.3 software (Applied Biosystems, Foster City, CA, USA).

DB 289 and d_8-DB 289 or DB 844 and d_4-DB 844 were eluted at room temperature using a Bonus RP 2.1 x 50 mm 3.5 µm analytical column. The flow rate was 500 µl/min.

Initial gradient conditions of 90% solvent A were held for 60 s. During this time, the column eluent was diverted from the mass spectrometer to waste. After these initial 60 s, the column eluate was directed to the mass spectrometer and the amount of solvent B (100% methanol) was increased linearly during 120 s until it reached 90%. The mobile phase composition was held constant (10 : 90, solvent A : solvent B) for the following 60 s. Initial conditions were reintroduced in a linear fashion over the next 60 s.

Samples were kept chilled (6º C) in covered 96-well plates with sample inserts. Injection volume was 5 µl. After each injection, the syringe was washed once with wash solvent 1 (50/50 methanol and water (v/v)) and wash solvent 2 (100% methanol). The injector valve and loop were washed three times with wash solvent 1 and three times with wash solvent 2.

Mass spectrometric conditions (user controlled voltages, gas pressures, and source temperature) were optimised for the maximum detection of either DB 289 and d_8-DB 289 or DB 844 and d_4-DB 844 using direct infusion of each compound in the manual tuning mode of Analyst 1.3.

solvents:

Solvent A: 10 mM ammonium acetate, 90% water (HPLC quality), 10% propanol

Solvent B: 100% Methanol

gradient:

% of A	% of B	time
90	10	0 – 1 min
10	90	3 – 4 min
90	10	4 – 5 min

flow rate: 0.5 ml/min.

2.5.2. Iontrap MS

Concentrations of the active compounds DB 75, DB 820 respectively were determined using reversed-phase HPLC with an HP API-ESI LC/MSD system (Palo Alto, CA, USA). The analytical unit consisted of an Agilent 1100 equipped with a binary pump (Palo Alto, CA, USA). Control of the equipment and calculation of the data was done using ChemStation Software (Agilent, Palo Alto, CA, USA).

The compounds were eluted through an Agilent Zorbax SB-CN column (2.1x 50 mm 5 µm particle) fitted with a guard column (Agilent 300SB-C3 2.1x 12.5 mm) holding a flow rate of 0.4 ml/min. Initial conditions with 25% solvent B was held for 5 min. Within 0.1 min the solvent B was raised to 100% and held up to 6 min. Then the gradient changes to 0% B immediately and continue into post-run time for 4 min (see table below). Selected ion monitoring (SIM) parameters were set as shown in the table below using the positive ion mode. Samples were kept chilled. Injection volume was 5 µl.

SIM Ion	Fragmentor	Gain EMV	SIM Resol.	Actual Dwell
305	150	1.0	62	62
306	150		62	62
310	150		15	15
313	150		15	15

solvents:

Solvent A: 5% acetonitrile, 95% water (HPLC quality), 0.025% TFA

Solvent B: 5% water (HPLC quality), 95% acetonitrile, 0.025% TFA

gradient:

% of A	% of B	time
75	25	0 – 5 min
	100	5.0 – 5.1 min
	100	5.1 – 6 min
		post run – 10 min

flow rate: 0.4 ml/min.

2.6. Data analysis

2.6.1. Prodrugs

Data acquisition was performed using multiple reaction monitoring *m/z* parent → *m/z* product of [DB 289+H] (365→334) and [d_8-DB 289+H] (373→342) or [DB 844+H] (366→319), and [d_4-DB 844+H] (370→323). Post-acquisition quantitative analyses were performed using Analyst 1.3.1. software.

Unknown sample concentrations of DB 289 were calculated from the weighted (1/x) quadratic curve, determined by the least-squares regression, constructed from the peak area ratios of DB 289 to d_8-DB 289, versus DB 289 concentration.

Unknown sample concentrations of DB 844 were calculated in a similar way using the weighted regression of the calibration curve constructed from the peak area ratios of DB 844 and d_4-DB 844.

2.6.2. Active compounds

Molecular ions of DB 75, DB 820 and their isotopic standards were monitored to determine the concentrations of the active compounds.

Concentrations of drugs were determined by matching peak area responses against a calibration curve of peak area responses of the internal standard.

Peak detection, peak area integration, peak area ratio calculation, calibration curve fitting (least-squares regression with weighting) and calculation of sample concentrations were carried out with ChemStation software.

The internal standard corrected for variation in the sample preparation steps used in the analysis of the plasma samples for the prodrugs as for the active compounds.

Data is represented as average concentration of plasma samples ± standard deviation obtained from three mice.

2.7. Determination of pharmacokinetic parameters

Values for C_{max}, t_{max} were obtained by visual analysis of the graphs.
To determine the AUC, AUMC, MRT, $t_{1/2}$ and k_{el} EXCEL software was used: AUC was calculated using the linear trapezoidal method. To determine the AUMC, the AUC is weighted by the integration over time. MRT is evaluated by dividing AUMC/AUC, $t_{1/2}$ =ln2*MRT, and k_{el} =1/MRT.

3. Results

3.1. Comparison of plasma levels in infected and uninfected mice

To estimate whether an infection with a parasite alters the plasma levels of a diamidine compound administered intraperitoneally or the diamidine formed from the prodrug administered orally, the same dose of drug or prodrug per body weight was applied to mice without and with a parasite load of 10^5 to 10^6 trypanosomes per ml. The concentration of drug in plasma was measured after appropriate preparation with HPLC/MS/MS techniques at specific times. Plasma courses of two active compounds, namely DB 75, DB 820, and of their corresponding prodrugs (DB 289, DB 844) were determined.

Pharmacokinetic profiles of active compounds were plotted in graphs from 0.25 hr up to 8 hr. Since only low amounts of drug could be detected after 8 hr, these values were neglected when representing the data graphically.

3.1.1. Active compounds

DB 75 in plasma of uninfected and STIB 900-infected mice after i.p. application (Fig. 1)

A slight difference in plasma levels and AUC could be observed between infected and uninfected animals. In both cases, the plasma level rose very rapidly, representing a very fast uptake of the drug from the abdominal cavity into the blood. The highest concentration could be measured at the first time, 15 min after application.

In infected animals a slightly higher C_{max}, AUC and therefore a slightly higher MRT and $t_{1/2}$ and a slightly lower k_{el} value were observed (see Chapter 10, Appendix B, Table 1).
The AUMC was 46% higher in infected as in uninfected animals (Chapter 10, Appendix B, Table 1).

DB 820 in plasma of uninfected and STIB 900-infected mice after i.p. application (Fig. 2)

This diamidine also showed a rapid uptake. Differences in plasma levels were detectable at 15 min and up to 2 hr after application with higher plasma levels and a less rapid elimination in infected mice. Highest drug concentration was found after 15 min whether an infection was present or not. At this time, around 30% more drug was found in the plasma of infected animals. The AUC showed a remarkable increase whereas the AUMC displayed only a slight rise (Chapter 10, Appendix B, Table 2).

3.1.2. Prodrugs

DB 289 in plasma of uninfected and STIB 900-infected mice after p.o. application (Fig. 3)

Plasma levels in the uninfected animals were higher than in the infected animals. In infected mice two plasma peaks at 2 and 6 hr after drug application were clearly noticeable. Quite contrary to the time-concentration curve for uninfected animals: the highest drug concentration (twice the amount as measured in infected animals) could be detected at the same time when the minimum between the 2 peaks at hour 4 in the infected occurred. The AUC and the AUMC over 24 h was nearly twice as high in the uninfected as in the infected animal. MRT, $t_{1/2}$ and k_{el} were comparable (Chapter 10, Appendix B, Table 3).

DB 844 in plasma of uninfected and STIB 900-infected mice after p.o. application (Fig. 4)

Plasma curves of infected and uninfected animals were almost identical. Both show 2 peaks at 1 and 8 hr after application. Because the time points were defined before the drug levels were determined it could not be assessed whether the drug level would still increase after 8 hr. Due to the missing time points between 8 and 24 hr the curve could have a course differing from Fig. 4.
A slight difference in AUC and AUMC was detectable (Chapter 10, Appendix B, Table 4).

3.2. Comparison of the conversion of the prodrug to the active drug

Conversion of DB 289 to DB 75 in uninfected (Fig. 5) and STIB 900-infected (Fig. 6) mice after p.o. application of DB 289

In uninfected mice on average around one third of the prodrug was converted to the active compound within 24 hr, whereas in the infected mice the amount of active compound within the same period of time constituted only one fourth of the administered prodrug. After 24 hr, more DB 75 than DB 289 was detectable in the blood in either case.

Conversion of DB 844 to DB 820 in uninfected (Fig. 7) and STIB 900-infected (Fig. 8) mice after p.o. application of DB 844

Comparing the plasma levels within 24 hr, on average 14% of the prodrug was converted to the active compound in infected mice. Slightly less, on average 10%, was activated in uninfected mice.

Converted DB 75 in uninfected vs. STIB 900-infected mice after orally administered DB 289 (Fig. 9)

DB 75 levels were determined after oral DB 289 application. The infected animals showed a lower drug level over the whole assessment period than the uninfected animals. Two weak peaks after 4 and 6 hr were identifiable in infected mice, whereas the uninfected animals reached their highest plasma concentration after 4 hr and no second peak could be detected. This pattern was already observed when DB 289 levels were measured. There too, the time point with the highest drug concentration between infected and uninfected animals differed and the number of peaks within the plasma course were not the same.
AUC and AUMC were around 30% lower in the infected animal (Chapter 10, Appendix B, Table 5).

Converted DB 820 in uninfected vs. STIB 900-infected mice after orally administered DB 844 (Fig 10)

Plasma curves of either infected and uninfected animals showed a similar pattern. No difference between both courses was clearly detectable: t_{max} occurred at the same time, AUC, AUMC, MRT, $t_{1/2}$, and k_{el} were within the same range (Chapter 10, Appendix B, Table 6). There was a not significant trend to higher values in the infected mice.

3.3. Comparison of analogues

This analysis was performed to assess differences in the pharmacokinetic parameters when the structure of the compound is modified slightly and therefore changes in the physical-chemical properties are obtained. Knowledge gained from this part of the study is of major importance for further drug development.

3.3.1. Active compounds

DB 75 vs. DB 820 in plasma of uninfected mice after i.p. application (Fig. 11)

Uptake of drug took place to a higher extent within the first 15 min if DB 820 was applied. From 30 min on, already, plasma concentration in DB 75 and DB 820 treated mice were comparable. Other pharmacokinetic parameters like AUMC, MRT, $t_{1/2}$ and k_{el} differed slightly, whereas no difference in AUC could be assessed (Chapter 10, Appendix B, Table 7).

DB 75 vs. DB 820 in plasma of STIB 900-infected mice after i.p. application (Fig. 12)

Also, in infected mice a higher amount of drug available in the plasma was obtainable within the first 15 min after DB 820 administration. Over a period of 24 hr DB 75 was eliminated slightly slower resulting in a higher plasma level after 24 hr compared to DB 820. The AUC of infected DB 820 treated mice was around 30% higher, whereas the AUMC (~ 30%), MRT, and $t_{1/2}$ was decreased by around 40% compared to DB 75. A difference in the k_{el} value clearly was detectable, as it was 80% higher in DB 820 treated animals (Chapter 10, Appendix B, Table 8).

3.3.2. Prodrugs

DB 289 vs. DB 844 in plasma of uninfected mice after p.o. application (Fig. 13)

In general, DB 844 drug levels were higher than levels of DB 289, except at the trough at 4 hr between the two peaks (at 1 and 8 hr). At this time the DB 289 course showed the highest drug concentration over the observed 24 hr period. Interestingly, the times where the C_{max} was detected differed completely: 1 hr for DB 844 and 4 hr for DB 289. Comparing the pharmacokinetic parameters, the highest measured plasma level was 4,5 times higher in DB 844, which was reflected in an AUC and an AUMC 3,5 times higher. No differences were detectable in MRT, $t_{1/2}$ and k_{el} (Chapter 10, Appendix B, Table 9).

DB 289 vs. DB 844 in plasma of STIB 900-infected mice (Fig. 14)

During the whole observation period, except at the 6th hr, clearly more drug could be detected after DB 844 administration. The time of the highest measurable plasma level differed in the infected mice, too. Interestingly, a trough was observable in the DB 289 as well as in the DB 844 treated animals. Compared to the trough at hour 4 in the DB 289 treated animal the one in the DB 844 treated mice was delayed, occurring at hour 6.

An eight fold higher plasma level could be detected in DB 844 treated animals, what resulted in a five times higher AUC and AUMC. No differences in MRT, $t_{1/2}$ and k_{el} were assessable (Chapter 10, Appendix B, Table 10).

3.3.3. Converted analogues

Converted DB 75 vs. converted DB 820 in plasma of uninfected mice after prodrug application p.o. (Fig. 15)

No difference in levels of DB 75 and DB 820 were detected over a period of 24 hr after application of 100 mg/kg p.o. of the corresponding prodrug. Pharmacokinetics differed, since C_{max} did not occur at the same time (Chapter 10, Appendix B, Table 11).

Converted DB 75 vs. converted DB 820 in plasma of STIB 900-infected mice after prodrug application p.o. (Fig. 16)

Around 60% more converted DB 820 was available in infected mice compared to converted DB 75. Differences in the elimination process were not detectable. As described for uninfected animals, t_{max} differed clearly (Chapter 10, Appendix B, Table 12).

3.4. Comparison i.p. – s.c. application in uninfected animals

Plasma levels of drugs applied via i.p. and s.c. route were determined to show the difference of the retarding effect based on different application routes.

DB 75 in plasma of uninfected mice i.p. vs. s.c. application (Fig. 17)

In both cases the C_{max} was achieved at 15 min, the first time point. In general, plasma levels after subcutaneous administration were higher than after intraperitoneal application. The difference between the C_{max} was around 30%. AUC and AUMC were 3 times higher after subcutaneous administration (Chapter 10, Appendix B, Table 13).

DB 820 in plasma of uninfected mice i.p. vs. s.c. application (Fig. 18)

Clearly higher plasma levels could be observed after subcutaneous injection of DB 820 compared to its intraperitoneal application. Highest measured level of DB 820 s.c. was about two times the i.p. value. Also AUC and AUMC were 4 to 5 times higher, whereas no difference in MRT, $t_{1/2}$ and k_{el} was observed (Chapter 10, Appendix B, Table 14).

DB 75 s.c. vs. DB 820 s.c. in plasma of uninfected mice (Fig. 19)

Within the observation period of 24 hr plasma levels and AUMC of DB 820 were always higher as compared to the ones of DB 75. The AUCs differed more than 60%, and the AUMC in DB 820 was around 40% higher. MRT, $t_{1/2}$ and k_{el} were within the same range (Chapter 10, Appendix B, Table 15).

4. Discussion

Pharmacokinetics helps to understand the dynamic movement of the drug within the body. It characterises the relationship of the drug concentration to dose, time and characteristics of the individual body to which the drug was applied. So, the optimal dosage -efficient but not yet toxic- can be determined. From pharmacokinetic parameters quantitative information about the disposition of drug can be gained, but no answer is obtained in the way the drug acts and how the body responds to the drug.

Important processes in pharmacokinetics are the adsorption of the drug from the application site, the distribution within the body, its metabolism concerning the transformation of the drug and its excretion. Key parameters to describe the fate of the drug in the body are the half-life ($t_{1/2}$), the AUC, C_{max} and t_{max}.

In this study C_{max}, t_{max}, AUC, AUMC, MRT, $t_{1/2}$ and k_{el} were calculated from measured plasma levels. The plot of the plasma drug concentration versus time represents its pharmacokinetic profile. The highest plasma level observed within the assessment period is represented by the peak concentration C_{max} at the time t_{max}. The extent of drug availability and exposure following a dose is reflected by area under the curve (AUC). This is a measurement for the total amount of drug absorbed. By plotting concentration-time versus time the area under the first moment curve (AUMC) is obtained. The ratio of AUMC and AUC results in the mean residence time, MRT. This value represents how long in average molecules of the applied compound stay in the body without being modified. This constant is rather not a pharmacokinetic constant than a constant for the in vivo liberation of the drug from its matrix.

Parameters which are useful to describe the elimination of the drug are rate constant of elimination (k_{el}) and the half-life ($t_{1/2}$), the time which is required to reduce the amount of drug in the plasma by half. This parameter is generally used to determine dosing intervals of drugs.

Method:
Two different methods of preparing the plasma samples were used to analyse the concentration in the plasma samples due to the different properties of the drugs to dissolve in liquids. Both methods have in common that the proteins of the plasma were precipitated either with acetonitrile or with acidic methanol and the compounds in the supernatant were measured after a conditioning process with HPLC/MS/MS techniques. Since a valid analytic method for the determination of the active compound was not yet established for the triple quadrupole MS, plasma samples were assessed using iontrap MS.

The pharmacokinetic profiles represent the result of all the processes considering the fate of a drug in the body from its absorption to its elimination. Different effects contribute to the observed courses. The following discussion is an attempt to explain these courses.

4.1. Plasma levels of infected and uninfected mice

Active drugs

DB 75 treatment yielded slightly higher plasma concentrations in infected than uninfected animals. Plasma levels in infected and DB 820 treated mice were higher than in their uninfected counterparts during the first two hours after application, whereas afterwards no difference between uninfected and infected plasma levels could be seen.
Accumulation of active compounds in trypanosomes is well known for DB 75 as well as for DB 820. Like pentamidine, transport of diamidines is mediated via a saturable P2 transporter. In vitro experiments showed an inverse proportional relationship between the drug level outside of the trypanosome and the accumulation factor. E.g., in vitro an average accumulation factor of around 100 000-fold for DB 75 after 4 hr incubation time with compound ranging from 250 ng/ml to 10000 ng/ml could be determined. In vivo an accumulation factor of around 57000 for DB 75 and around 38000 for DB 820 was found in trypanosomes (strain S427, *T. b. brucei*) after treatment of mice with 7.5 µmol/kg i.v. after

24 hours (corresponding to 2.8 mg/kg DB 75 dihydrochloride and 3.68 mg/kg DB 820 diacetate) [Mathis M. A., personal communication].

Interestingly, the effect of this accumulation on plasma concentrations could not be observed in either case. Probably, the absorption processes were so extensive and therefore effects of the accumulation processes in trypanosomes on plasma concentrations simply are not visible.

Another issue that has to be taken into account is, that infection inhibits metabolism. Shertzer H. G. et al. reported a reduced capability of host mixed-function oxidases to metabolise drugs during infections [Shertzer, H.G. (1981)]. By decreasing the rate of metabolism, drugs are transformed to a lesser extent and so plasma levels decline slower. The inhibition could be observed in the elimination process of DB 75 in infected animals showing a prolongation, which was not found for DB 820. A possible explanation could be that this effect is just not detectable in the case of DB 820, because other effects like an accelerated elimination process are stronger. It can be assumed that the elimination process will be accelerated similarly to the adsorption process. In the same matter as the drug is absorbed quickly from the application site, it also will be absorbed quickly from the excretory organs and eliminated rapidly.

Prodrugs

In the case of DB 289 approximately twice the amount of prodrug (represented by the AUC) was available over the whole observation period if animals were uninfected. Interestingly, the pharmacokinetic profile looked completely different, and plasma peaks occurred at totally different times when uninfected mice were compared to infected mice. The reason for the change in the pharmacokinetic profile after DB 289 treatment is not known.
After DB 844 treatment neither a different pharmacokinetic profile nor differences in the occurrence of plasma peaks could be detected. However, a small surplus in the total amount of drug detected was also assessable in uninfected animals.
Accumulation processes may be the reason for lower plasma levels in infected animals. However, accumulation of the prodrug itself in trypanosomes is rather inconsiderable, since prodrugs are not known to be concentrated within the parasite. Since a massive accumulation of active compound after low dose treatment was observed in trypanosomes in vivo and in vitro, the plasma concentrations of the prodrug will be reduced by shifting the balance of the

transformation process from the prodrug to the active compound, and therefore lower plasma levels of the original prodrug are obtained.

Since uptake of DB 820 by trypanosomes was observed to be less excessive than uptake of DB 75, one would expect differences also– but to a lesser extent- in the plasma concentrations of DB 844. Indeed, this difference was detectable, but not as big as expected. The reason for this rather unexpected small difference may be the inhibition of the metabolism resulting in a higher amount of the original, non-metabolised drug.

Transformation of the prodrug to the active drug

In general, the ratio of active compound to non-converted prodrug differed obviously between the analogues. One third to one fourth was metabolised from DB 289 to DB 75, whereas only an inconsiderable fraction was metabolised from DB 844 to DB 820. However, more DB 844 compared to DB 289 was found in the plasma.

After treatment with DB 289 the total amount of DB 75 in the blood was around one third smaller in infected as compared to uninfected mice, whereas no significant difference of DB 820 levels after DB 844 treatment between infected and uninfected animals could be detected.

There is evidence that infection lowered the plasma level of DB 75 in infected DB 289-treated animals by accumulation of the converted DB 75 in trypanosomes. Plasma concentrations of DB 820 after DB 844 treatment were more or less the same as in infected animals showing a trend to higher levels of active drug in infected mice. A better uptake of DB 844 compared to DB 289 from the application site was observed, which led to the expectation that an increased conversion of DB 844 molecules to DB 820 would take place and accumulation of the active compound in the trypanosome would occur. Interestingly, only a trend to higher plasma levels of DB 820 but no significant elevated conversion of DB 844 was observed as the plasma concentrations in uninfected mice show (Fig. 10). Additionally, it must be considered that the accumulation factor for DB 820 is lower than for DB 75, and will decrease additionally, when the concentration of drug increases.

Here again, the inhibition of metabolism must make a considerable contribution to the plasma concentrations of DB 820 in infected mice, otherwise bigger differences would be expected.

Analogues

Modifications of drug structures result in changes of physicochemical properties of the original compounds. By using ACD Scholar software, predictions about distribution and membrane permeability can be done. This program is able to estimate the partition coefficient (P, respectively log P) and the distribution coefficient (D, respectively log D) at different pH values based on the chemical structure. This program uses partial structures of the molecule to estimate these values. Good correlation between prediction and actual measured values were obtained for neutral drugs, but not for drugs like diamidines showing high pK_as. Recently, Saulter determined pK_a, log P and log D values for DB 75, DB 820, DB 289 and DB 844 [Saulter, J.Y. (2005)].

In general, a drug should be hydrophilic enough to get distributed by the bloodstream and also lipophilic enough to pass membranes. Too lipophilic drugs stick to membranes and are not able cross to them. Depending on the compartment in which a drug should act, different requirements have to be met. To treat second stage trypanosomosis penetration of the blood brain barrier would be essential.

Optimal log P values differ from organ to organ and from application route to application route. Assuming passive adsorption, best log P values to achieve CNS penetration are around 2 ± 0.7. For excellent oral absorption log P should be around 1.8 [www.raell.demon.co.uk/chem/logp/logpka.html].

Active drugs

Except for the first 15 min, plasma levels of DB 75 and DB 820 treated mice were comparable in uninfected animals. Infection created a difference between the two drugs. Up to 1 hr DB 820 treated animals displayed higher levels of the diamidine. After this period, elimination of DB 75 occurred less rapidly than in the DB 820 treated mice, resulting in higher drug levels. In vivo plasma levels above 250 nM DB 75 and above 180 nM were measured during the 24 hr observation period. In vitro data showed that the IC_{50} for DB 75 is 4 nm and 7 nM for DB 820.

Physicochemical properties of DB 820, a slightly more polar molecule, seemed to favour adsorption processes. Better membrane permeability could be observed during the absorption process from the application site into the blood, as well as in the membrane passage into excretory organs, as is indicated by the amount of absorbed drug and by the slopes of the curves.

Prodrugs

Pharmacokinetic profiles of DB 289 and DB 844 looked quite similar. Except for DB 289 in uninfected mice, all pharmacokinetic profiles showed two peaks and a minimum in between. These highs and this minimum did not correspond chronologically at all when DB 289 was compared with DB 844. Additionally, the ratio of the first to the second peak differed clearly: after DB 289 treatment both peaks were more or less of equal height, whereas in the case of DB 844 the first peak dominated.

The absorption of DB 844 compared to DB 289 was favoured by far, independent of the infection status. Differences in the physicochemical properties may be the reason for different pharmacokinetic profiles, showing peaks and the minimum not at the same time.

Converted active drugs

Since no difference was detectable in the amount of converted drug in uninfected animals, it was concluded that the converting enzyme(s) did not favour the structure of one prodrug over the other. In infected animals, higher levels of DB 820 were obtained, possibly because of the better accumulation of DB 75 in the parasites (higher accumulation factor) and/or because inhibition of metabolism was stronger by DB 844, DB 820 respectively.

Application routes: i.p. vs. s.c.

Both routes of administration, i.p. and s.c., had a certain slow release effect. Drug was stored at the application site and released slowly from there. In either case, DB 75 and DB 820, more drug was released via the s.c. route and plasma levels stayed longer on a higher level. This effect was based only on the site of application. The formulation of the drug was equal referring to the slow release effect. The pharmacokinetic profiles showed a dominant absorption particularly within the first 2 hr for DB 844 compared to DB 289 regardless of the route of administration.

5. Conclusion

The graphs representing the pharmacokinetic profile of each compound showed a high variability. The reason for this variability originates from the non-serial sampling method in mice which had to be used to perform this experiment. The big differences concerning the

standard deviation represent the inter-individual variability. Each mouse had a different genetic background as well as polymorphisms of single genes may occur.
Different factors are involved from the point when a drug is applied to the body till its excretion: below this factors will be discussed in a more general matter:

From in vitro experiments DB 75 was expected to be slightly more potent with an IC_{50} of 1.7 ng/ml as compared to DB 820 with an IC_{50} of 3.3 ng/ml. Unexpectedly, in vivo DB 820, DB 844 respectively, seemed to be more active than DB 75 or DB 289. In the acute mouse model 4 x 20 mg/kg DB 820 i.p. cured 4 out of 4 mice, after DB 75 application 0/4 mice were cured at the same dose, but the mean survival time was prolonged by at least 44 days compared to control mice. In the chronic model a cure of 5 out of 5 mice was achieved with 6 x 100 mg/kg DB 844 p.o., whereas after application of the same dose of DB 289 only 3 out of 5 mice could be cured. The reason for this discrepancy in vitro versus in vivo must be a better availability of the compound within the mouse.

5.1. Uptake of drug

5.1.1. Adsorption of drug from the application site

Transport proteins, like P-glycoprotein, are not yet known to transport diamidines from the abdominal cavity or the lumen of the intestine into the blood. So passive diffusion plays an important role in drug uptake. Membrane passage is a major obstacle for drugs to get to the site where they can act. First of all, membranes have to be crossed for the drugs to be distributed within the body by the blood stream. Therefore, physicochemical properties of compounds may be a limiting factor. As mentioned above, log P values characterise the distribution of a compound as neutral molecule between an immiscible solvent and an aqueous phase. Ideal log P values for penetrating the intestinal membrane are around 1.35. Calculated log P values for DB 75 and DB 820 are 2.681 and 1.2 ± 0.4, respectively.
For oral absorption best log P values are around 1.8. Therefore, DB 844 will cross membranes easier with a log P value of 3.2 compared to log P of 3.78 for DB 289. Taking these calculated values into account, it is quite easy to predict, which of those molecules will pass the blood brain barrier to a bigger extent. Optimum log P value for CNS penetration are around 2 ± 0.7. And indeed, DB 844 was observed to cross the blood brain barrier in a sufficient amount and therefore was able to cure second stage trypanosomosis in mice better than DB 289.

Different routes of passing membranes were observed in vitro experiments comparing DB 75 to DB 289. DB 75 was considered to be transported predominantly paracellular across a CACO-2-monolayer. This monolayer mimicks the human gastro-intestinal epithelium. Masking the diamidine group with methoxy moieties resulted in a higher uptake predominantly via a transcellular transport [Zhou, L. (2002)]. Transport of DB 820 and DB 844 is expected to behave in the same manner.

5.1.2. Transport of compound within the body

After crossing the membranes, a certain amount of the drug binds to plasma proteins. Basic drugs like diamidines are expected to bind on either lipoproteins or on alpha-1-acid glycoprotein (α-AGP). So far, it is not known to which plasma protein diamidines do bind. α-AGP belongs to the acute phase proteins which are known to be increased during an acute infection or inflammation [Piafsky, K.M. (1980)], [Baumann, H. (1994)]. It is assumed that a trypanosome infection increases the amount of α-AGP, and therefore higher plasma levels for DB 75 are expected in infected animals since the total amount of drug, free and bound, is estimated using this determination method.

Active drugs were bound to 70-75%, whereas protein binding of prodrugs was 99%. Whether prodrugs also bind to α-AGP and therefore plasma concentration would be higher in infected animals has to be clarified. It could also be possible that another protein which is not increased by an infection is involved in drug transport and thus no increase in plasma concentration would be detected.

5.2. Status of infection

5.2.1. Accumulation

Infection may influence the plasma concentration of active drugs, since trypanosomes are able to transport and store diamidines in their organism. The P2 transporter is one of the transporters who is considered to be involved in this process, besides LAPT (low-affinity pentamidine transporter) and HAPT (high-affinity pentamidine transporter) [de Koning, H.P. (2001)].

Accumulation factors were assessed in a similar mouse model for DB 75 and DB 820 in mice infected with trypanosome strain S427. With the aid of these values and the grade of parasitaemia determined microscopically by tail blood examination, it is possible to estimate the amount of accumulated drug in infected animals roughly. At the day the experiment was

performed two thirds of the mice showed a parasitaemia of 10^5 trypanosomes/ml and one third of the animals even had 10^6 trypanosomes/ml. Assuming an infection with 10^5 trypanosomes/ml, 57 nM of DB 75, respectively 38 nM of DB 820 per 100 nM DB measured could be withdrawn in infected animals compared to uninfected.

5.2.2. Inhibition of metabolism

Drug metabolism consists of two major steps performed by the liver. In a first step, molecules are made more hydrophilic by introduction or by liberation of functional groups with the aid of enzymes. Processes which take place within this step are oxidations, reductions or hydrolysis reactions. Hydrolysis often is catalysed by specific or unspecific esterases, whereas the other mentioned reactions are performed by mixed-function oxidases. These enzymes are called mixed-function oxidases, because one atom oxygen of the O_2 molecule is transferred to the substrate (in this case to the drug to metabolise) and the other is transferred to hydrogen atoms to produce water.

It could be shown that a present trypanosome infection was able to lower metabolism by decreasing the activity of those mixed-function oxidases. In addition, drugs used to treat trypanosomosis like melarsoprol or suramin were shown to inhibit these oxidases in vitro, too [Shertzer, H.G. (1981)]. If diamidines per se are also potent oxidase inhibitors has to be subject of further investigations. Taking the data of this study into consideration, one would expect that DB 820, DB 844, respectively exhibits a bigger inhibitory effect on metabolism than DB 75, DB 289 respectively.

The second phase of metabolism is the so called conjugation. Molecules obtained in Phase I get linked with highly polar, mostly inactive molecules for rapid elimination. Glucuronic acid is one of those polar molecules to which the activated Phase I molecules can bind. It is known, that DB 844, DB 820 and all intermediates are conjugated intensively during phase II metabolism and therefore are no longer available for the organism. This may explain the low rate of the converted active compound found after prodrug application. A certain amount of prodrug got lost through conjugation and was eliminated without being subject to metabolism.

5.2.3. Conversion of prodrugs to active compound

Differences in metabolism of the conversion DB 289 and DB 844 to their active compounds are expected. DB 844, as an asymmetric molecule, shows a more complex metabolism, since O-demethylation can occur on the methoxyamidine related to the phenyl or to the pyridyl

group first. Seven different phase I intermediates are obtained during the metabolism of DB 844, whereas only four different intermediates occur during the metabolism of DB 289 to DB 75 [see metabolic pathways of DB 289 (Fig. 20) and DB 844 (Fig. 21)].

To round up, pharmacokinetic profiles are the sum of complex interactions ranging from absorption processes, differences in physicochemical properties of the drug, status of infection associated with a possible accumulation of compounds in trypanosomes, inhibition of metabolism and differences in excretory processes.
In this study we could show that there is a difference between plasma levels in infected and uninfected mice.

Although accumulation of diamidines in trypanosomes may lower the plasma level, the inhibition of metabolism seems to be more relevant for plasma levels of the drugs analysed in this study. Probably, these compounds themselves show inhibitory effects on metabolism. When comparing two different compounds under the same conditions (route of application, status of infection) differences were obtained from the physical-chemical properties of the drugs and their influences.

Fig. 1: DB 75 in plasma of uninfected and STIB 900-infected mice after i.p. application

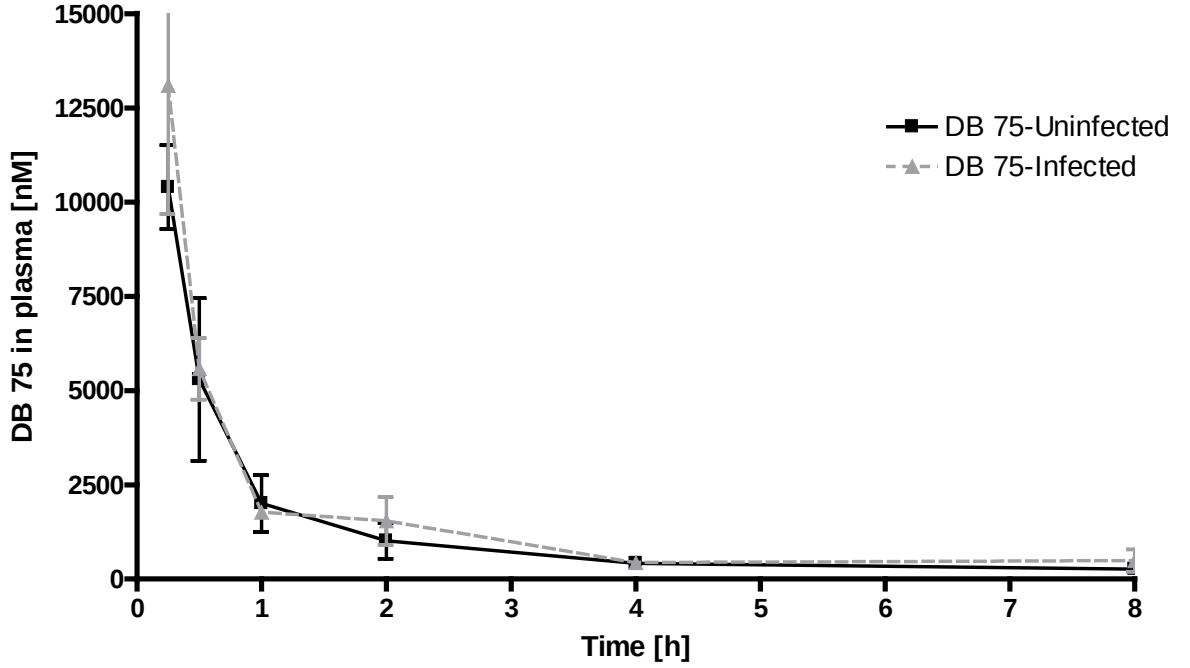


Fig. 2: DB 820 in plasma of uninfected and STIB 900-infected mice after i.p. application

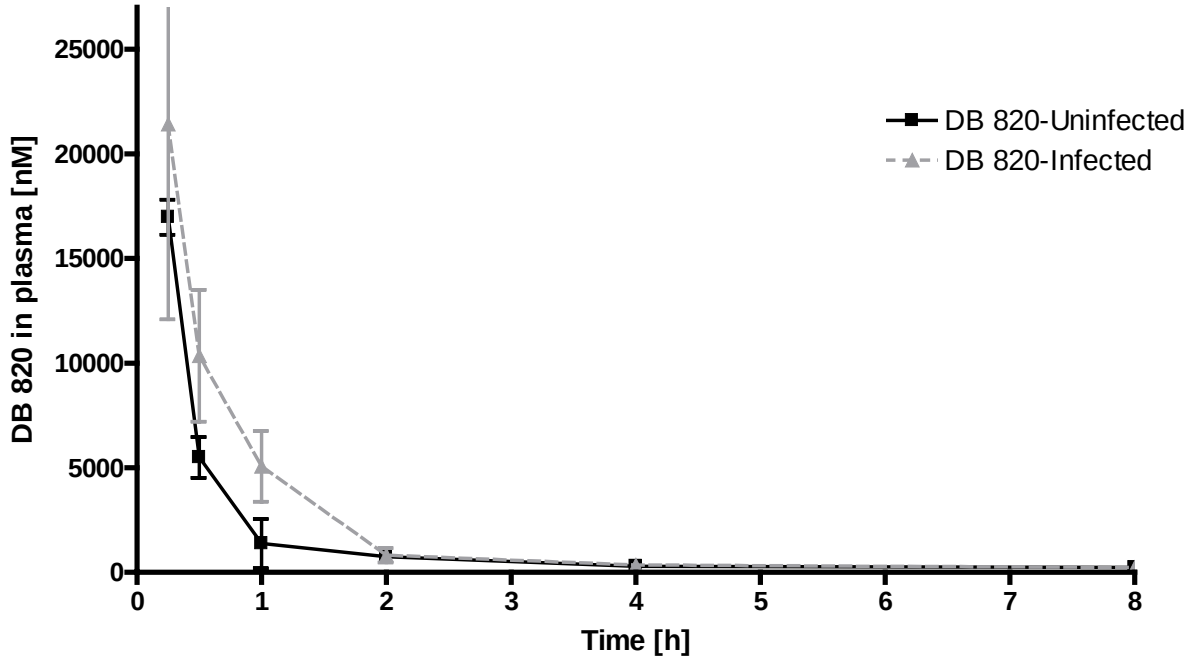


Fig. 3: DB 289 in plasma of uninfected and STIB 900-infected mice after p.o. application

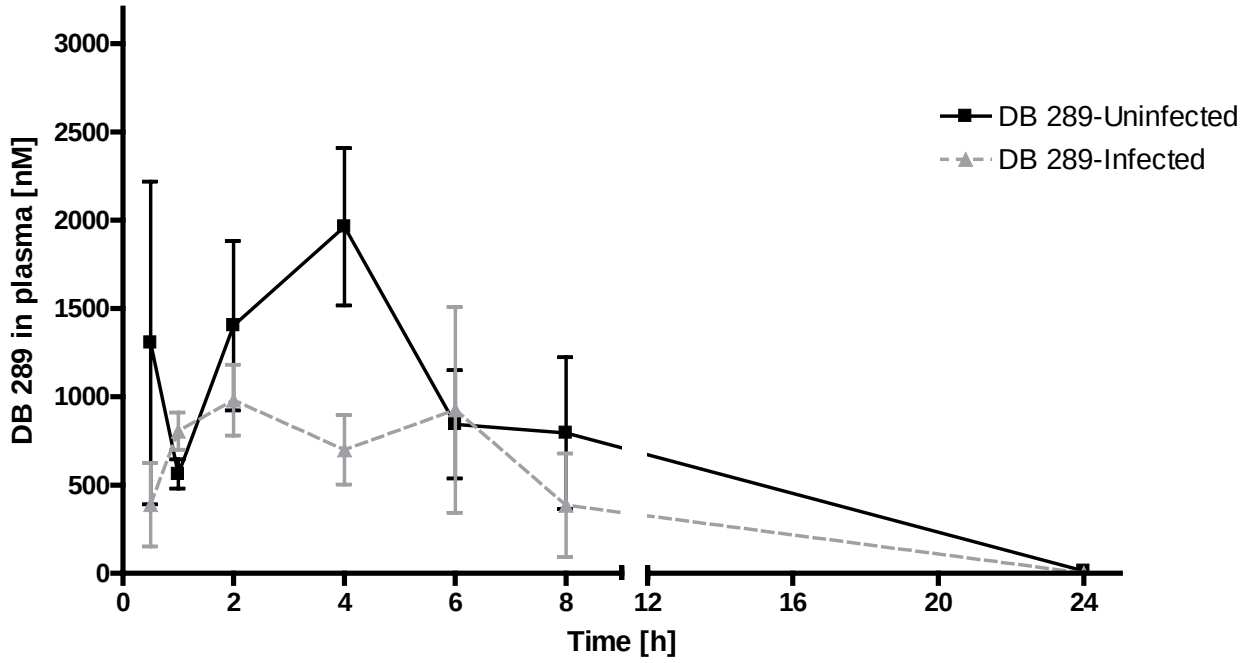


Fig. 4: DB 844 in plasma of uninfected and STIB 900-infected mice after p.o. application

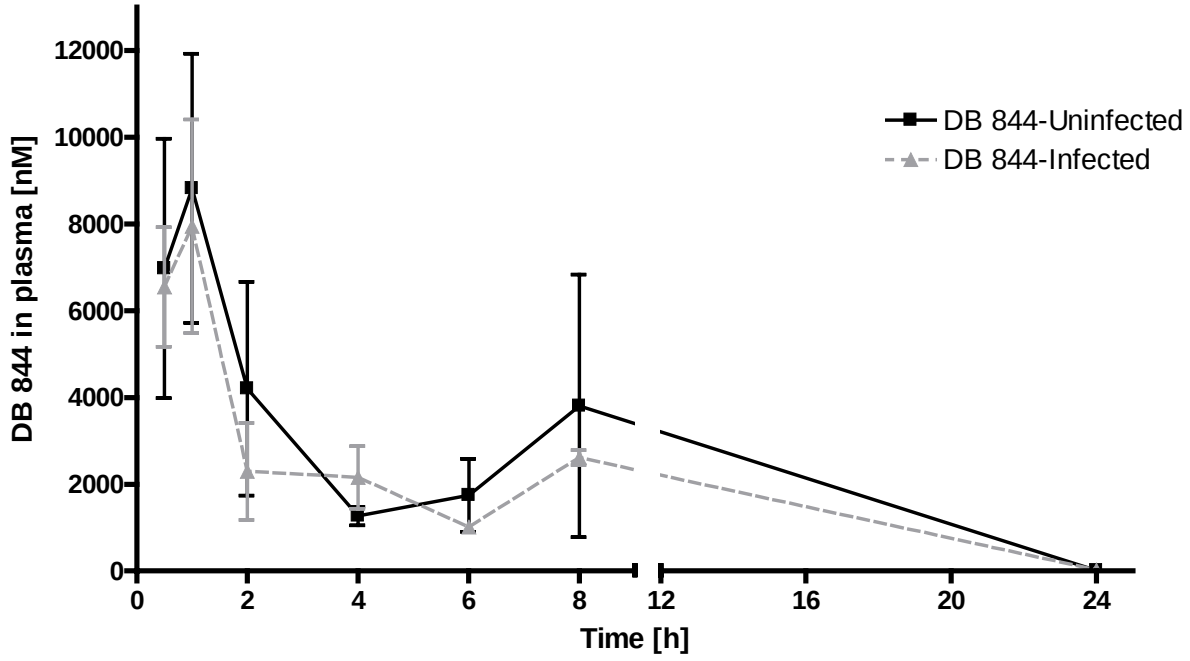


Fig. 5: Conversion of DB 289 to DB 75 in uninfected mice after p.o. application of DB 289

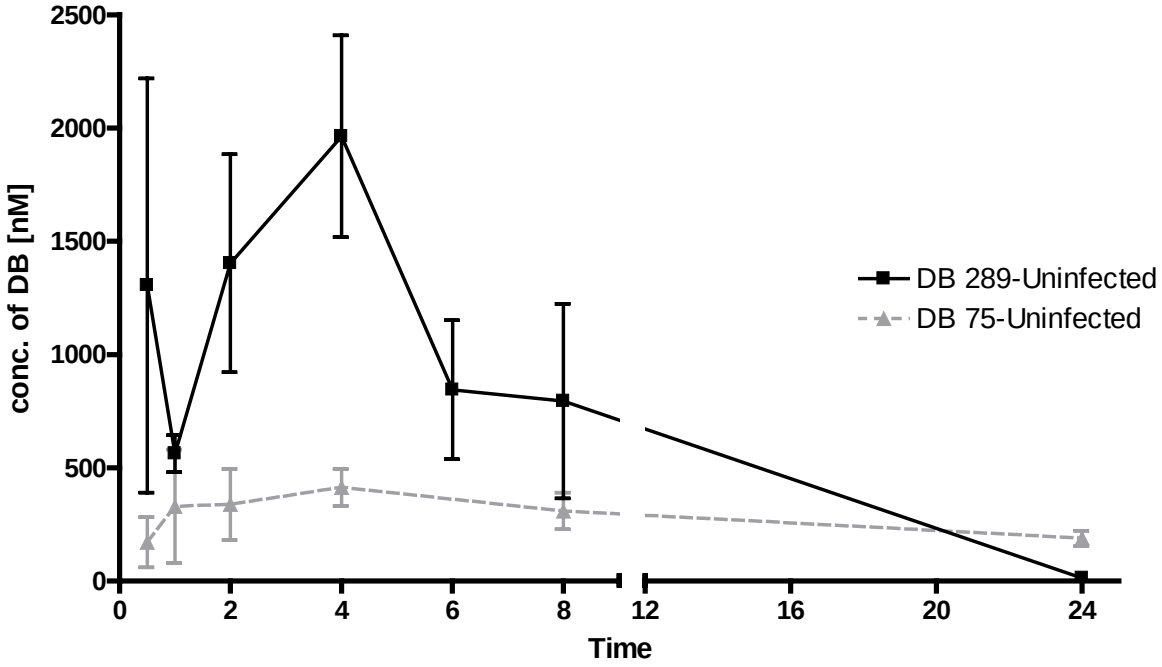


Fig. 6: Conversion of DB 289 to DB 75 in infected mice after p.o. application of DB 289

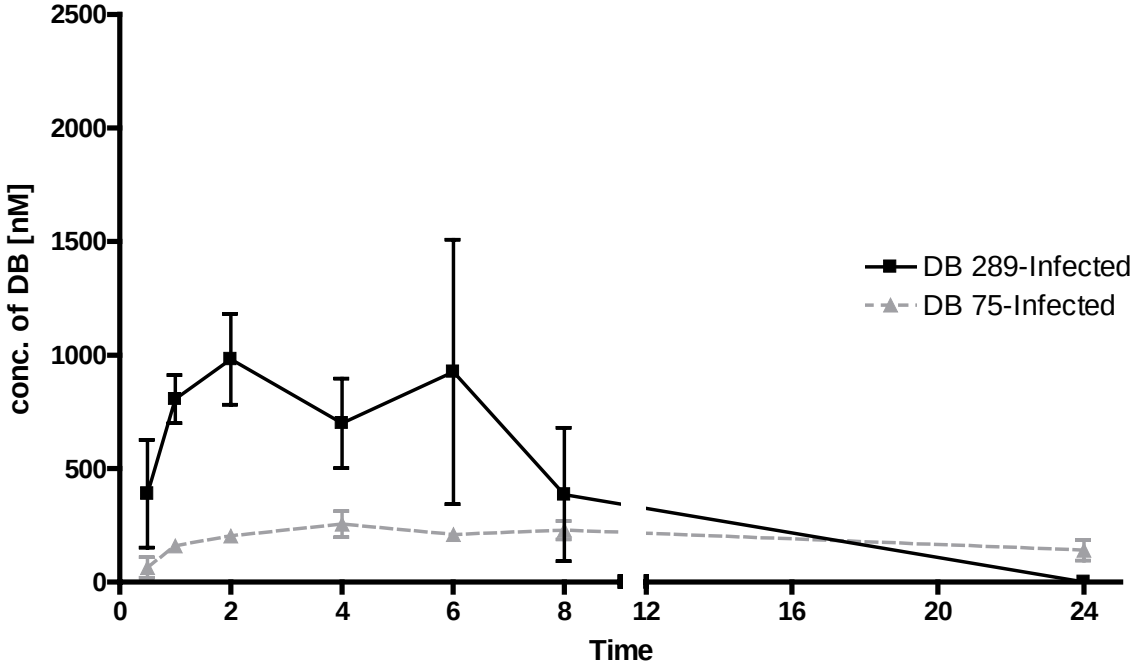


Fig. 7: Conversion of DB 844 to DB 820 in uninfected mice after p.o. application of DB 844

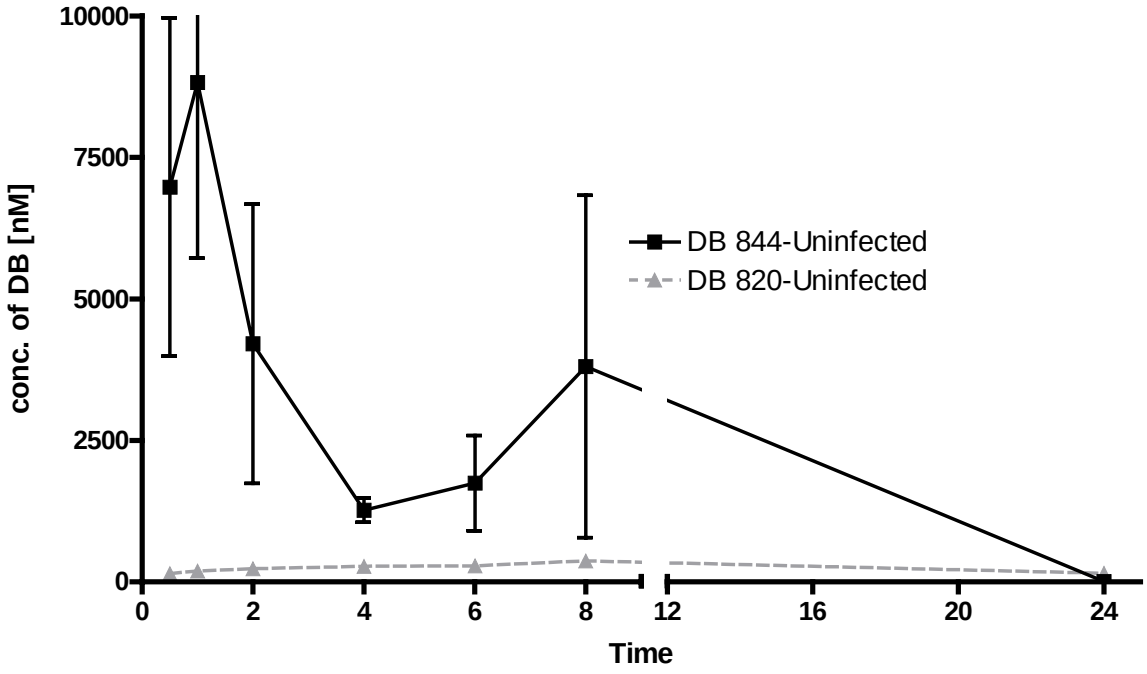


Fig. 8: Conversion of DB 844 to DB 820 in infected mice after p.o. application of DB 844

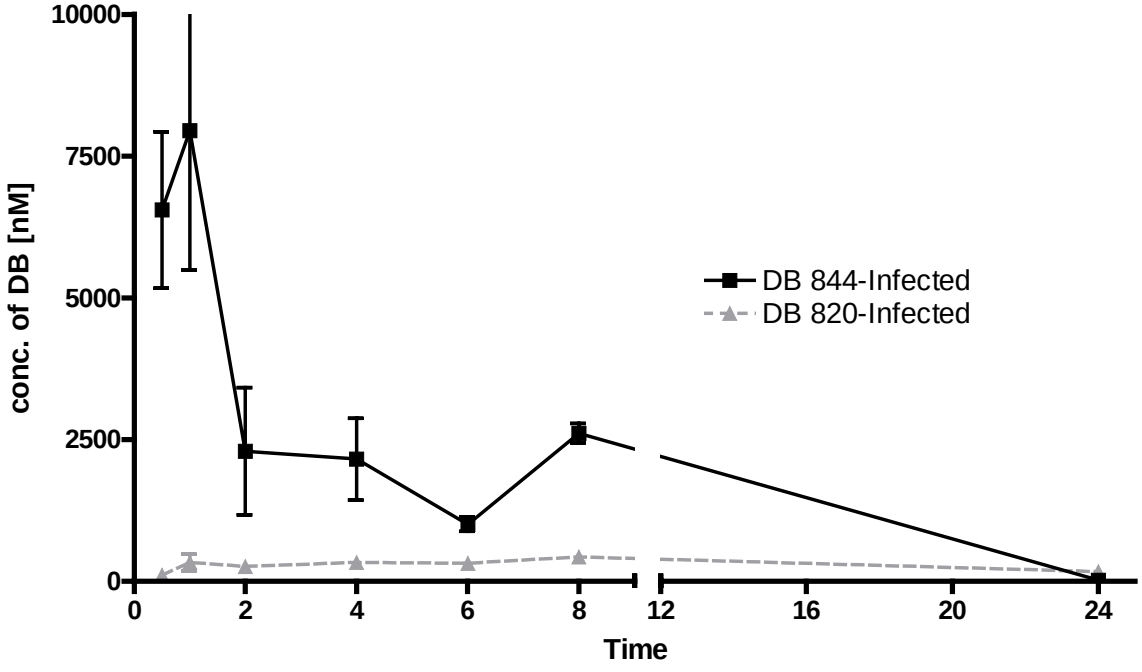


Fig. 9: DB 75 in plasma of uninfected and STIB 900-infected mice after treatment with DB 289 p.o.

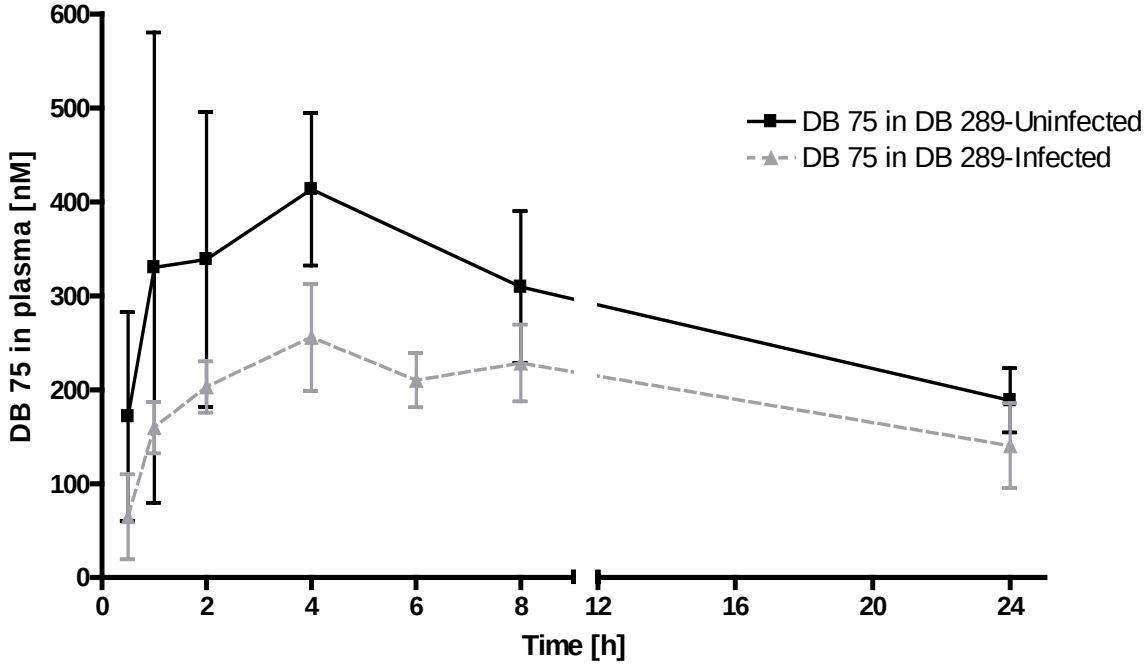


Fig. 10: DB 820 in plasma of uninfected and STIB 900-infected mice after treatment with DB 844 p.o.

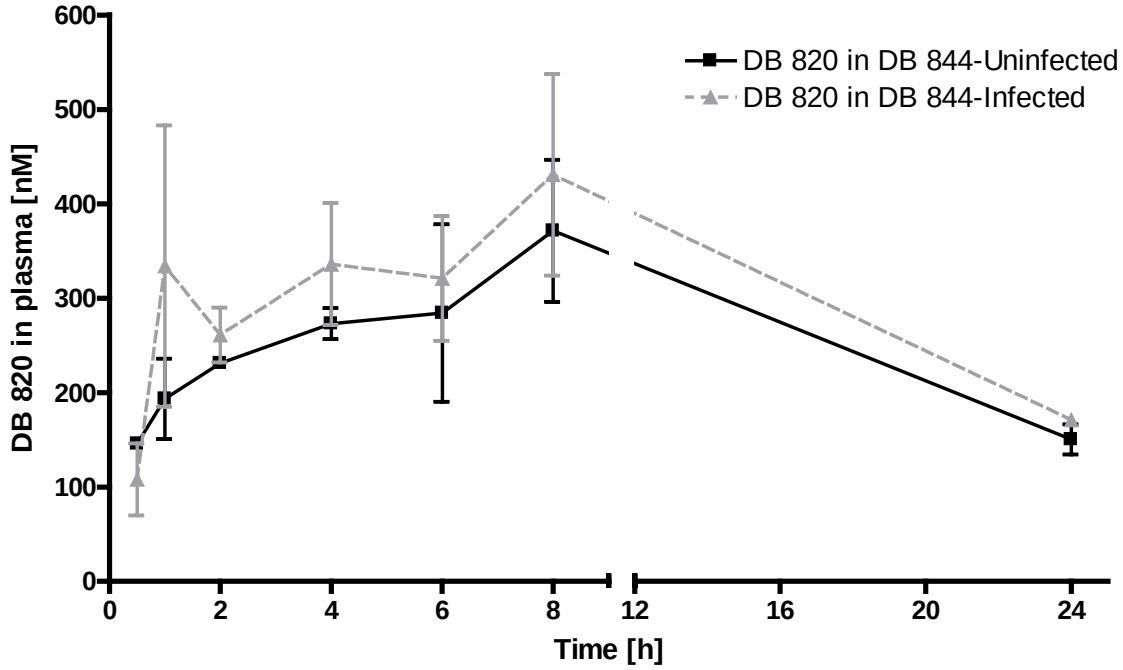


Fig. 11: DB 75 and DB 820 in plasma of uninfected mice after i.p. application

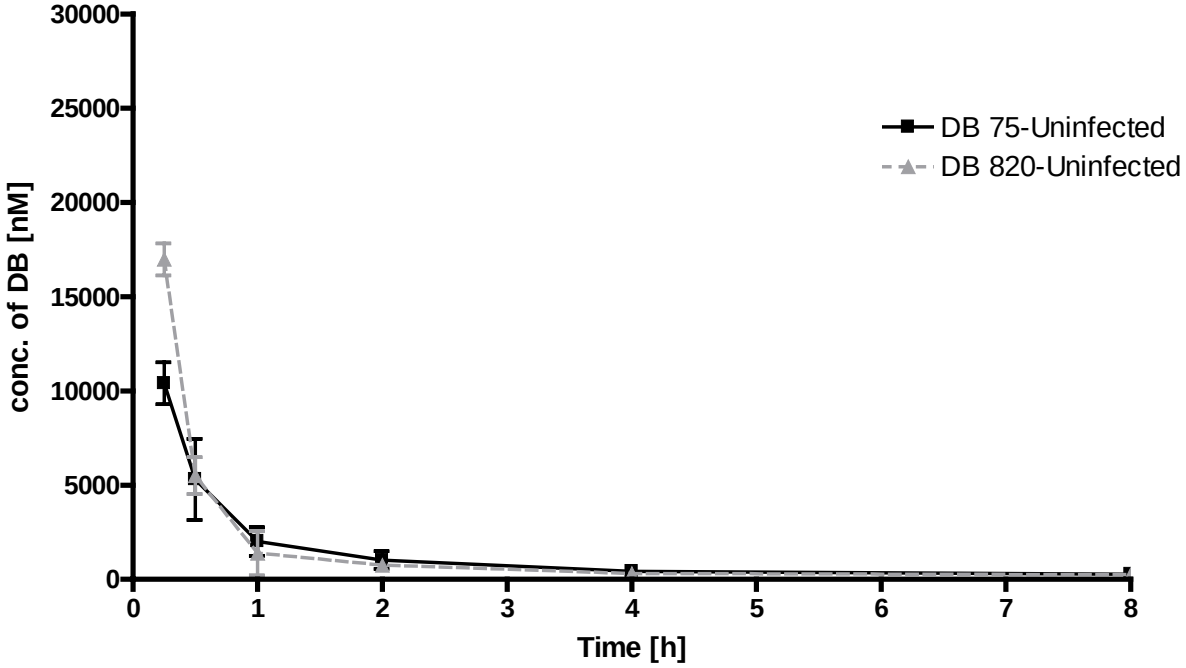


Fig. 12: DB 75 and DB 820 in plasma of STIB 900-infected mice after i.p. application

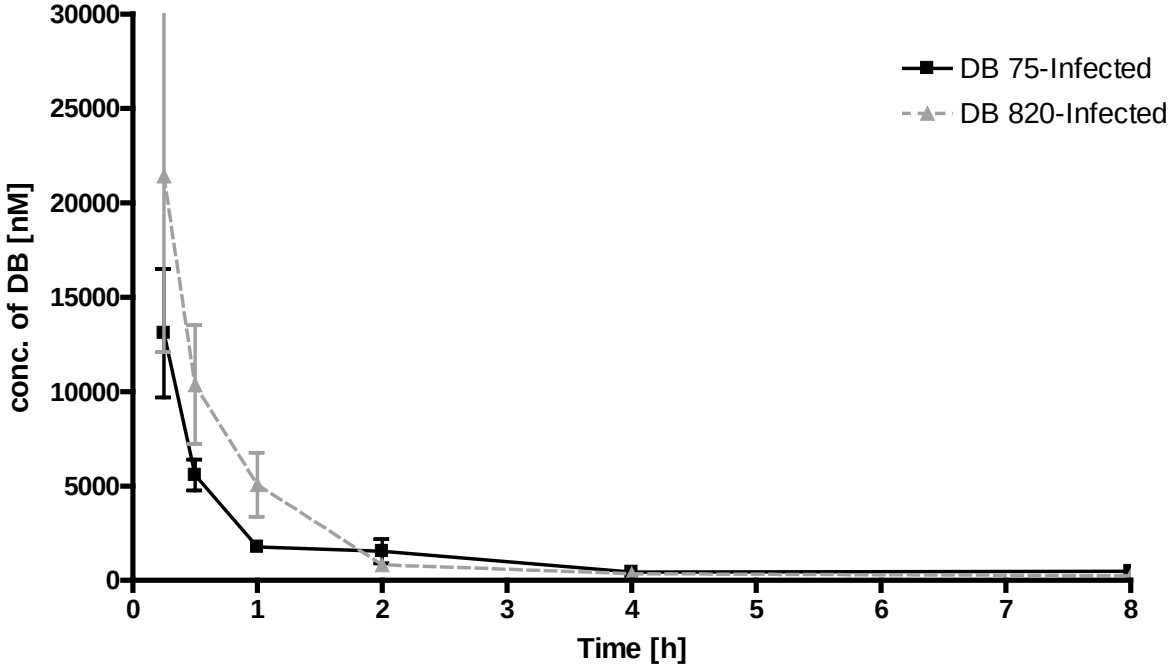


Fig. 13: DB 289 and DB 844 in plasma of uninfected mice

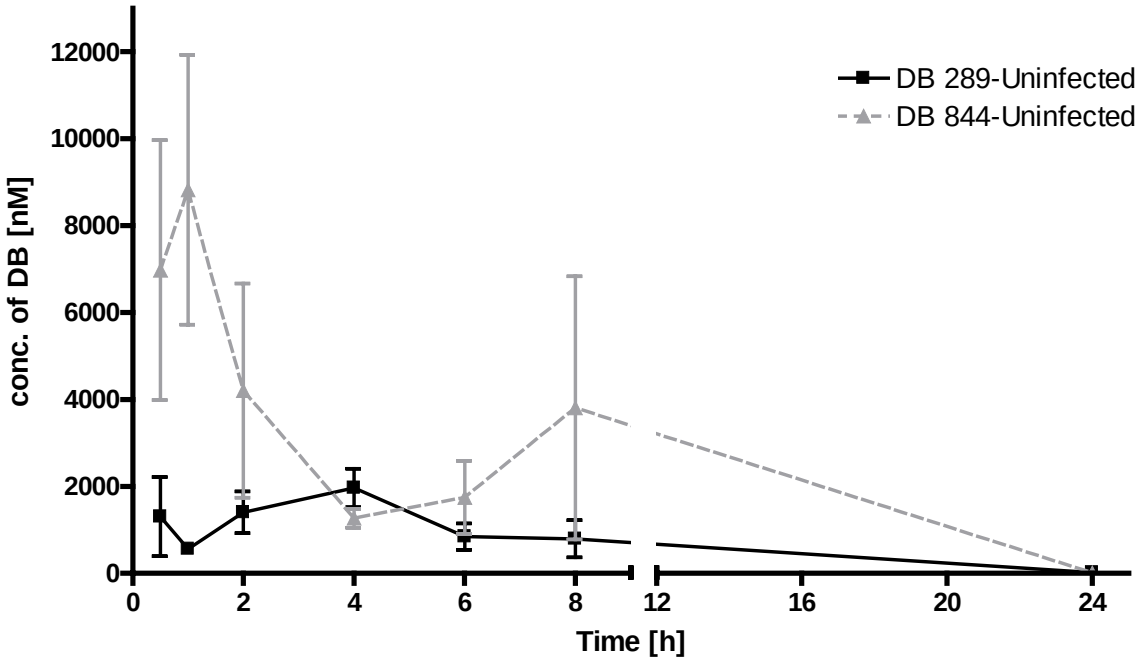


Fig. 14: DB 289 and DB 844 in plasma of STIB 900-infected mice

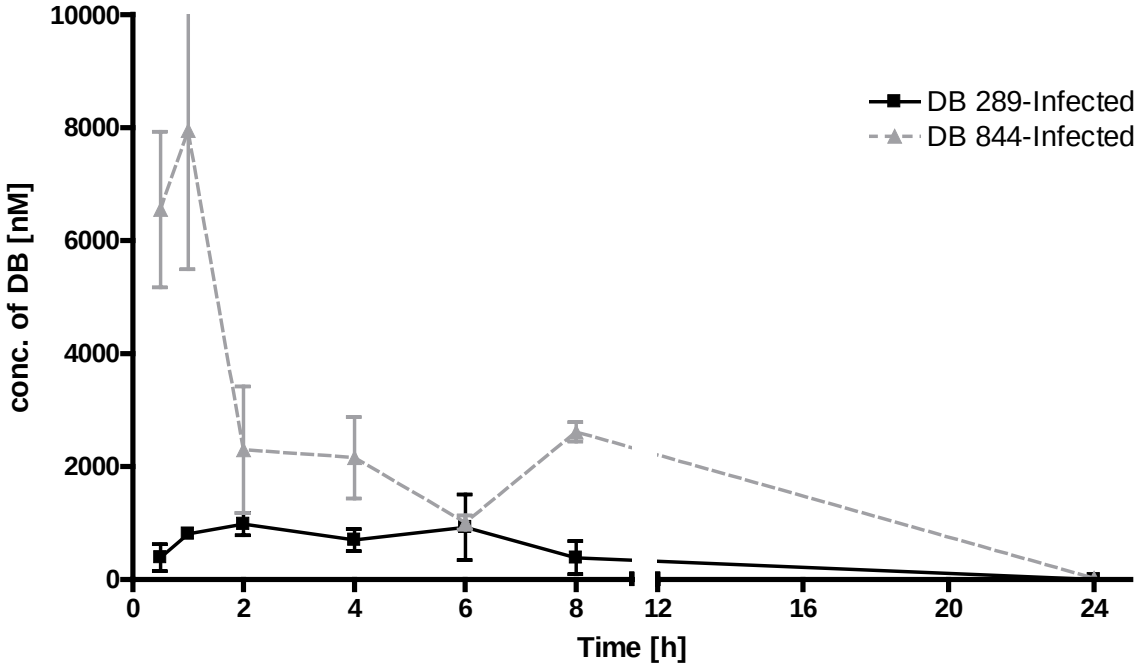


Fig. 15: Converted DB 75 and DB 820 in plasma of uninfected mice after orally administered prodrug

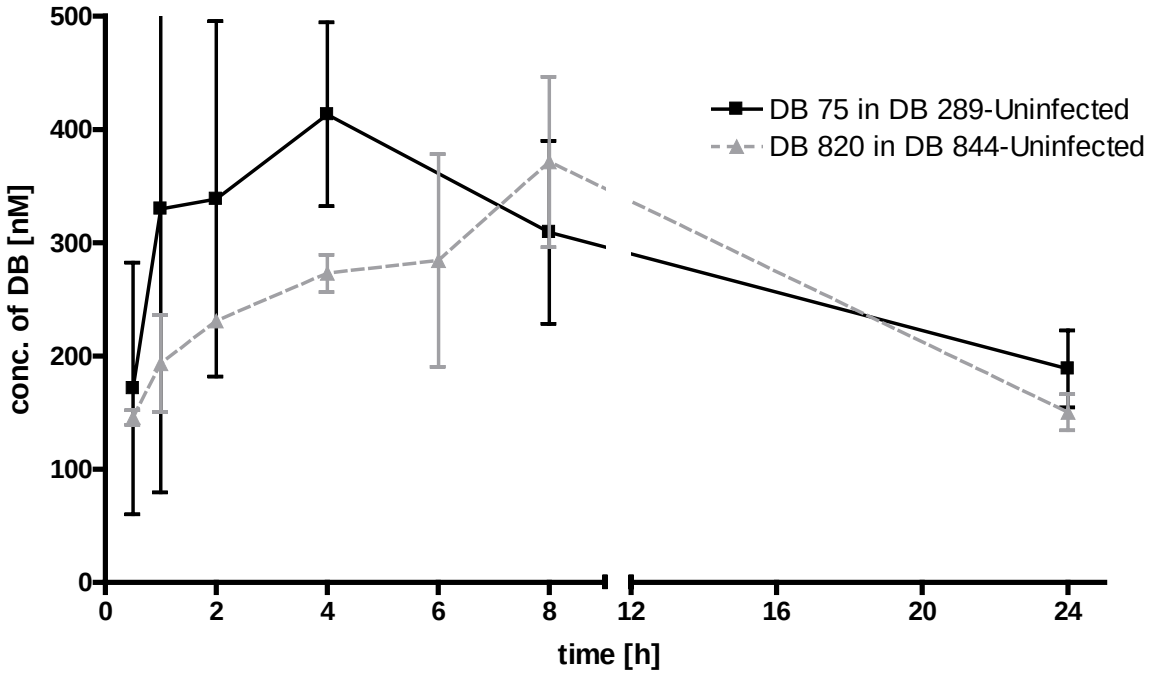


Fig. 16: Converted DB 75 and DB 820 in plasma of STIB 900- infected mice after orally administered prodrug

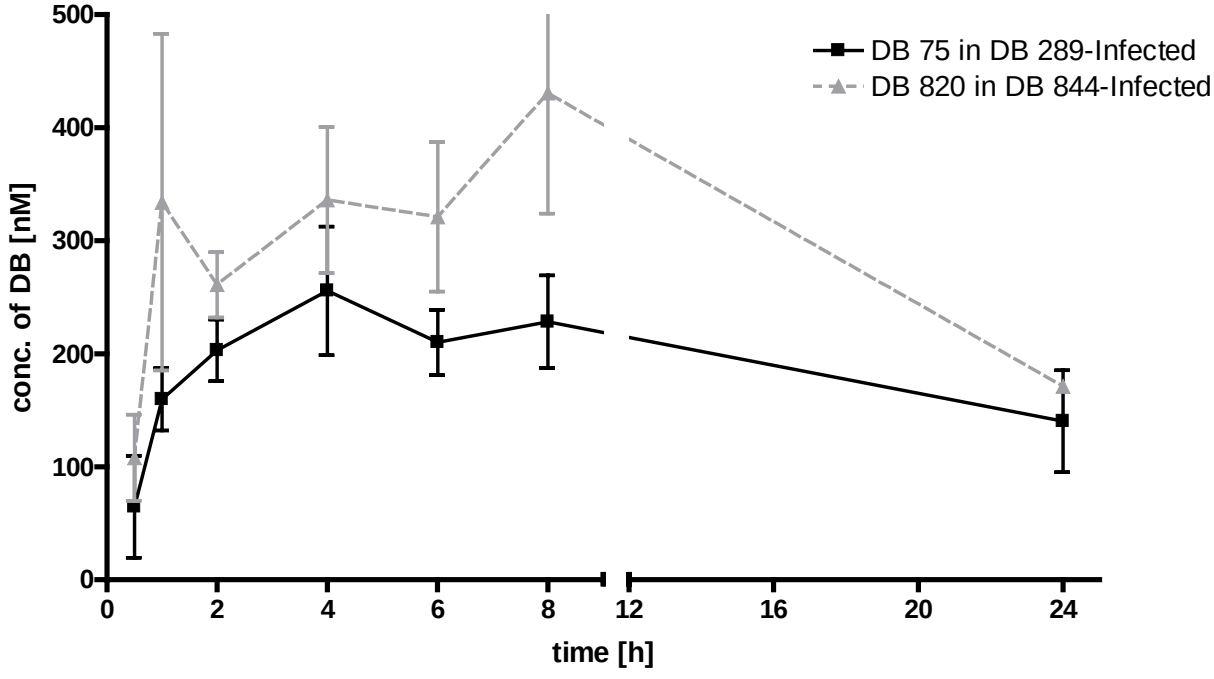


Fig. 17: DB 75 in plasma of uninfected mice i.p. vs. s.c.

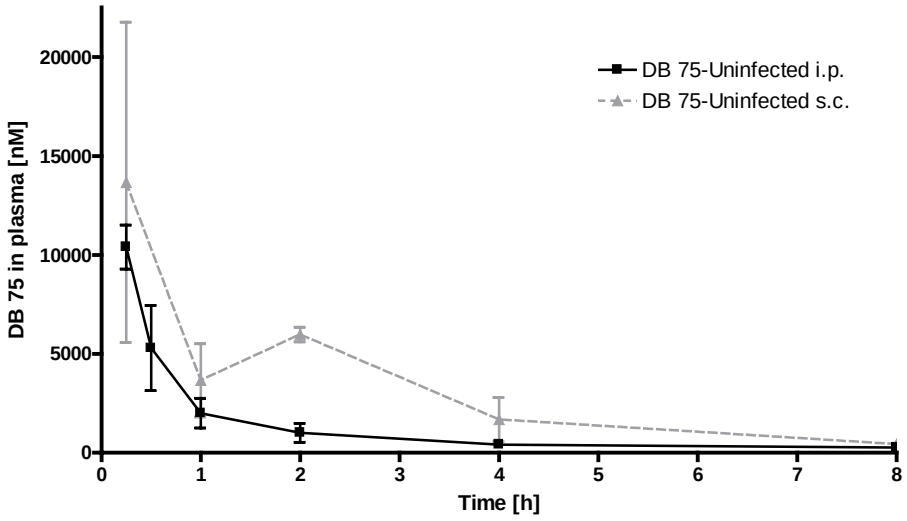


Fig. 18: DB 820 in plasma of uninfected mice i.p. vs. s.c.

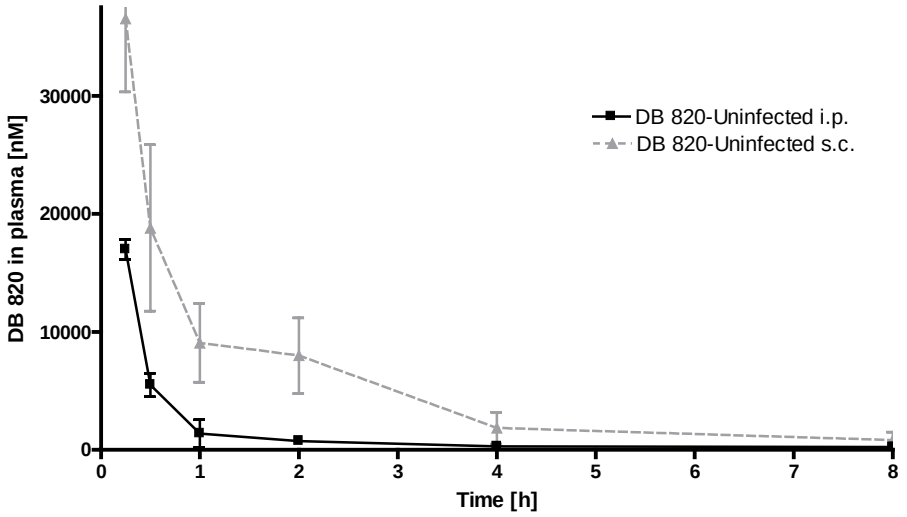


Fig. 19: DB 75 vs. DB 820 s.c. in plasma of uninfected mice

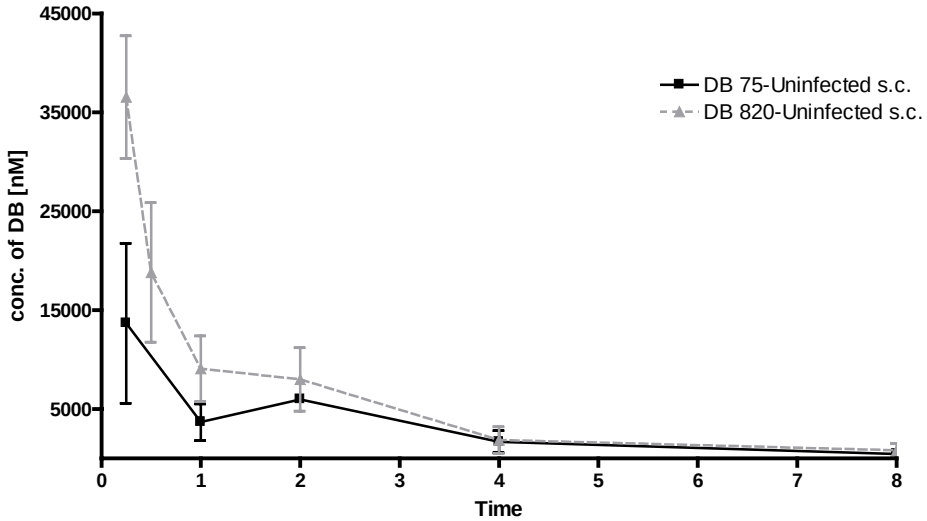


Fig. 20: Metabolism of DB 289

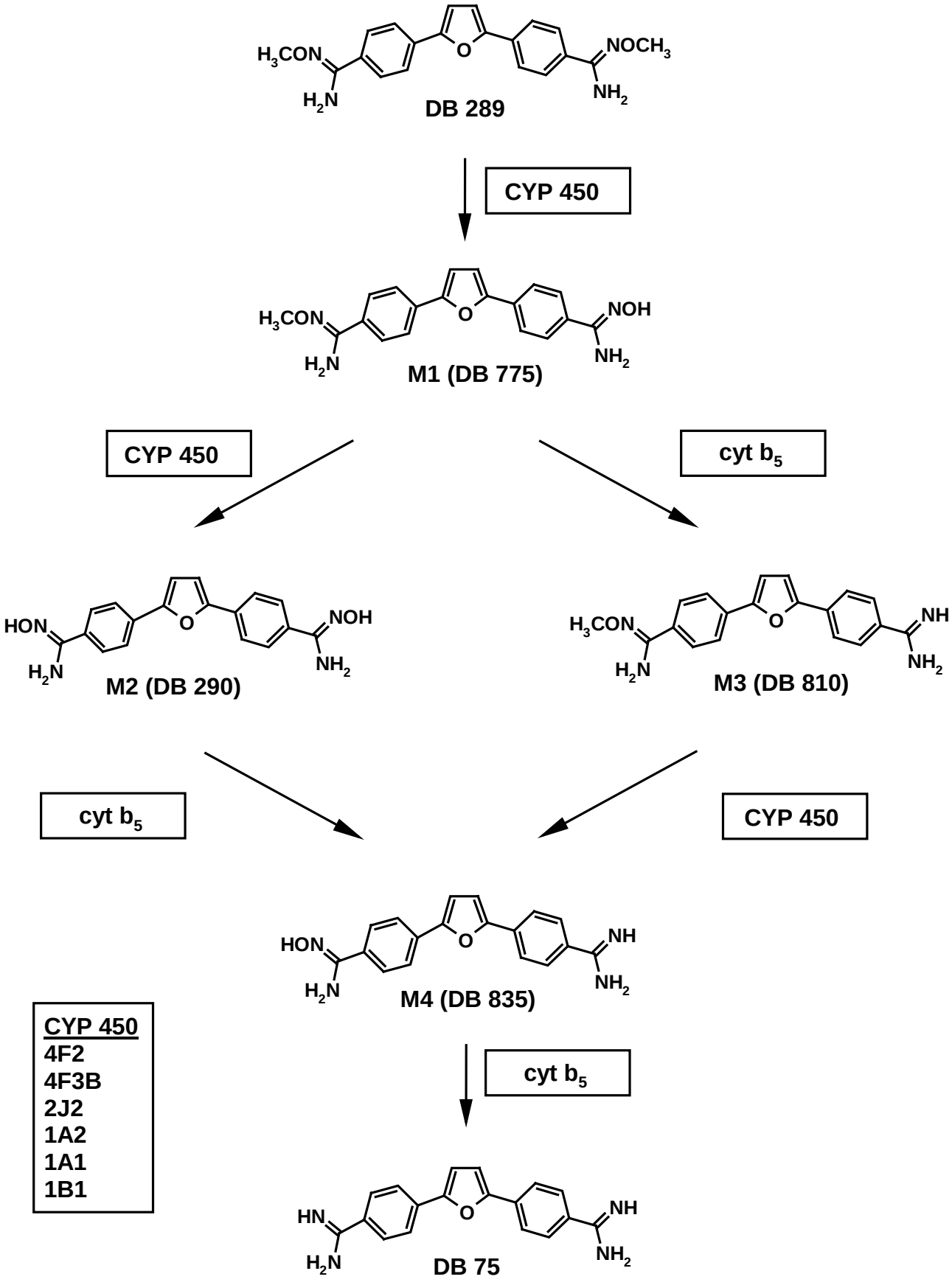
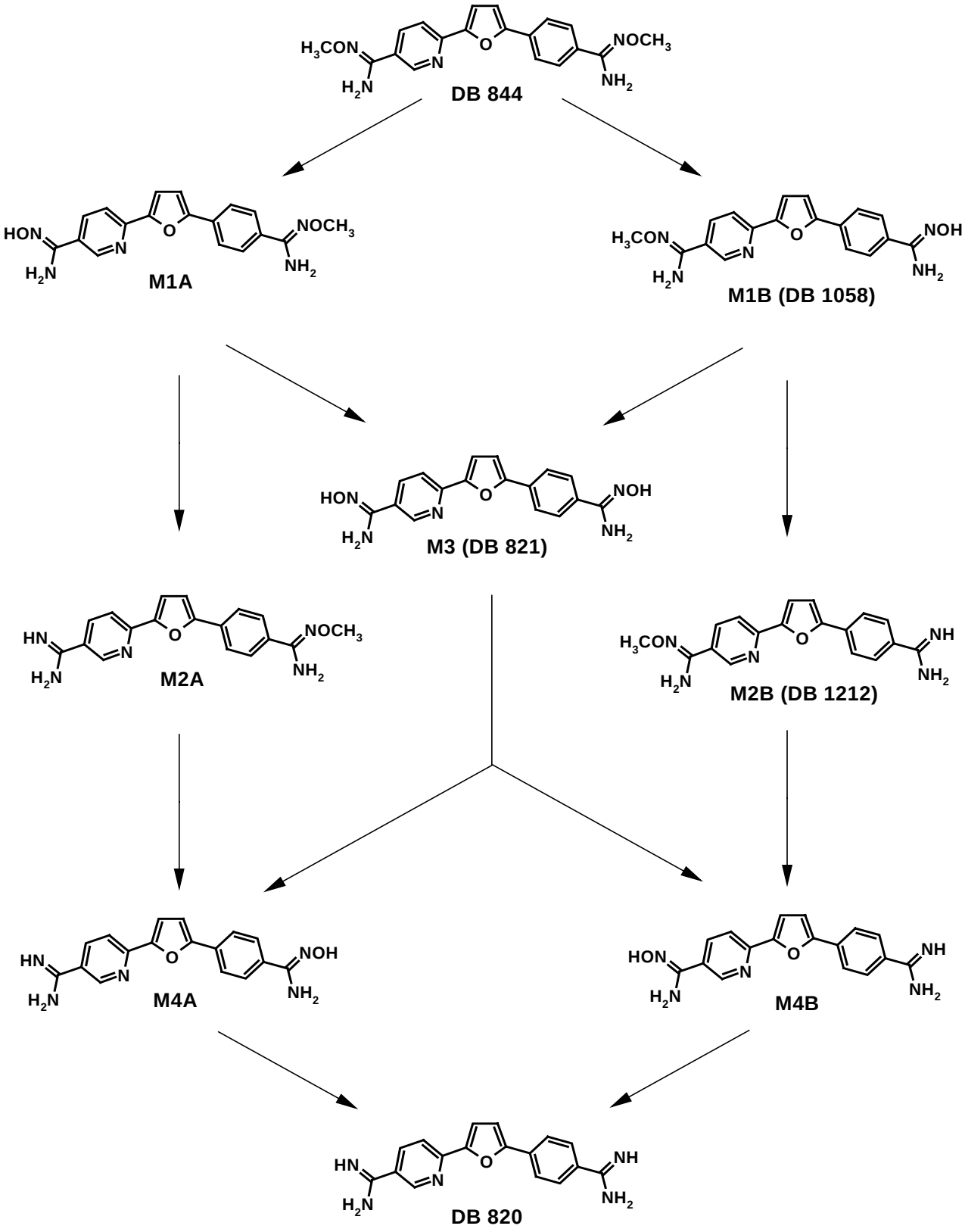


Fig. 21: Metabolism of DB 844



References:

1. Anbazhagan M, Boykin DW. A facile synthesis of the prodrug 2,5-Bis(4-*O*-methoxyamidinophenyl)furan and analogs. Heterocyclic Communications 2003; 9:117-8.

2. Baumann H, Gauldie J. The acute phase response. Immunol Today 1994; 15:74-80.

3. Blum J, Nkunku S, Burri C. Clinical description of encephalopathic syndromes and risk factors for their occurrence and outcome during melarsoprol treatment of human African trypanosomiasis. Tropical Medicine and International Health 2001; 6:390-400.

4. Boykin DW, Kumar A, Hall JE, Bender BC, Tidwell RR. Anti-Pneumocystis activity of Bis-amidoximes and Bis-O-Alkylamidoximes prodrugs. Bioorg Med Chem Letters 1996; 6:3017-20.

5. Das BP, Boykin DW. Synthesis and antiprotozoal activity of 2,5-bis(4-guanylphenyl)furans. J Med Chem 1977; 20:531-6.

6. de Koning HP. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol Pharmacol 2001; 59:586-92.

7. Ismail MA, Boykin DW. Synthesis of deuterium-labelled 6-[5-(4-amidinophenyl)furan-2-yl]nicotinamidine and *N*-alkoxy-6-{5-[4-[*N*-alkoxyamidino)phenyl]-furan-2-yl}-nicotinamidines. J Labelled Cpd and Radiopharm 2004; 47:233-42.

8. Ismail MA, Brun R, Easterbrook JD, Tanious FA, Wilson WD, Boykin DW. Synthesis and antiprotozoal activity of aza-analogues of furamidine. J Med Chem 2003; 46:4761-9.

9. Piafsky KM. Disease-induced changes in the plasma binding of basic drugs. Clin Pharmacokinet 1980; 5:246-62.

10. Saulter, J. Y. Permeability and metabolism of potential prodrugs for the antimicrobial agent 2,5 Bis(4-amidinophenyl)furan (DB75). 2005. University of North Carolina, School of Pharmacy. Ref Type: Thesis/Dissertation

11. Shertzer HG, Hall JE, Seed JR. Hepatic mixed-function oxidase activity in mice infected with *Trypanosoma brucei gambiense* or treated with trypanocides. Mol Biochem Parasitol 1981; 3:199-204.

12. Stephens CE, Patrick DA, Chen H, Tidwell RR, Boykin DW. Synthesis of Deuterium-Labelled 2,5-Bis(4-amidinophenyl)furan, 2,5-Bis[4-(methoxyamidino)phenyl]furan, and 2,7-Diamidinocarbazole. J Labelled Cpd and Radiopharm 2001; 44:197-208.

13. Zhou L, Lee K, Thakker DR, Boykin DW, Tiwell RR, Hall JE. Enhanced permeability of the antimicrobial agent 2,5-bis(4-amidinophenyl)furan across Caco-2 cell monolayers via its methylamidoxime prodrug. Pharm Res 2002; 19:1689-95.

Accumulation of DB 75 in trypanosomes in vitro

Abstract

Accumulation of DB 75 in trypanosomes was assessed in vitro. Thereto, solutions containing various numbers of trypanosomes were incubated with different concentrations of DB 75 for 1, 2 and 4 hours. After the incubation period, trypanosomes were separated from supernatant by centrifugation. The concentration of remaining drug in the supernatant was determined using a fluorescence reader. The obtained fluorescence signal was converted to a DB 75 concentration with the aid of a calibration curve for DB 75. Differences between the amount of drug in solution before trypanosomes had been added and the concentration determined in the supernatant, represented the amount of DB 75 taken up by trypanosomes.
Accumulation of DB 75 in trypanosomes was observed to be inverse linear to the number of trypanosomes and direct linear to the amount of drug present. With the aid of this study it could be shown that the differences between plasma levels of DB 75 in uninfected and infected mice after conversion of DB 289 to DB 75 seen in “Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice” could be explained by the accumulation of the drug in the trypanosome.

1. Introduction

When designing new compounds acting against parasites, one strategy to prevent severe adverse effects on the host but exhibiting an excellent activity on the parasite is to select molecules which interfere particularly with the metabolism of the parasite and not of the host. One of many examples for such selectivity is the antimalarial Fansidar® (sulfadoxine + pyrimethamine), that acts on the folate pathway, which exists only in the parasite. A similar selectivity was found for the antitrypanosomal pentamidine, which accumulates up to millimolar concentrations in African trypanosomes but not in mammalian host cells [Berger, B.J. (1995)], [Damper, D. (1976)]. The uptake of pentamidine into trypanosomes was shown to be mediated by three distinct transporters [de Koning, H.P. (2001b)]. These transporters are necessary for the uptake of nucleobases. In contrasts to humans, trypanosomes have to rely on salvage pathway of nucleobases, since they are not able to synthesise them de novo. Therefore, drugs containing the same recognition motif as nucleobases, like melaminoarsenicals and diamidines such as pentamidine, are transported into the

cell by the same carriers. After internalisation of the drug, pentamidine is stored by binding to specific sites in the parasite and is therefore no longer available free in the cell plasma. This leads to further drug uptake until an equilibrium is reached.
The complete mode of action of pentamidine is not revealed yet, but binding to trypanosomal DNA is proposed to be one of multiple cellular targets [Pepin, J. (1994)], [Nok, A.J. (2003)]. Additionally to the accumulation of the compound, the curvature and AT content of the trypanosomal DNA differs from the human one and therefore binding to trypanosomal DNA is favoured over binding to human DNA.
Not seldom, adverse effects like irritations at the application site, hypoglycaemia, renal failure, acute pancreatitis, hypotension and rarely cardiac arrhythmias were reported after i.v. or i.m. application of pentamidine [Pharmaceutical information “Pentamidine Isethionate” Rhône-Poulenc Rohrer]. The “UNC lead consortium to discover new drugs for the treatment of parasitic diseases” was funded with the aim to develop pentamidine analogues which exhibit the same or even better activity against parasites but exert less toxicity and are orally bioavailable. One of those analogues synthesised within this project, namely DB 75, showed an excellent activity in vivo and in vitro. Additionally, fewer adverse effects were reported for the orally applicable prodrug DB 289 (which is metabolised to the active compound DB 75) in clinical trials phase I and II compared to pentamidine [C. Burri, personal communication]. In mice, 50 mg/kg DB 75 administered i.p. was as well tolerated as the same dose of pentamidine.

Due to structural similarities, transport and accumulation processes are expected to be similar for DB 75 compared to pentamidine. An important tool to visualise the distribution of DB 75 is its fluorescent property. Studies may be performed, tracing the path of the drug from the application site through the body to the target: the parasite. Additionally, studies concerning uptake and distribution within the parasites are also easily possible due to this innate fluorescence.

DB 75 - Way through the body:
Investigations were performed for DB 75 itself and its prodrug DB 289 to estimate the penetration through cells imitating the intestine epithelium (CACO-2 cells). Within these studies two ways of crossing were observed: transcellular for DB 289 and paracellular crossing events for DB 75 [Zhou, L. (2002)].

Under physiological conditions, the diamidine groups of DB 75 are both protonated (pK_a: 11.8 and 10.4 [Saulter, J.Y. (2005)]) and therefore the dicationic compound is very polar. Generally, uptake of polar compounds through membranes representing lipophilic barriers is expected to be very little. Accumulation of polar drugs may therefore only occur when one or even several specific transporter/s and/or an extensive intracellular binding site is/are present.

Import and storage of DB 75 in trypanosomes

Transport processes for DB 75 are not yet fully elucidated. So far, the P2 transporter, one of the nucleobase transporters, was shown to be involved in the uptake of this diamidine [De Koning, personal communication]. In vitro studies using a DNA dodecamer representing the minor groove DNA demonstrated that DB 75 is a potent minor groove binder [Laughton, C.A. (1996)], [Trent, J.O. (1996)]. The enhanced DNA affinity to the minor groove is in large part due to nonbonded interactions between the furan group and the walls of the narrow AT-rich minor groove [Trent, J.O. (1996)]. Additionally, strong binding effects of an intercalating nature were shown for GC and GC/AT rich sequences of the DNA and RNA [Wilson, W.D. (1993)]. To date it is not known, if the intercalation with GC/AT mixed sequences of DNA and intercalation with RNA is of big importance for the mode of action of DB 75 or not, since the complete mode of action is not revealed yet.

When inspected under a fluorescence microscope, several structures were observed after an incubation period of trypanosomes with DB 75. It is possible to visualise organelles containing DNA like the nucleus and kinetoplast (blue fluorescence), and also organelles showing yellow fluorescence which were identified as acidocalcisomes [Docampo, R. (1999)] (Fig. 1). These organelles are cytoplasmic vacuoles containing very high concentrations of releasable Ca^{2+} and a Ca^{2+}-H^+ translocating ATPase activity. They further contain high concentrations of phosphorus as polyphosphates for possible energy storage, Mg^{2+}, Na^+, Zn^{2+} and very little of Cl^-, K^+ and sulfur.

In vivo observations

In mice, lower plasma levels of DB 75 were observed in infected animals compared to uninfected animals when a low dose of DB 75 was administered, or when DB 75 had been converted after application of the prodrug DB 289. It is possible that the accumulation of

DB compound in trypanosomes may be the reason for diminished plasma levels in parasitised animals. Possible consequences may be treatment failures and generation of resistant trypanosome populations due to unintended underdosing. To prevent this and to estimate the amount of active compound which may be accumulated in a trypanosome during treatment, the amount of drug bound by trypanosomes was determined in vitro and based thereon, accumulation factors of DB 75 in trypanosomes were calculated.

2. Materials and methods

2.1. Compounds and chemicals

2.1.1. DB 75

DB 75 was obtained from Dr. D. W. Boykin, Georgia State University, USA.

2.1.2. Culture medium (BMEM)

Trypanosomes were cultivated in Minimum Essential Medium (MEM) with Earle`s salts (powder, GIBCO, Invitrogen, Basel, Switzerland), supplemented with 25 mM HEPES (GIBCO, Invitrogen, Basel, Switzerland), 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM nonessential amino acids (100x, GIBCO, Invitrogen, Basel, Switzerland). The medium was further supplemented with 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine and 15% heat inactivated horse serum [Baltz, T. (1985)].

2.2. Drug dilutions

A stock solution of 1 mg/ml DB 75 in water was prepared. By diluting the stock solution with the appropriate amount of culture medium (BMEM), a calibration curve containing two times the final drug concentration was obtained. This calibration curve ranged from 500 to 20000 ng (1325 – 53000 nM) DB 75 per ml without any further dilution. By adding trypanosome solutions of various concentrations 1:1, dilutions containing 250, 500, 1000, 2500, 5000 and 10000 ng/ml DB 75 in culture medium were obtained.

2.3. Trypanosome solutions

Solutions containing trypanosomes of the strain STIB 900 (*Trypanosoma brucei rhodesiense*) in 3 different concentrations were prepared. Thereto, the density of trypanosomes from

culture was determined with the Neubauer chamber and solutions containing $1*10^5$, $2*10^6$ and $1*10^7$ parasites per ml in culture medium (2x final trypanosome density) were prepared.

2.4. Plate layout

For each incubation time one 96-well plate was used and an additional one for the calibration curve. Analysing three different incubation times (1, 2 and 4 hr), four plates in total were needed.

2.4.1. Plate layout for plates representing one incubation period (Fig. 2)

Into each 96-well plate, 100 µl of the drug solution containing the highest concentration of DB 75 (20000 ng/ml) was added to wells of row A, B and H of column 1. The second highest drug dilution (10000 ng/ml) was pipetted into wells of row A, B and H of column 2 as described for the highest drug concentration. The same was done with the other drug dilutions. BMEM without DB 75 was added into wells of column 7 row A, B, and H.
To wells of row A (column 1-7), 100 µl of the trypanosome solution with the lowest number of parasites was added. The solution containing $2*10^6$ parasites per ml was placed into wells of row B column 1-7, and the trypanosome solution containing the highest parasite density was pipetted into wells of row H column 1-7.
This arrangement of the plate was chosen, because it easily allowed removal of the supernatant after centrifugation.
All plates were mixed well for 2 min using a plate shaker (IKA MTS 2/4 digital, VWR International, Dietikon, Switzerland) performing 600 shakes/min and were incubated at 37° C and 5% CO_2 for 1, 2 or 4 hr, respectively.

2.4.2. Plate layout calibration curve and transferred supernatants (Fig. 3: column 1)

100 µl of each drug solution (2x the final drug concentration) was pipetted into wells of column 1, starting with the highest concentration in row A and descending down to row E. In row F screening medium without DB 75 was added. To achieve the same drug dilution (1:1) as in the plates representing an incubation period, 100 µl BMEM was added to each well containing already the drug solutions and additionally to the well with the blank medium.

2.5. Sample preparation (see Fig. 3)

After being incubated 1, 2 or 4 hr, plates were centrifuged at 2750 rpm (Jouan CR 4 11, Johnson Controls, Basel, Switzerland) and 150 µl supernatant was transferred into wells of the plate containing the calibration curve: supernatants obtained after an incubation period of 1 hr were placed into the wells of column 2-4: the supernatants of the wells with the lowest number of trypanosomes were transferred into wells of column 2, the supernatants of the wells containing the second highest number of trypanosomes were transferred to column 3 and the supernatants of the wells containing the highest trypanosome concentrations were transferred to row 4. Supernatants obtained after a 2 hr incubation were transferred into wells of column 5-7 and supernatants obtained after a 4 hr incubation period were transferred into wells of column 8-10. All supernatants were arranged in a way, that the highest drug concentration was placed in row A, the second highest drug concentration was in row B and so on.

2.6. Instruments and data analysis

Plates were read with the fluorescence reader (SpectraMAX Gemini XS, Bucher Biotech AG, Basel, Switzerland) at excitation wavelength 370 nm and emission wavelength 460 nm. Data analysis was performed with SOFTmaxPRO 3.1.2 and EXCEL.

Reduction of the fluorescence signal of a solution of DB 75 when incubated with trypanosomes was determined (= fluorescence signal of calibration curve – fluorescence signal of supernatant). The accumulation factor resulted from the ratio of the concentration of DB 75 bound by trypanosomes to the ratio of concentration in the solution taking the corresponding volume into account. To assess the magnitude with which the drug got concentrated in trypanosomes, the following equation was used:

$$f = (vol_{tot} - vol_{tryps}) * conc_{tryps} / (conc * vol_{tryps})$$

f: accumulation factor

vol_{tot}: total volume

vol_{tryps}: volume of trypanosomes (*)

conc: measured concentration of DB 75 in solution

$conc_{tryps}$: calculated concentration of DB 75 in trypanosomes

(*) volume of 1 trypanosome = 37.4 fl, data obtained from " Studies of kinetics of parasitaemia and trypanosome disappearance after drug treatment of first infection and relapses"

3. Results

The experiment was performed three times independently, so that the obtained values resulted from three independent binding studies. A table showing the average concentration of DB 75 can be found in Chapter 10, Appendix C.

3.1.1. Calibration curves

The average correlation factor of the three determined calibration curves was 0.9991 with a standard deviation of 0.000404.

3.1.2. Determination of the accumulation factor

The obtained accumulation factor ranged from around 2000 to 1`000`000-fold, depending on the amount of drug and number of trypanosomes incubated (Table 1).

3.2. Binding of DB 75

3.2.1. Uptake of DB 75 in trypanosomes (Fig. 4)

The smaller the number of trypanosomes, the higher was the uptake of DB 75 per trypanosome independent of the amount of DB 75 present. Trypanosomes exhibiting a density of $5*10^4$ trypanosomes/ml incorporated almost 20 times more DB 75, than trypanosomes of a density of $1*10^6$ trypanosomes/ml, which took up around five times more DB 75 than trypanosomes of a density of $5*10^6$ trypanosomes/ml.

3.2.2. Kinetics of the uptake (Fig. 5)

Independent of the numbers of trypanosomes, highest concentrations of DB 75 in trypanosomes were achieved after 2 hr. After 4 hr, levels of bound drug dropped below the 1 hr values in the case of $5*10^4$ and $1*10^6$ trypanosomes per ml. Interestingly, in the solution containing the highest trypanosome concentration accumulation of DB 75 after 1 hr was lower than after 4 hr.

3.3. Accumulation factors

3.3.1. Dependence of the accumulation factor on the trypanosome density (Fig. 6)

Independent of the duration of the incubation period, it could be shown that the higher the numbers of trypanosomes, the lower the accumulation factor was. The decrease of the value of the accumulation factor correlates with the increase of the number of trypanosomes.

3.3.1. Dependence of the accumulation factor on the amount of DB 75 present (Fig. 7)

Regardless of the number of parasites, the following observation was made: the higher the concentration of DB 75 present, the lower the accumulation factor was.

4. Discussion

The fluorescent property of DB 75 facilitated the assessment of the amount of drug accumulated in trypanosomes compared to other uptake studies performed with pentamidine, also a diamidine. First of all, no radiolabelled counterpart had to be synthesised de novo. Additionally, one could circumvent all the critical aspects and handling with radioactive material. No other indicators were needed, because of the ability to measure the presence of the compound directly due to the native fluorescence.

The proportionality between the measured intensity of the fluorescence signal and the concentration within the selected range of 250 ng/ml to 10000 ng/ml was given, what was represented by the correlation factor of the calibration curve of nearly 1 and its small deviation. Therefore, reproducible and accurate results were granted when determination of drug concentrations in a sample of unknown DB 75 concentration was performed by fluorescence spectroscopy.

Binding of DB 75

Uptake of DB 75 could be shown to be an inverse linear process. Trypanosomes of the lowest density ($5*10^4$ trypanosomes/ml) bound around 20 times (mean: 18.33, stand. dev.: 1.26) more DB 75 compared to the solution containing 20x more trypanosomes ($1*10^6$ trypanosomes/ml). The same applied for the solution containing $1*10^6$ trypanosomes/ml where around 5x (mean: 6.11, stand. dev.: 1.16) more bound drug could be detected compared to the solution with the highest trypanosome density which contained $5*10^6$ trypanosomes/ml.

Generally, the more drug present, the more drug bound. Graphs representing the uptake of DB 75 versus the drug concentration in the solution (Fig. 5), showed the characteristics of a saturated processes with a curve represented by a steep rise at low concentrations changing to a small slope and finally ending in a plateau at high concentrations. This is explainable by the fact, that the more compound is available for transport, the higher the chance for each single molecule to get transported into the cell.
Since the highest concentration at which the uptake was studied was 10000 ng/ml, the value at which the plateau was reached could be little higher than determined in this study. Higher concentrations of drug solutions were excluded from this study, since a killing effect on trypanosomes was expected.

Accumulation factors

Trypanosome suspensions of lower densities were observed to take up higher amounts of drug compared to trypanosome suspensions of higher densities. This may be explainable by the probability with which molecules were taken up. If there are fewer trypanosomes in a solution of DB 75 of a given concentration, the possibility for each molecule to be taken up is higher, than if a lot of trypanosomes compete for transport of this molecule. This only applies for solutions in which drug is not present to excess. Since the accumulation factor is based on both uptake and the amount of drug remaining outside the trypanosome, a higher accumulation factor is expected, when the trypanosome concentration is low.

The decrease in the value of the accumulation factor when the amount of drug present increases is not surprising since the accumulation factor represents the ratio of the concentration in trypanosomes to the amount of drug outside the trypanosomes. Assuming that trypanosomes accumulate always the same amount of drug, the ratio between the amount of drug accumulated in trypanosomes and the amount of drug remaining in the solution would be higher if the drug concentration of the solution is low than if the amount of drug in the solution is high.

Another reason for the decrease of the accumulation factor could be that the higher the concentration of drug present, the more damaged the trypanosomes are. It is assumable that if trypanosomes were damaged, their pumps do not work properly anymore and therefore influx and accumulation of DB 75 would be diminished. Additionally, if trypanosomes would die

within the incubation period and would get lysed, the amount of accumulated drug would be freed and would be measurable therefore in the supernatant.

General aspects

Two processes play an important role, when a substrate or drug is accumulated within an organism. First, a specific transport system is needed and second, a storage system for this accumulated substrate has to be available.

Transporters

Transporters recognising specific structures are responsible for active transport of substrates into the cell. One important transporter is the P2. Since *Trypanosoma brucei* is not able to synthesise purines de novo, parasites have to rely on the salvage of preformed purines from the host. Purine uptake is performed by specific nucleobase transporters. In trypanosomes, 11 nucleoside and nucleobase transporters were identified to date. The function and the substrates of all these carriers are not known yet [Landfear, S.M. (2004)].

Which transporters are involved in the uptake of DB 75 is not revealed totally at this time. There is strong evidence that the P2 transporter plays an important role in uptake of DB 75. Comparing the uptake mechanisms of the drugs on the market to treat trypanosomosis, exhibiting the same recognition motif than DB 75, conclusions are not easy to draw:

a) Melarsoprol

Uptake of melarsoprol is mediated via two transporters [de Koning, H.P. (2004)]. One of them, the P2 transporter recognises nucleosides and nucleobases that are structurally related to adenosine. The recognition motif of this transporter consists of a) an amidine group that may be integrated into a pyridine or pyrimidine ring, b) an aromatic system associated or integrated with the amidine group and c) an electronegative group attached to the aromatic ring in para position to the amidine, that is able to contribute with a lone electron pair to the π-system [de Koning, H.P. (1999)]. These criteria are all in agreement with the structures of diminazene, pentamidine and the melaminophenylarsenicals.

Mutations within the P2 transporter leading to reduced uptake of substrate, what is thought to contribute to melarsoprol resistance [Maser, P. (1999)].

b) Pentamidine

Pentamidine, is taken up also by the P2 transporter and at least by two alternative carriers as well [de Koning, H.P. (1999)]. Complete cross resistance between melarsoprol resistant strains and pentamidine does not occur, because these other carriers are involved in uptake of pentamidine: the P2 transporter, also called ASPT 1 (adenosine-sensitive pentamidine transporter 1), showed the highest affinity to pentamidine responsible for 50-70% of the total uptake of the drug. The other carriers show different affinities to pentamidine: the so called HAPT (high affinity pentamidine transporter) exhibits high affinity to pentamidine, but is saturated at very low concentrations what leads to little contribution on the total uptake of the drug. The LAPT (low affinity pentamidine transporter) contributes little to the total uptake, but still a considerable amount, due to its very high capacity and due to its lack of sensitiveness to inhibitors like the diamidines propamidine, stilbamidine and diminazene [de Koning, H.P. (2001a)].

c) Diminazene

Recent studies about uptake of diminazene into trypanosomes showed, that this compound is more or less exclusively accumulated by the P2 transporter [de Koning, H.P. (2004)].

All these structurally related compounds show differences in their uptake into the trypanosome. Therefore, DB 75 may be mediated also by several transporters. Further experiments on the internalisation of DB 75 are urgently needed.

Storage of accumulated drug

Pentamidine was reported to rapidly reach high intracellular levels due to binding to intracellular sites, such as kinetoplast DNA [Carter, N.S. (1995)]. The same was observed for DB 75, that is stored by binding to AT rich sites at the minor groove. Additionally, accumulated DB 75 was detected in acidocalcisomes when analysing by fluorescence microscopy. Why this organelle is able to accumulate DB 75 is not known (binding to DNA as a reason may be excluded, since DNA is absent from acidocalcisomes). Possibly, the protonated drug may form complexes with negatively charged ions like chloride, sulphate or phosphate anions. Also, it is not elicited to date what transporters are used to get the drug in there.

5. Conclusion

Accumulation factors determined in an in vitro system are only able to give a rough estimation about the magnitude of accumulation that may be achieved in vivo.
Theoretically, mice showing a parasitaemia of $1*10^6$ trypanosomes/ml would be able to bind 292 nM of administered compound based on the amount of DB 75 in pM bound by 1 trypanosome obtained in this in vitro assay at an incubation concentration of 250 ng/ml, 662.5 nM respectively DB 75 (average value for 1, 2 and 4 hr: 0.29 pM/trypanosome).
Accumulation plays a significant role only when plasma levels are low. As seen for plasma levels of converted DB 75 after application of DB 289, a difference of about 135-170 nM infected vs. uninfected mice were found within the first 4 hr after treatment (see "Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice").
In vivo, other factors have to be taken into consideration which will reduce the calculated amount of the bound DB 75. One of them would be the protein binding of the drug that reduces the amount of free and therefore active drug in the blood. Drug bound by plasma proteins would not be transported into trypanosomes, if the affinity to the protein would be higher than the affinity of the trypanosomes to the transporter. The amount of drug determined in "Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice" represented the amount of free and protein bound drug in the plasma. Additionally, drug levels will be reduced constantly by metabolism and therefore less DB 75 is expected to be present for accumulation in trypanosomes in vivo.

Fig. 1: Fluorescence of trypanosomes incubated with DB 75

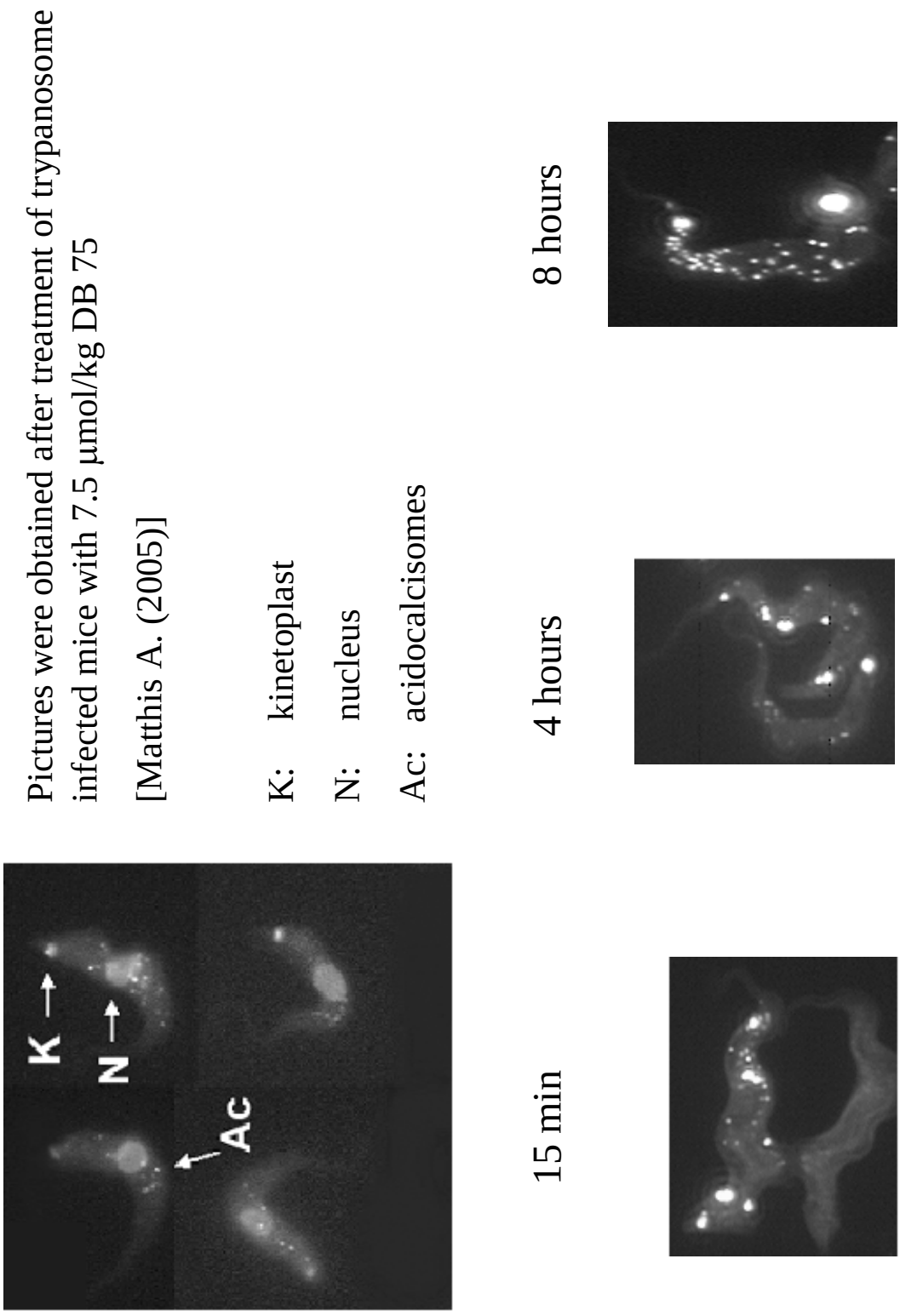


Fig. 2: Plate representing an incubation period

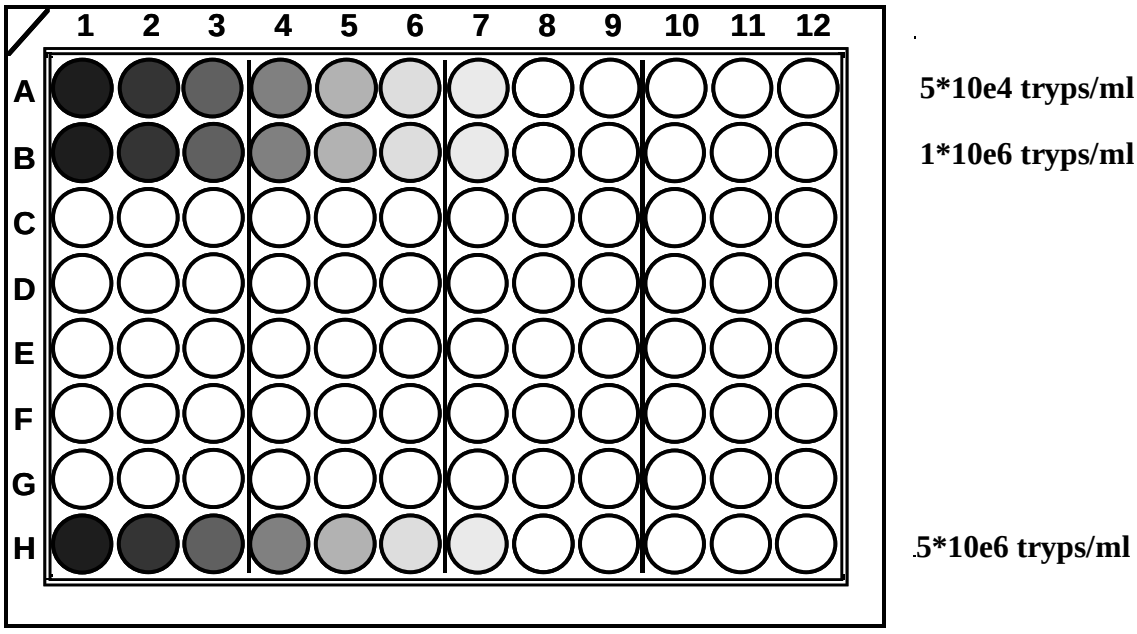


Fig. 3: Plate with calibration curve and transferred supernatant

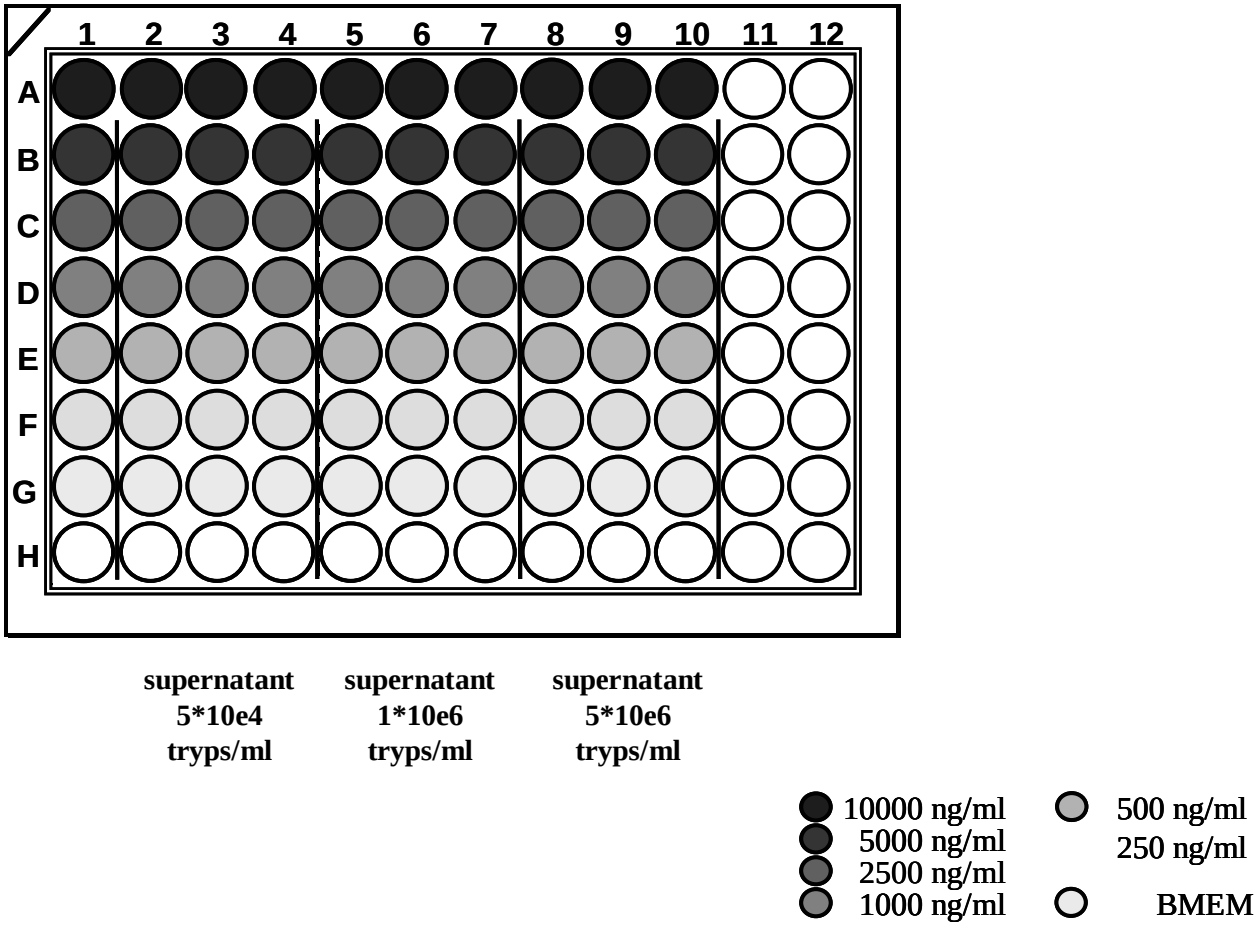


Fig. 4: Binding of DB 75 in trypanosomes

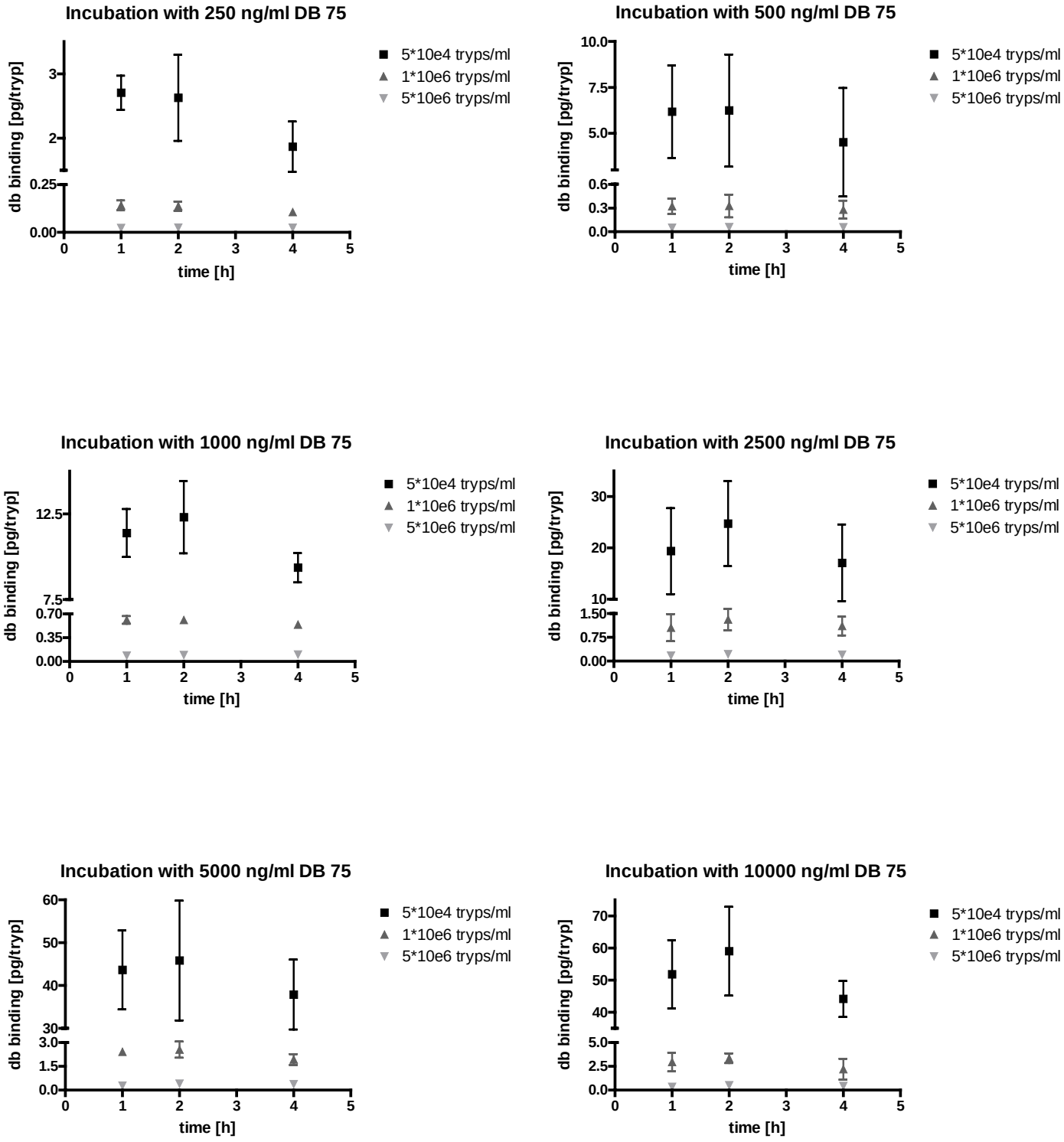


Fig. 5: Binding of DB 75 in trypanosomes

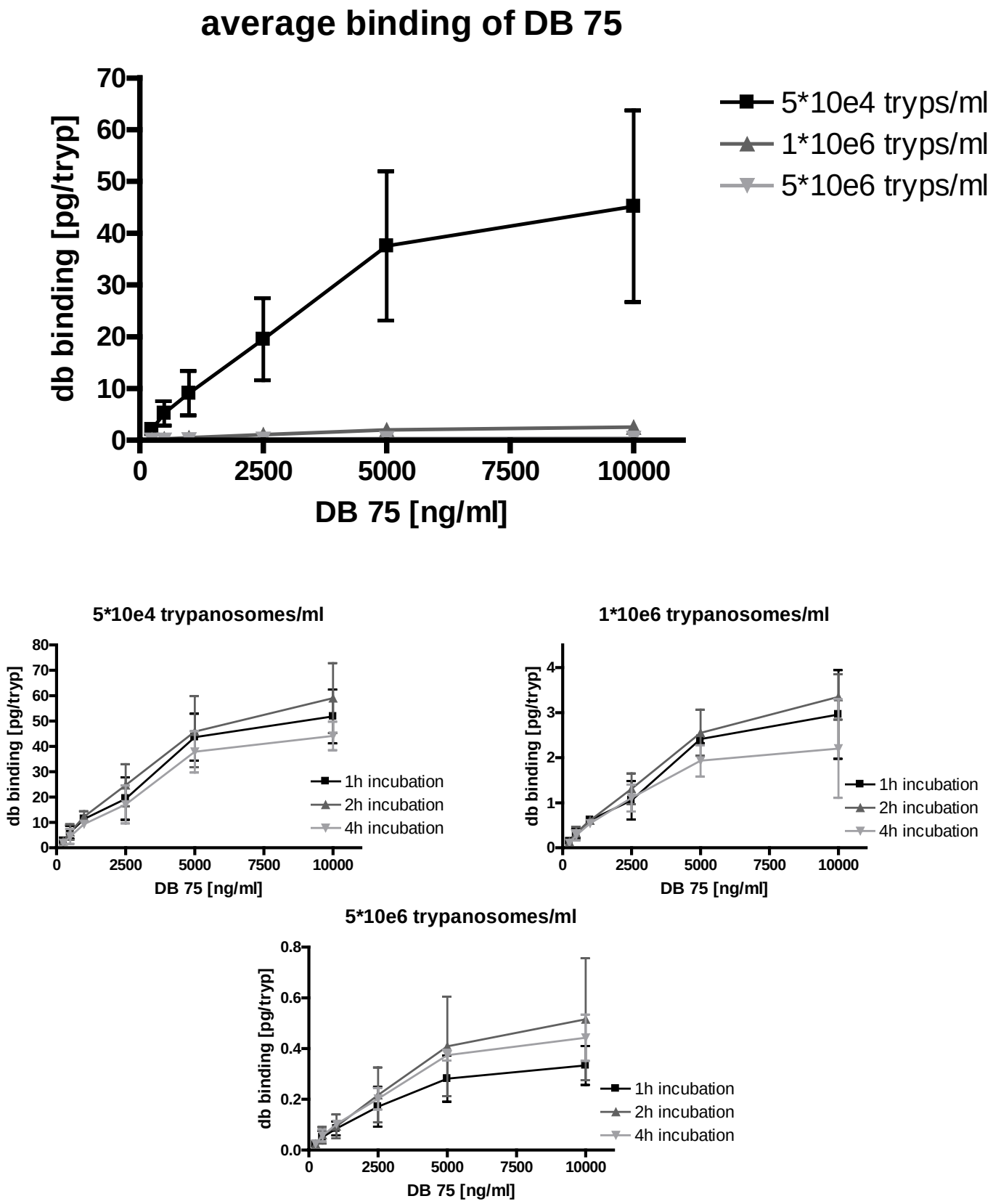


Fig. 6: Accumulation factor of DB 75

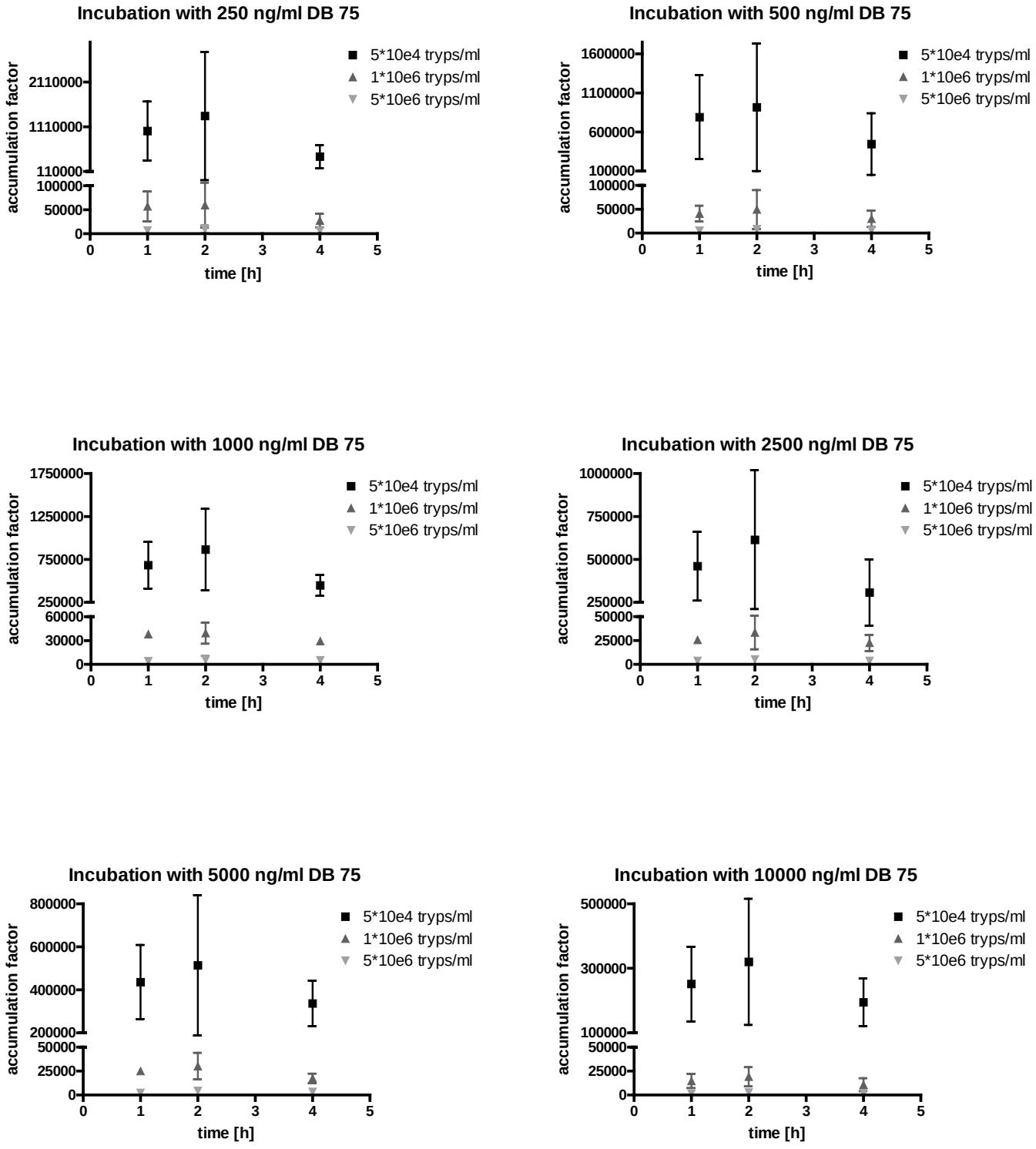


Fig. 7: Accumulation factor of DB 75

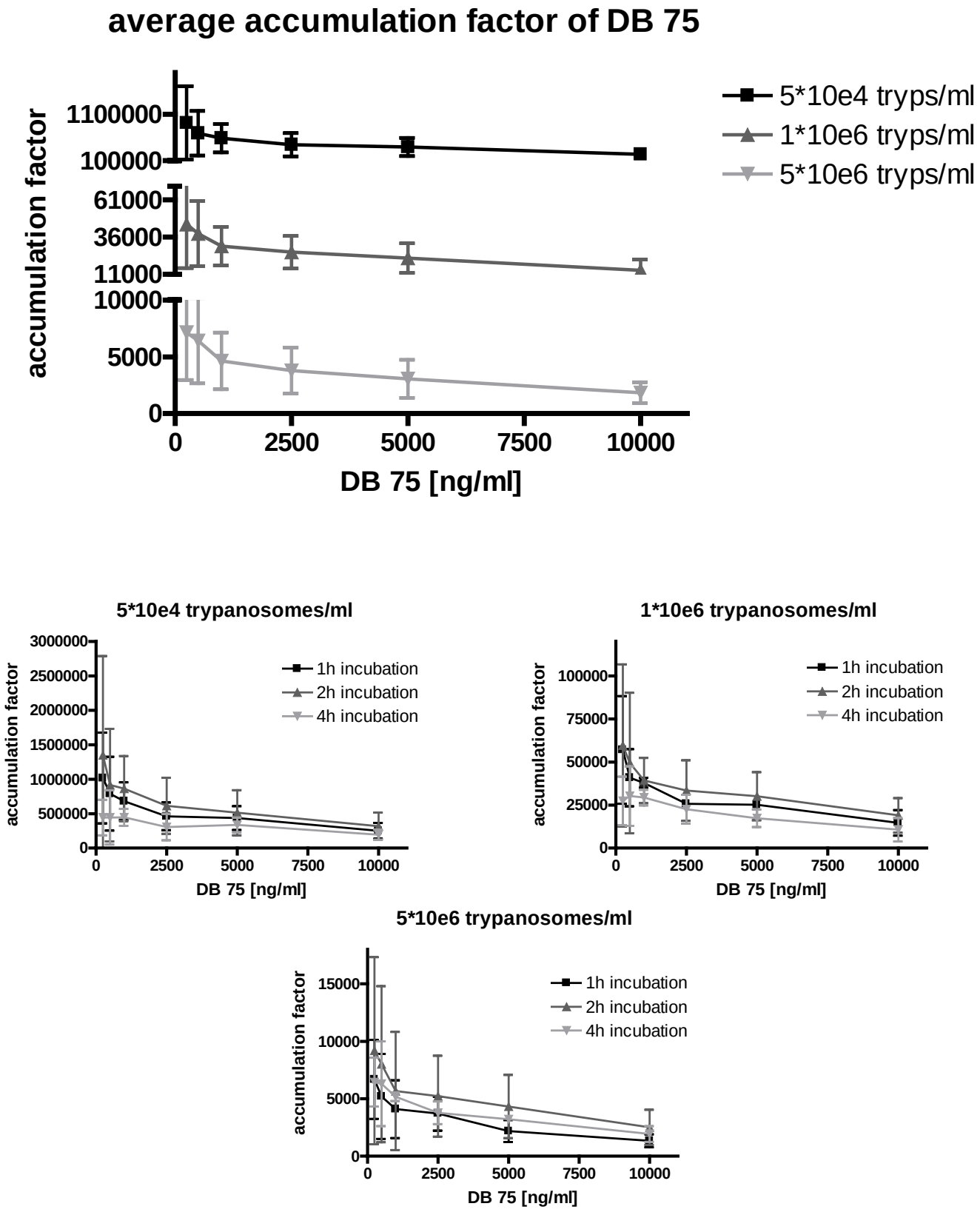


Table 1: Binding and accumulation factor of DB 75

binding in pg/trypanosome

250 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	2.09	0.85
1*10e6 tryps/ml	0.11	0.05
5*10e6 tryps/ml	0.02	0.01

500 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	5.20	2.38
1*10e6 tryps/ml	0.28	0.11
5*10e6 tryps/ml	0.05	0.02

1000 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	9.08	4.29
1*10e6 tryps/ml	0.47	0.24
5*10e6 tryps/ml	0.08	0.04

2500 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	19.51	7.94
1*10e6 tryps/ml	1.09	0.40
5*10e6 tryps/ml	0.18	0.07

5000 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	37.54	14.44
1*10e6 tryps/ml	2.00	0.79
5*10e6 tryps/ml	0.32	0.14

10000 ng/ml	average (1-4h)	standard dev.
5*10e4 tryps/ml	45.20	18.54
1*10e6 tryps/ml	2.51	1.11
5*10e6 tryps/ml	0.40	0.17

binding in pM/trypanosome

662.5 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	5.54	2.26
1*10e6 tryps/ml	0.29	0.12
5*10e6 tryps/ml	0.06	0.02

1325 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	13.77	6.29
1*10e6 tryps/ml	0.74	0.29
5*10e6 tryps/ml	0.14	0.06

2650 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	24.05	11.37
1*10e6 tryps/ml	1.25	0.63
5*10e6 tryps/ml	0.21	0.10

6625 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	51.70	21.05
1*10e6 tryps/ml	2.88	1.06
5*10e6 tryps/ml	0.47	0.20

13250 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	99.48	38.25
1*10e6 tryps/ml	5.31	2.09
5*10e6 tryps/ml	0.86	0.38

26500 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	119.77	49.12
1*10e6 tryps/ml	6.66	2.93
5*10e6 tryps/ml	1.05	0.45

accumulation factor

250 ng/ml, 662.5 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	916235.71	790420.89
1*10e6 tryps/ml	44622.65	29246.54
5*10e6 tryps/ml	7144.59	4192.58

500 ng/ml, 1325 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	693994.79	482874.29
1*10e6 tryps/ml	38383.77	21764.06
5*10e6 tryps/ml	6413.97	3754.15

1000 ng/ml, 2650 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	589101.92	306961.30
1*10e6 tryps/ml	29910.78	12973.99
5*10e6 tryps/ml	4629.56	2497.54

2500 ng/ml, 6625 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	447433.86	250037.64
1*10e6 tryps/ml	25885.14	10922.85
5*10e6 tryps/ml	3790.07	2034.18

5000 ng/ml, 13250 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	395627.39	195553.05
1*10e6 tryps/ml	21868.78	9905.57
5*10e6 tryps/ml	3062.15	1670.48

10000 ng/ml, 26500 nM	average (1-4h)	standard dev.
5*10e4 tryps/ml	236973.84	121720.48
1*10e6 tryps/ml	13815.32	7163.51
5*10e6 tryps/ml	1848.68	926.20

References:

1. 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; 4:1273-7.

2. Berger BJ, Carter NS, Fairlamb AH. Characterisation of pentamidine-resistant *Trypanosoma brucei brucei*. Mol Biochem Parasitol 1995; 69:289-98.

3. Carter NS, Berger BJ, Fairlamb AH. Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. J Biol Chem 1995; 270:28153-7.

4. Damper D, Patton CL. Pentamidine transport in *Trypanosoma brucei*-kinetics and specificity. Biochem Pharmacol 1976; 25:271-6.

5. de Koning HP. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol Pharmacol 2001a; 59:586-92.

6. de Koning HP, Anderson LF, Stewart M, Burchmore RJ, Wallace LJ, Barrett MP. The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insights on diamidine resistance in African trypanosomes. Antimicrob Agents Chemother 2004; 48:1515-9.

7. de Koning HP, Jarvis SM. Adenosine transporters in bloodstream forms of *Trypanosoma brucei brucei*: substrate recognition motifs and affinity for trypanocidal drugs. Mol Pharmacol 1999; 56:1162-70.

8. de Koning HP, Jarvis SM. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. Acta Trop 2001b; 80:245-50.

9. Docampo R, Moreno SN. Acidocalcisome: A novel Ca2+ storage compartment in trypanosomatids and apicomplexan parasites. Parasitol Today 1999; 15:443-8.

10. Landfear SM, Ullman B, Carter NS, Sanchez MA. Nucleoside and nucleobase transporters in parasitic protozoa. Eukaryot Cell 2004; 3:245-54.

11. Laughton CA, Tanious F, Nunn CM, Boykin DW, Wilson WD, Neidle S. A crystallographic and spectroscopic study of the complex between d(CGCGAATTCGCG)2 and 2,5-bis(4-guanylphenyl)furan, an analogue of berenil. Structural origins of enhanced DNA-binding affinity. Biochemistry 1996; 35:5655-61.

12. Maser P, Sutterlin C, Kralli A, Kaminsky R. A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. Science 1999; 285:242-4.

13. Nok AJ. Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. Parasitol Res 2003; 90:71-9.

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

15. Saulter, J. Y. Permeability and metabolism of potential prodrugs for the antimicrobial agent 2,5 Bis(4-amidinophenyl)furan (DB75). 2005. University of North Carolina, School of Pharmacy. Ref Type: Thesis/Dissertation

16. Trent JO, Clark GR, Kumar A, Wilson WD, Boykin DW, Hall JE et al. Targeting the minor groove of DNA: crystal structures of two complexes between furan derivatives of berenil and the DNA dodecamer d(CGCGAATTCGCG)2. J Med Chem 1996; 39:4554-62.

17. Wilson WD, Ratmeyer L, Zhao M, Strekowski L, Boykin D. The search for structure-specific nucleic acid-interactive drugs: effects of compound structure on RNA versus DNA interaction strength. Biochemistry 1993; 32:4098-104.

18. Zhou L, Lee K, Thakker DR, Boykin DW, Tiwell RR, Hall JE. Enhanced permeability of the antimicrobial agent 2,5-bis(4-amidinophenyl)furan across Caco-2 cell monolayers via its methylamidoidme prodrug. Pharm Res 2002; 19:1689-95.

Investigations of plasma samples by bioassay

Abstract

To assess active metabolites in plasma samples of trypanosome infected and uninfected mice treated with DB 75, DB 820 and their corresponding prodrugs DB 289, DB 844, anti-trypanosomal activity of plasma samples were determined by bioassay. Activity was evaluated as equivalent to DB 75, DB 820 respectively and compared to the concentration determined by HPLC/MS/MS. Differences bigger than a factor of two (amount of drug determined by bioassay to amount of drug obtained from HPLC/MS/MS) suggested the presence of at least one additional active metabolite.

Results indicated active metabolites for each of those compounds. Interestingly, occurrence of active metabolites differed completely among the active drugs and also among the prodrugs.

1. Introduction

With the aid of chemical, analytical methods courses of compounds in plasma can be determined after suitable preparation steps. These methods are generally very accurate. To assess an actual concentration of a specific compound, a calibration curve of this compound has to be generated and a linear or other relationship between the concentration and the signal obtained by the analytical detector has to be granted within a specified range. Thereto, probes representing specific concentrations have to undergo the same preparation procedure as the plasma samples which have to be analysed. After having obtained the corresponding analytical signal to each probe of the calibration curve, it is possible to calculate the calibration curve with the corresponding correlation factor. Then, plasma samples containing an unknown concentration of this drug can be analysed, and concentrations may be calculated using the determined calibration curve. The disadvantage of such an analytical evaluation method is that only concentrations of compounds can be determined which are chemically identified, stable and synthesisable.

Bioassays in contrast, assess the activity of all active principles (active compounds and metabolites) that occur in a plasma sample in total. A biological principle is the basis of this analysis, and therefore the obtained results are subjected to fluctuation.

In this case, trypanosomes cultured in vitro were used for the assessment of the anti-trypanosomal activity of active compounds in plasma samples of mice obtained after

treatment with DB compounds. A method similar to the bioassay described by Burri and coworkers was used [Burri, C. (1992)]. Instead of determining the minimal inhibitory concentration (MIC), the IC_{50}, the concentration at which 50% of the parasites compared to a control population were killed, was assessed. Thereto, adaptations as described by Räz were made [Raz, B. (1997)].

Parasites were incubated in a serial dilution of DB 75 or plasma sample of unknown DB 75 concentration, and the IC_{50}s were determined using a viability indicator. Based on the IC_{50}s obtained for the reference drug DB 75 and for the unknown plasma samples, the concentrations of DB 75 in the plasma samples were calculated and compared to concentrations obtained in the chemical, analytical method by HPLC/MS/MS (see "Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice"). The same was done with plasma samples collected after treatment with DB 820, where DB 820 was used as reference drug.

Since prodrugs showed a rather negligible activity in the bioassay (IC_{50} in the µmolar range), it was not possible to assess their concentrations. Instead, the amount of converted active drug was assessed.

This system based on viable organisms is very sensitive to changes in the environment (temperature, CO_2%, medium...), which may result in variations when determining the same sample independently another time. Generally, a difference of a factor of two is acceptable when the same plasma sample is analysed twice.

2. Material and methods

2.1. Drugs and Chemicals

2.1.1. DB compounds

DB 75 was synthesised as described by Das and Boykin [Das, B.P. (1977)], [Boykin, D.W. (1996)], DB 820 according to Ismail [Ismail, M.A. (2003)].

2.1.2. Dilutions of reference drugs

A solution of 100 ng/ml DB 75, DB 820 respectively in BMEM was prepared (= two times highest starting concentration).

2.1.3. Resazurin

A solution of 0.0125% resazurin (Fluka, Buchs, Switzerland) in phosphate buffered saline (PBS) was used to detect viability of the trypanosomes.

2.2. Plasma samples

Plasma samples were identical to the ones used for “Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice”. For further information about trypanosomes, animal work and collection of the plasma samples, see chapter 5.

2.2.1. Dilution of the plasma samples

Based on the amount of drug determined by the HPLC/MS/MS method, suitable dilutions of the plasma samples in BMEM were prepared, resulting in solutions containing between 50 and 30 nM active drug (= two times the highest concentration). To minimise the inhibitory effect of the blank plasma sample on the bioassay, each plasma sample was pre-diluted at least 1:3. Additionally, determination of the IC_{50} of blank plasma samples pre-diluted 1:3 (= two times the highest concentration) was performed to assess the extent of their inhibitory effect.

2.3. Bioassay (Fig.1)

In each 96-well plate, one reference drug (DB 75 or DB 820 of known concentration) and 3 plasma samples containing the same compound as represented by the reference drug were tested. Thereto, serial drug dilutions for each drug/plasma sample were performed in triplicate (3 columns), starting with wells of row H, containing the highest concentration. Dilution steps were performed (1:3) up to wells of row B. Wells of row A did not contain any drug or plasma sample, serving as control. To two columns a trypanosome solution with a density of $3*10^4$ trypanosomes/ml was added, whereas the volume of the other column was complemented with medium and served as background. After an incubation period of 68 hr, the viability indicator resazurin was added and another incubation period of 4 hr took place.

2.4. Instruments and data analysis:

The fluorescence reader SpectraMAX Gemini XS (Bucher Biotech AG, Basel, Switzerland) was used to read the plates (excitation wavelength: 536 nm, emission wavelength 588 nm). Data analysis was performed with SoftmaxPRO 3.1.2. and EXCEL.

The obtained IC_{50} of the reference drug was set in relation to the IC_{50} of the unknown plasma sample (see equations below). The unknown concentration of the plasma sample was calculated and compared with the concentration obtained from the HPLC/MS/MS studies. Thereto, results obtained in ng/ml were transformed into nM [molecular weight DB 75 (dihydrochloride salt) 377.27 g/mol, DB 820 (diacetate salt) 490.71 ng/mol].

f:	factor		
IC_{50}ref:	IC_{50} of reference	starting conc. of reference:	50 ng/ml
IC_{50}plasma:	IC_{50} of plasma sample	starting conc. in plasma:	x ng/ml
n:	pre-dilution of plasma sample		

$f = IC_{50}ref / IC_{50}plasma$

$x = 50\ ng/ml * f * n*2*3$

Multiplication with the factor 2 and 3 takes the dilution obtained by pipetting 2x the highest concentration and the pre-dilution 1:3 into account.

3. Results

In chapter 10, Appendix D, a table showing the concentrations of DB 75 determined by bioassay and HPLC/MS/MS can be found.

3.1. Plasma course of DB 75 in trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay (Fig. 2, Fig. 3)

Results of the bioassay showed clearly, that in infected animals the amount of DB 75 determined by HPLC/MS/MS was lower than the one calculated for the bioassay. Except for plasma samples collected at 1 and 24 hr, the ratio between the amount determined by the analytical method and the biological determination in infected mice was bigger than 2. In uninfected animals, this was only the case for the plasma samples collected 15 min after drug application.

3.2. Plasma course of DB 75 after application of DB 289 in trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay (Fig. 4, Fig. 5)

At 30 min, determined DB 75 concentration were manifold bigger in the bioassay than values determined by HPLC/MS/MS independent of the status of infection (uninf: 5x, inf.:7x, both results showed big deviations, but all obtained ratios were bigger than 2). Thereafter, values for DB 75 obtained in the bioassay were more or less similar in uninfected animals than the ones obtained by HPLC/MS/MS. At 4, 6 and 8 hr, plasma samples of infected animals showed higher activity in the bioassay compared to the DB 75 concentration determined by the analytical method.

3.3. Plasma course of DB 820 in trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay (Fig. 6, Fig. 7)

Interestingly, uninfected plasma samples showed higher anti-trypanosomal activities in the bioassay compared to the amount of DB 75 found with the HPLC/MS/MS method after 0.5, 1, 8 and 24 hr, whereas plasma levels of infected animals had only slightly increased levels of DB 820 (ratio >2) after 8 and 24 hr when determined by bioassay.

3.4. Plasma course of DB 820 after application of DB 844 in trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay (Fig. 8, Fig. 9)

Plasma samples of uninfected animals showed higher activity at 0.5, 6 and 24 hr when determined by bioassay. A similar pattern of the plasma activity curve was observed in infected animals exhibiting a ratio higher than factor 2 at 30 min and 6 hr.

3.5. Inhibitory concentration of blank plasma samples

The anti-trypanosomal activity of each five blank plasma samples - 5x infected, 5x uninfected- were determined by bioassay. The factors calculated from the IC_{50} of the reference drug to the IC_{50} obtained from the blank plasma sample did not differ considerably between infected (mean: 0.49, stand. dev.: 0.23) and uninfected mice (mean: 0.37, stand. dev.: 0.07).

4. Discussion

Influence of blank plasma on trypanosomes: background

Blank plasma samples pre-diluted 1:3 (= two times highest concentration), with an actual plasma concentration of 16.67% (1:6) in the well of the highest concentration, showed an inhibitory effect on trypanosomal growth. This effect was of major importance, when plasma samples to be tested had not been further pre-diluted to reduce the amount of plasma and the concentrations of active compounds were low.

Based on an average factor of 0.4 obtained from IC_{50}blank_plasma/IC_{50}ref, the inhibitory effect would correlate with an starting concentration of 2*3*50*0.4 = 120 ng/ml DB compound, what would be 318 nM DB 75, 244 nM DB 820 respectively. However, concentrations below these levels were measured, indicating variations of the inhibitory effects of the plasma.

Therefore, plasma concentrations determined by bioassay exhibiting a concentration of DB compound below 320 nM should be interpreted with caution if they had not been further pre-diluted than the minimum (16.67% plasma in wells of highest concentration).

Active metabolites after DB 75 treatment

Within the first 15 min, ratios of DB 75 concentrations determined in the bioassay to the ones determined by HPLC/MS/MS were higher than factor 2 in all mice. At 30 min, only infected mice showed ratio higher than 2. These samples were all diluted additionally more than 10-fold (most of them 30x), which decreases the amount of plasma in the well of highest concentration to below 0.83%. Therefore, inhibitory effects based on the plasma itself can be excluded. The obtained differences indicated, that an active metabolite may be formed very early. Then, the ratio drops below 2 at 1 hr. Thereafter, a parallel course can be detected, when ratios for uninfected animals were compared to infected animals. Infected animals showed ratios higher than factor 2 from 2 to 8 hr. Especially, samples collected at 2 hr had been further diluted. For the other samples, showing a ratio higher than 2, concentration of active DB 75 was calculated to be by far higher than what may be achieved by the inhibitory effect of plasma on its own. Probably, another active metabolite or even several active metabolites were formed within this period. Since these differences were obtained only in infected mice, one could speculate if the degradation of this metabolite/s is inhibited in infected animals as metabolism was shown to be inhibited in parasitised animals when

concentration of plasma samples were determined by HPLC/MS/MS. Rather unlikely, but still possible, is the assumption that infection may cause changes in metabolic pathways and therefore different metabolites were obtained in infected compared to uninfected animals.

Active metabolites after application of DB 289

After 30 min, a ratio higher than 2 was obtained in infected and in uninfected animals. Since these plasma samples were not further pre-diluted and showed a low concentration of active DB 75 when assessed by HPLC/MS/MS, the inhibitory effect of the plasma may be the reason for these differences. After 4 hr, only infected animals showed a small increase with a ratio higher than 2, which rose up a little bit to the 8th hr. Plasma samples collected during this period were not diluted further, but the concentration calculated may not be caused totally by inhibitory effects of the plasma sample.

Interestingly, ratios higher than factor 2 were mainly observed in infected animals only, although courses of converted DB 75 determined by HPLC/MS/MS clearly showed that those levels were lower in infected animals compared to uninfected animals.

Active metabolites after DB 820 treatment

Neither in infected nor in uninfected animals a ratio higher than 2 was obtained when determination by bioassay to the one by HPLC/MS/MS was compared within the first 8 hr. The peak obtained for uninfected animals at the 1st hr may be explained by one plasma sample which showed an extremely low concentration of DB 820 when assessed by HPLC/MS/MS and the inhibitory effect of plasma since no further dilution was performed. After 8 hr, the ratio bioassay/HPLC/MS/MS was slightly higher than 2 in infected animals and even more higher in uninfected animals. Inhibitory effects of plasma samples have to be taken into account since almost all of the plasma samples were not further diluted. However, not the total activity obtained in the bioassay may be caused by the inhibitory effect of the plasma, therefore the presence of an active metabolite may be possible.

Active metabolites after application of DB 844

As already described for DB 75 after application of DB 289, differences were seen in the case of DB 844/DB 820 within the 1st hr. These differences were probably due to the inhibitory effect of the plasma and low concentration of the converted active drug.

Remarkably, a ratio higher than 2 was obtained for uninfected animals at the 6th hr. Plasma samples were not further pre-diluted, and differences which occurred may not totally be due to inhibitory effect of plasma. Therefore, it is possible that an active metabolite may be formed. The time at which this metabolite occurred indicated that it has to be a different one than the one that may be built after DB 820 application, since it could be detected before the possible metabolite after DB 820 treatment which was observed after the 8th hr.

Why active metabolites were only found in uninfected animals after DB 820 application, respectively DB 844, and not in infected animals is not known to date. The same applies for metabolites found only in infected animals after treatment with DB 75, respectively DB 289.

Possible metabolites of DB compounds based on metabolism of pentamidine (Fig. 10)
Assuming that DB compounds were metabolised similarly to pentamidine, N-hydroxylation of the amidines could take place [Berger, B.J. (1991)], [Berger, B.J. (1992)]. In a first step, one of the amidine moieties could be hydroxylated resulting in an asymmetric compound (DB 75 → M4 (DB 835), DB 820 → M4A and/or M4B, metabolites identified when prodrug DB 844 got activated to the active compound DB 820). In a second step, the other diamidine moiety may be hydroxylated (DB 75 → → DB 290, DB 820 → → DB 821).
Another possible way of degradation would be the oxidation of the furan spacer, although furan rings of many drugs were reported to be metabolically stable. If a metabolic cleavage of furan is transformed, hydroxylation of the 5-position, followed by tautomerism and hydrolysis of the 5-hydroxyfuran derivative as reported for diclofurime, a Ca-antagonist [Le Fur, J.M. (1985)], would be imaginable.

In vitro data of possible metabolites
Some of the metabolites mentioned above were already synthesised within the project of the "UNC lead consortium to discover new drugs for the treatment of parasitic diseases" and tested for their in vitro activity against trypanosomes. The following IC_{50} values were obtained:
active compounds:
DB 75: IC_{50} = 1.5 ng/ml (= 4 nM)
DB 820: IC_{50} = 3.25 ng/ml (= 7 nM)

metabolites of DB 289 (Chapter 5, Fig. 20):

M1 (DB 775)	IC_{50} = 1.16 μg/ml (= 2.6 μM)
M2 (DB 290)	IC_{50} = 22.35 μg/ml (= 66.4 μM)
M3 (DB 810)	IC_{50} = 0.028 μg/ml (= 0.066 μM)
M4 (DB 835)	IC_{50} = 0.187 μg/ml (= 0.461 μM)*

*impurities with DB 75 due to synthesis

metabolites of DB 844 (Chapter 5, Fig. 21):

M1A:	not determined
M1B (DB 1058)	IC_{50} = 4.61 μg/ml (= 9.7 μM)
M2A	not determined
M2B (DB 1212)	0.030 μg/ml (0.066 μM)
M3 (DB 821)	IC_{50} = 55.4 μg/ml (= 120.2 μM)
M4A	not determined
M4B	not determined

Based on these in vitro results, the obtained active metabolites were not identical with DB 775, DB 290 and DB 835 in the case of DB 289; DB 810 would be possible, since a good activity was obtained in the in vitro assay. In the case of DB 844, possible active metabolites were not DB 821 and DB 1058; M2B would be possible due to its activity in vitro. Not yet determined metabolites should be tested in vitro to identify possible active metabolites.

5. Conclusion

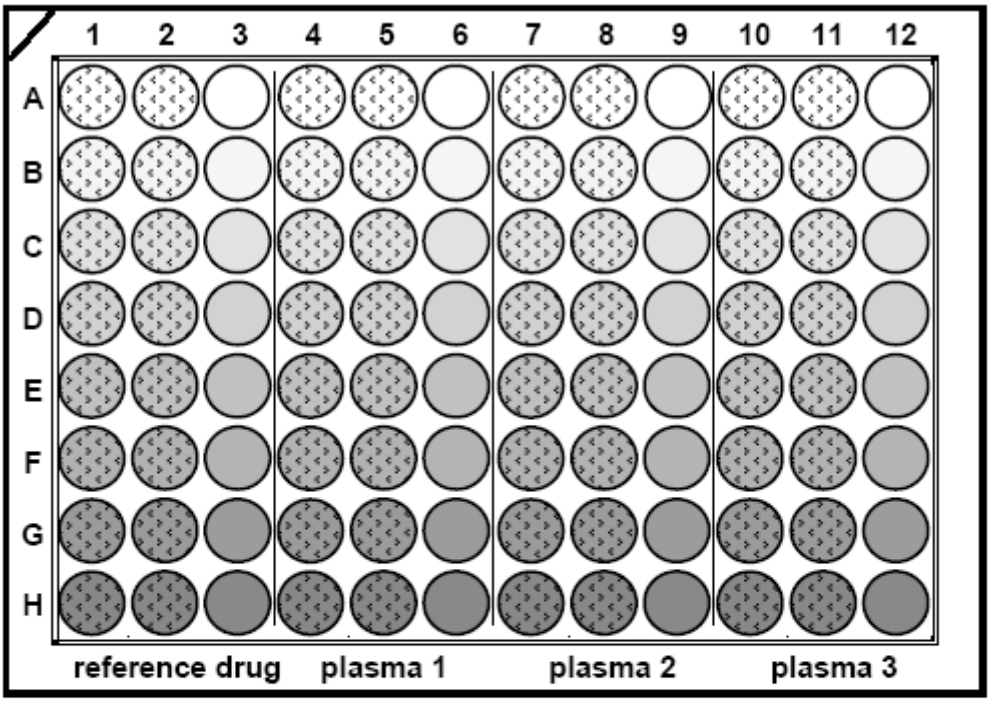
Hints for the existence of active metabolites were obtained in infected animals after DB 75 or DB 289 treatment, respectively. In the case of DB 75, possible metabolites occurred very early within the first 30 min and after 2 hr. Possible metabolites after treatment with DB 289 occurred only in infected animals after 4 hr and were detectable till the 8th hr. If these metabolites were the same as the ones which were seen after DB 75 treatment has to be clarified.

Also, metabolites may be formed after DB 820 or DB 844 application, respectively. After DB 820 treatment, possible metabolites may be formed after 8 hr up to 24 hr, especially in

uninfected animals. 6 and 24 hr after DB 844 treatment, results of the bioassay indicated the presence of possible metabolites in uninfected animals.

To gain more evidence on formed metabolites further studies are required. First of all, possible metabolites after incubation with liver microsomes have to be analysed and identified. Then a comparison with plasma samples is possible.

Fig. 1: Plate layout bioassay



control

background without trypanosomes

drug dilutions and trypanosomes

drug dilutions

Fig. 2: Concentration of DB 75 in plasma samples of trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay

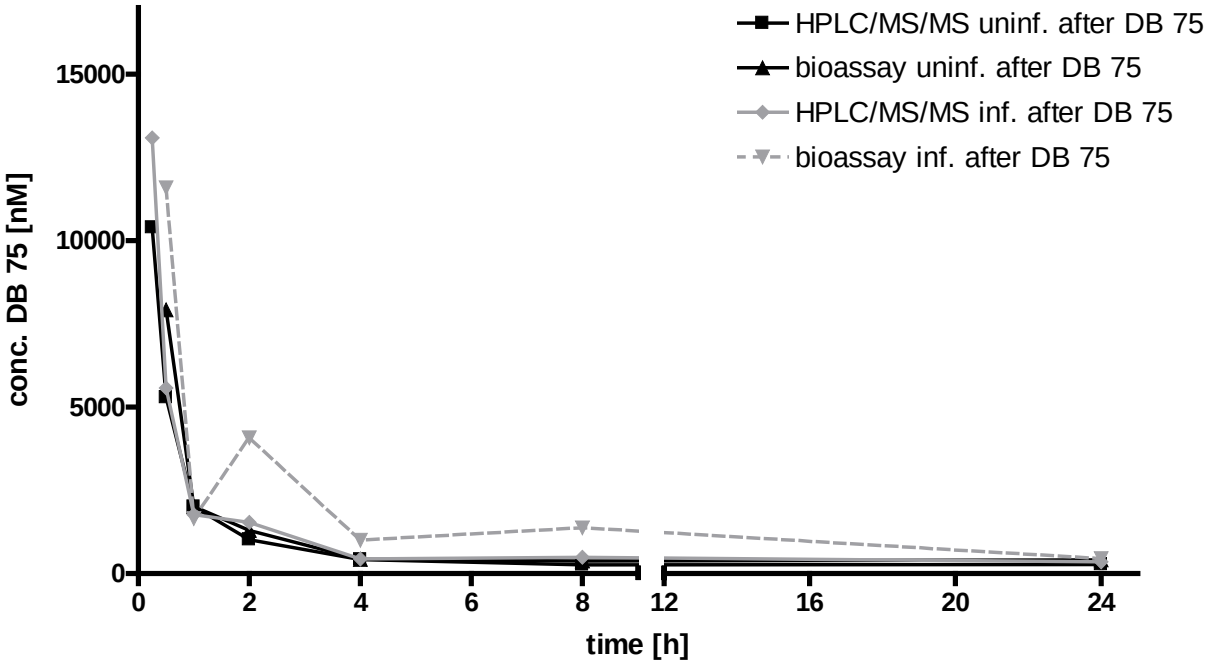


Fig. 3: Ratios of the DB 75 concentration in plasma samples determined by HPLC/MS/MS and bioassay

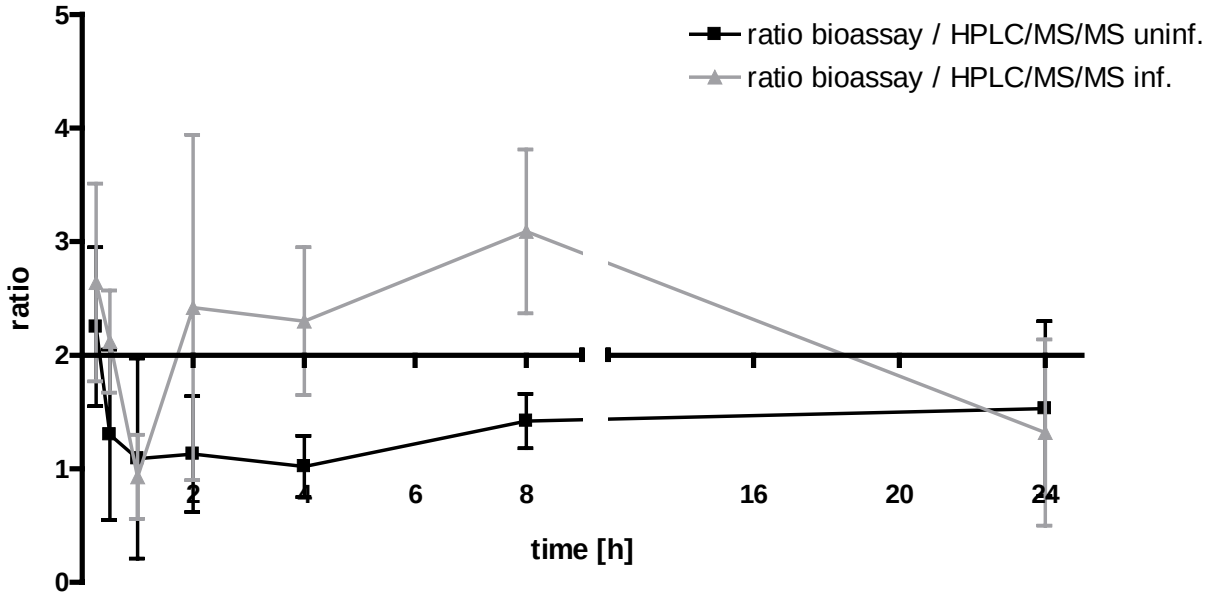


Fig. 4: Concentration of DB 75 after conversion of DB 289 in plasma samples of trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay

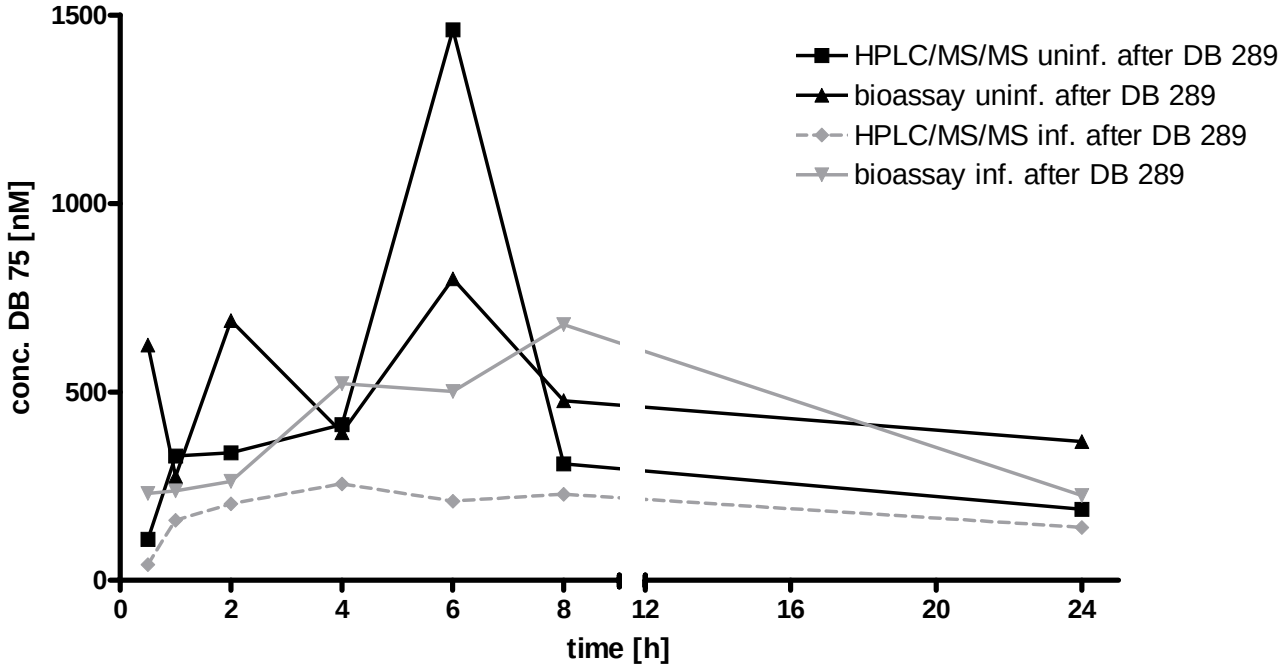


Fig. 5: Ratios of DB 75 concentration in plasma samples after conversion of DB 289 determined by HPLC/MS/MS and bioassay

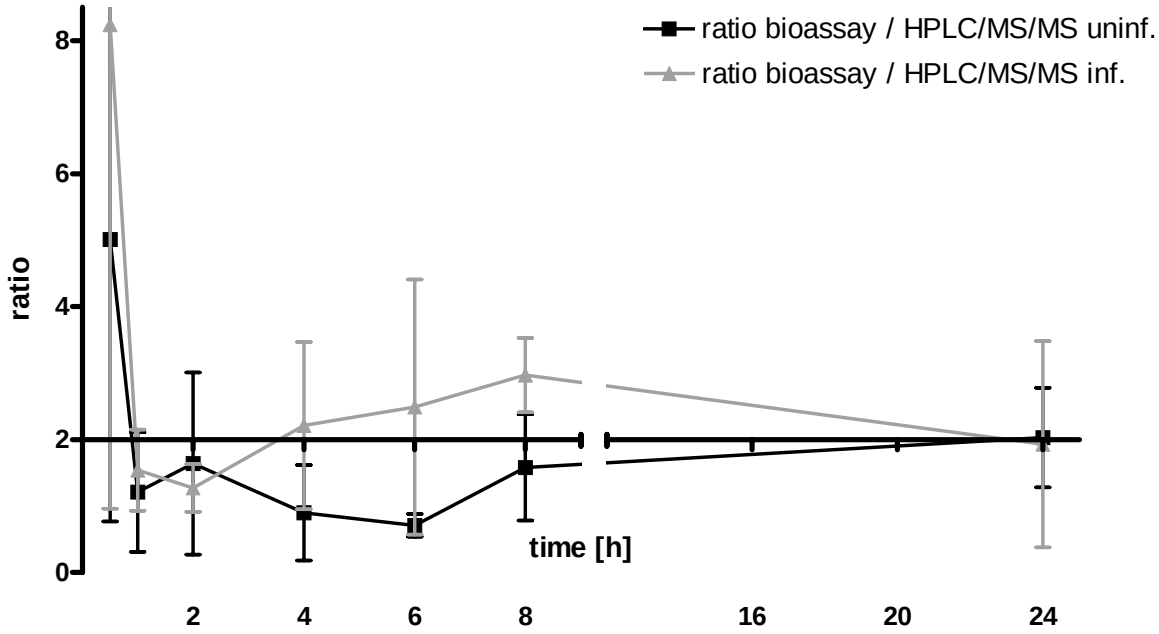


Fig. 6: Concentration of DB 820 in plasma samples of trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay

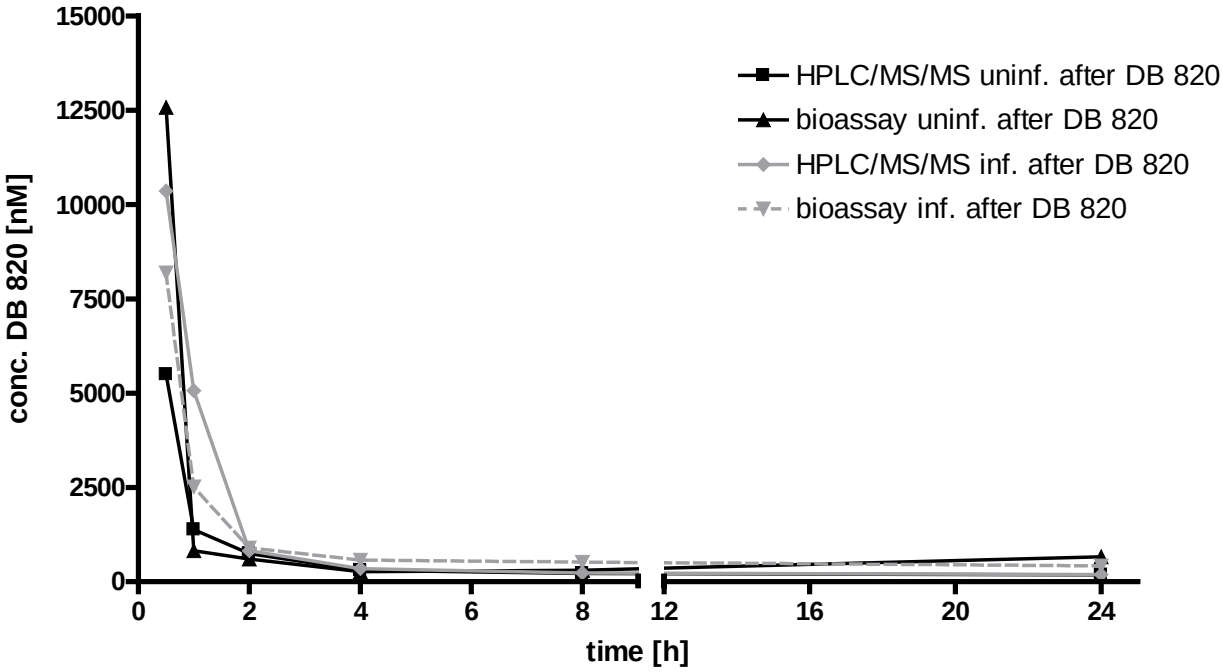


Fig. 7: Ratios of DB 820 concentration in plasma samples determined by HPLC/MS/MS and bioassay

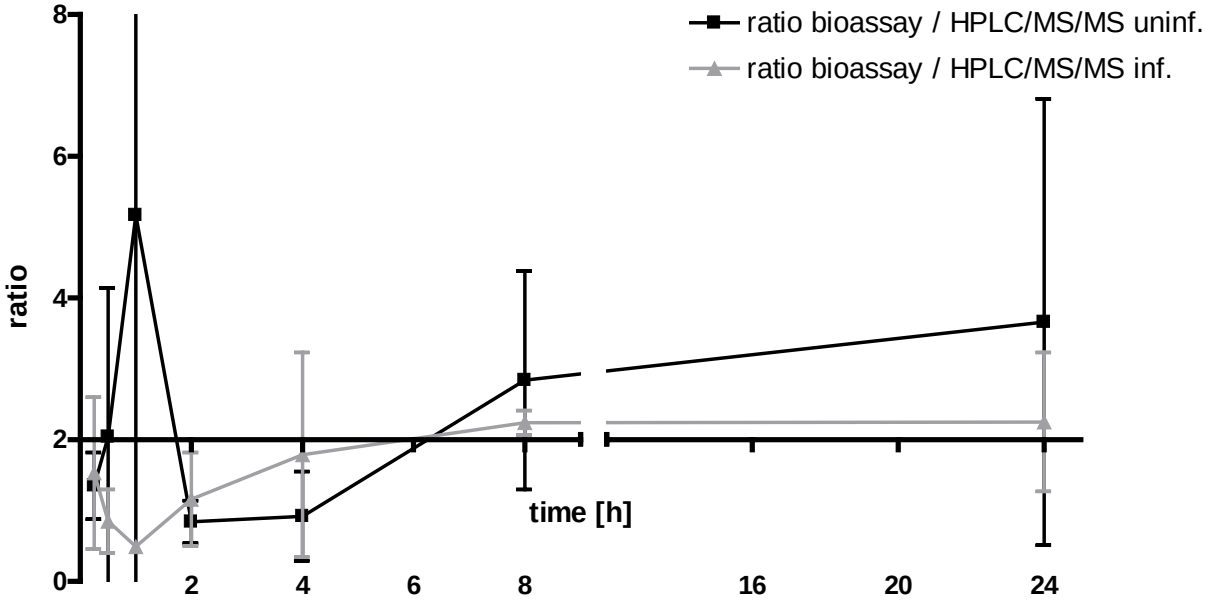


Fig. 8: Concentration of DB 820 after conversion of DB 844 in plasma samples of trypanosome infected and uninfected mice determined by HPLC/MS/MS and bioassay

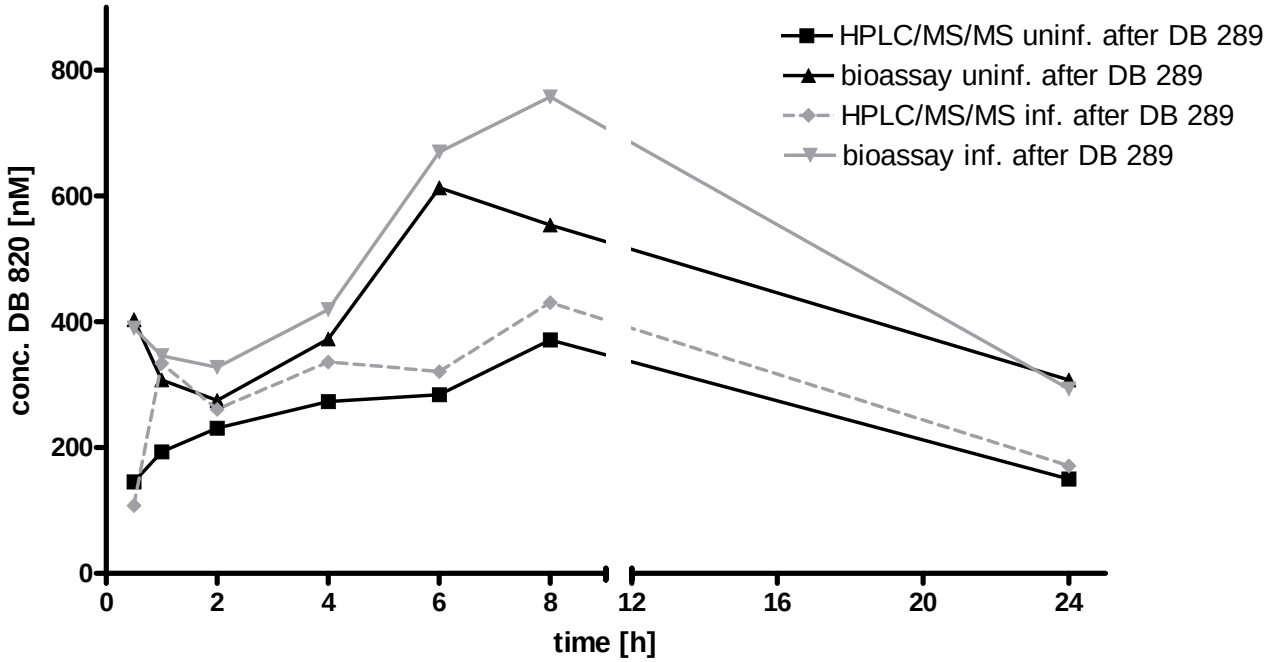


Fig. 9: Ratios of DB 820 concentration in plasma samples after conversion of DB 844 determined by HPLC/MS/MS and bioassay

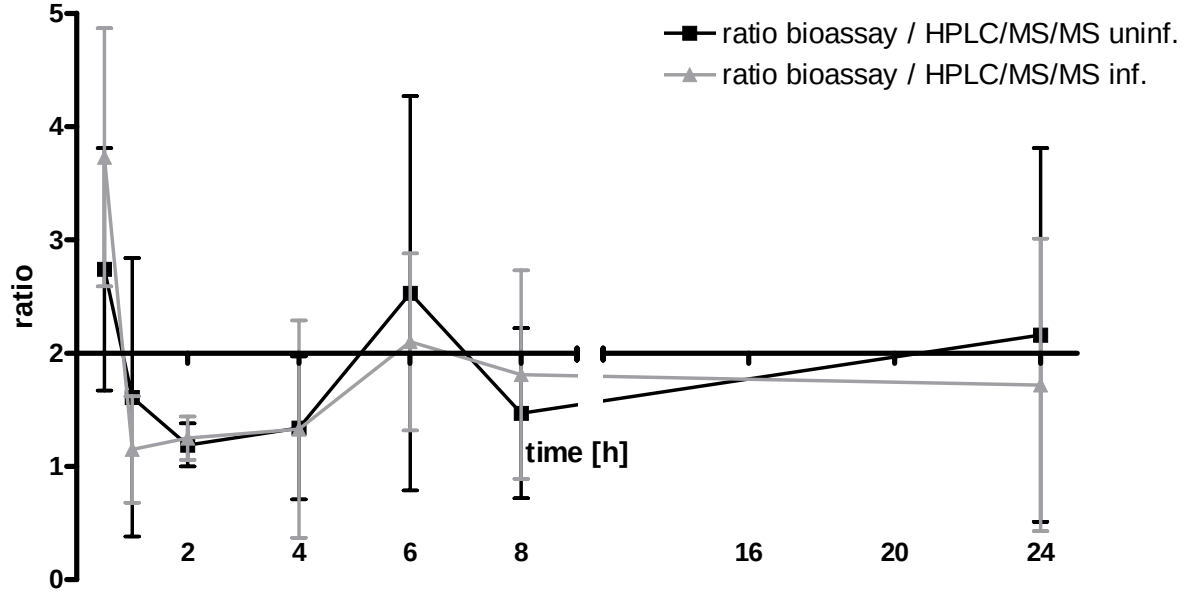
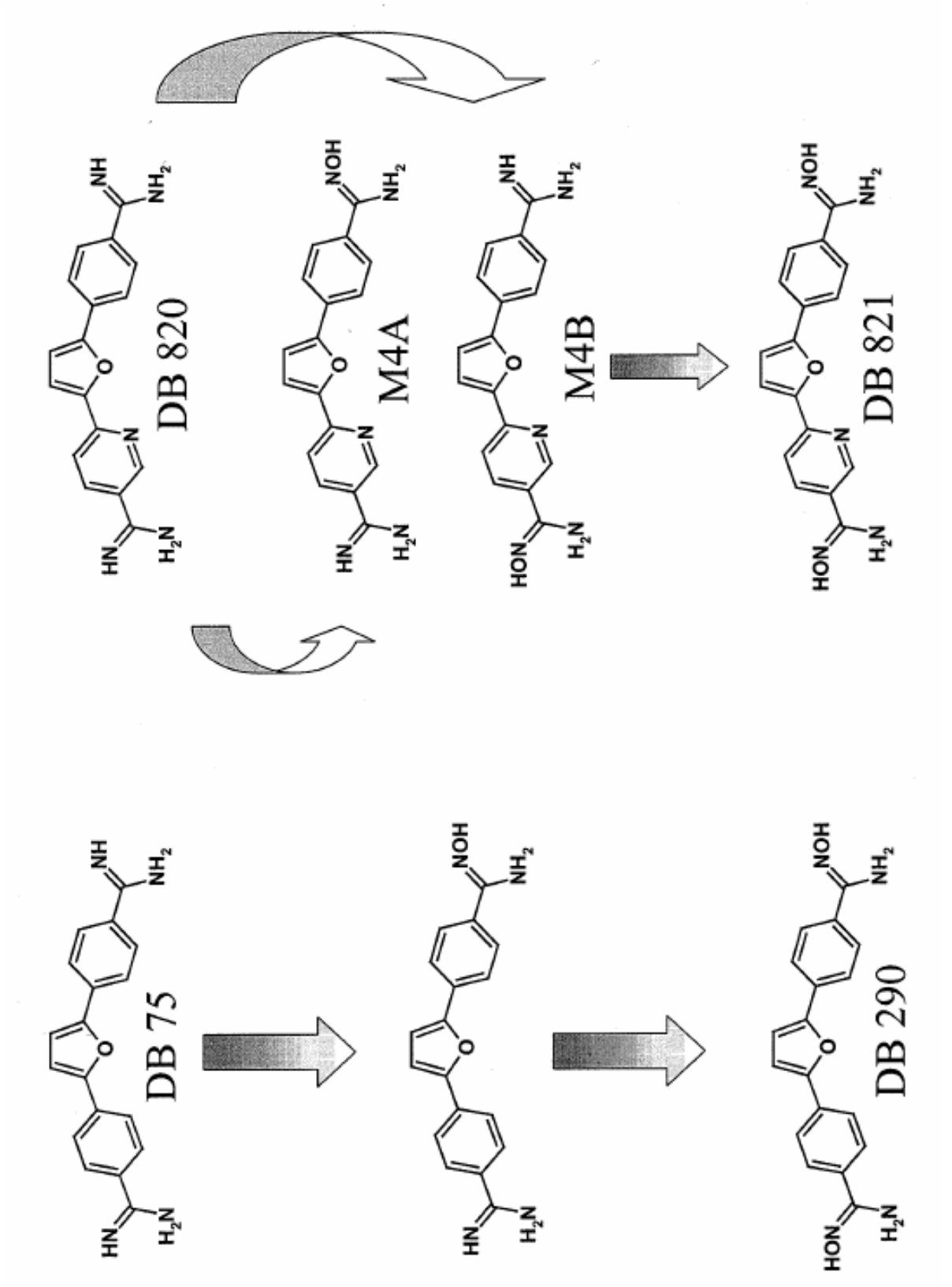


Fig. 10: Possible N-hydroxylation of DB compounds



References:

1. Berger BJ, Naiman NA, Hall JE, Peggins J, Brewer TG, Tidwell RR. Primary and secondary metabolism of pentamidine by rats. Antimicrob Agents Chemother 1992; 36:1825-31.

2. Berger BJ, Reddy VV, Le ST, Lombardy RJ, Hall JE, Tidwell RR. Hydroxylation of pentamidine by rat liver microsomes. J Pharmacol Exp Ther 1991; 256:883-9.

3. Boykin DW, Kumar A, Hall JE, Bender BC, Tidwell RR. Anti-Pneumocystis activity of Bis-amidoximes and Bis-O-Alkylamidoximes prodrugs. Bioorg Med Chem Letters 1996; 6:3017-20.

4. Burri C, Brun R. An in vitro bioassay for quantification of melarsoprol in serum and cerebrospinal fluid. Trop Med Parasitol 1992; 43:223-5.

5. Das BP, Boykin DW. Synthesis and antiprotozoal activity of 2,5-bis(4-guanylphenyl)furans. J Med Chem 1977; 20:531-6.

6. Ismail MA, Brun R, Easterbrook JD, Tanious FA, Wilson WD, Boykin DW. Synthesis and antiprotozoal activity of aza-analogues of furamidine. J Med Chem 2003; 46:4761-9.

7. Le Fur JM, Labaune JP. Metabolic pathway by cleavage of a furan ring. Xenobiotica 1985; 15:567-77.

8. Raz 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.

Pharmacokinetic studies of DB 75 and its prodrug DB 289 in *Plasmodium berghei* infected and uninfected mice

Abstract

In vivo observations showed that *P. berghei* infected mice treated with DB 75 could decrease their parasitaemia by 87% and prolong their survival time to 23 days compared to 6-7 days obtained for control animals. Unfortunately, when administering the prodrug DB 289, parasitaemia was reduced only by 42% and survival days were only slightly prolonged to 15 days. Pharmacokinetic studies were performed for DB 75 and its prodrug DB 289 in *P. berghei* infected and uninfected mice to reveal the reasons for those differences.
It could be shown that the rate of conversion of prodrug to its active principle was the same in uninfected animals than in infected. Taking the plasma protein binding into consideration, insufficient concentration of free active compound was found in infected and in uninfected mice as the comparison with an in vitro time dose assay showed. Increase of the plasma proteins as reported for infected animals might lead to further reduction of free active drug in plasma. Another factor which may reduce the concentration of free active drug, may be the accumulation of DB 75 in parasites as seen when parasites incubated with DB 75 were inspected under the UV microscope.
This study showed that the amount of active drug after application of the prodrug in plasma was subcurative.

1. Introduction

Today, approximately 40% of all people, mostly people living in the developing countries, are at risk of malaria. There, this infectious disease is still one of the leading causes of morbidity and mortality. It is estimated that infections with plasmodia cause 1.2 millions deaths per year, of which 90% occur in Sub-Saharan Africa. Children under the age of five, as well as pregnant women and their unborn child are particularly vulnerable to malaria [http://www.who.int/mediacentre/factsheets/fs094/en/index.html].

Malaria is a parasitic disease caused by protozoans of the genus *Plasmodium*, which is transmitted by the female anopheles mosquito. Four species are infectious to humans: *P. vivax, P. ovale, P. malariae* and *P. falciparum*. Infections can be distinguished based on

morphological criteria and their development in the host. *P. falciparum* causing malaria tropica is by far the most life threatening disease of all plasmodial infections.

Parasites undergo a complex life cycle containing sexual and asexual reproduction and changes of hosts. When injected by the bite of an infected mosquito, the parasites in the stage of sporozoites invade liver cells. There, maturation to schizonts and segregation to merozoites takes place (asexual reproduction). Merozoites are released and invade erythrocytes. In the red blood cell, erythrocytic schizogeny takes place: merozoites develop via immature trophozoites (ring stage) into mature schizonts which again release merozoites. Some of the parasites differentiate to gametocytes which have to be taken up by a mosquito to complete their lifecycle. In the insect they mature to micro- and macrogametes and fuse to a zygote (sexual reproduction). Zygotes become motile ookinetes which invade the midgut wall of the mosquito where they develop into oocysts which grow and release sporozoites. Sporozoites invade the salivary glands, from where they are transmitted into a new host.

Since the plasmodium life cycle differs a lot from the human one, drug targets are manifold. However, only about eight drugs are available for treatment of malaria [Hatz, C. (1998)]. Additionally, spreading resistance of the parasite to some anti-malarials weakened treatment success (most important example: chloroquine resistance). Therefore, new drugs are urgently required.

The efficacy of aromatic diamidines against human protozoal infections was known since 1930, when activity against trypanosomes was observed.
During the 1940´s anti-plasmodial activity of aromatic diamidines in monkeys infected with *Plasmodium knowlesi* was demonstrated [Bell, C.A. (1990)]. However, diamidines were never used clinically in the treatment of malaria.
Potent anti-malarial activity was reported for pentamidine, which served then as lead compound. The parasitic DNA is considered to be the main cellular target of pentamidine and its analogues. It could be shown, that these compounds bind to the minor groove with preference to AT-rich sites. By this binding, an interference with DNA-interacting enzymes is achieved. Since the plasmodium genome contains up to 80% adenine and thymidine, compared to 50% in humans, binding to the DNA of the pathogen is favoured. Additionally,

the curvature of the DNA double helix differs from parasite to host, and therefore molecules show different binding capacities.

Chemical modelling and subsequent screening activities revealed highly active pentamidine analogues against trypanosomes. One of the most promising molecules is the diphenylfuran DB 75, which showed an excellent activity against trypanosomes in vitro and in vivo. When tested on *P. falciparum* strain K1 in vitro, it exhibited a potent anti-malarial effect with an IC_{50} of 5.8 ng/ml.
Testing this compound in vivo in the *P. berghei* mouse model, a moderate to good activity of 87% with 23 days survival was achieved after application of 4 x 50 mg/kg s.c. After oral application of 4 x 100 mg/kg DB 289, the corresponding methoxyamidine prodrug, no significant effect on the parasite could be demonstrated (activity 42.4%, survival days 15.2 on average). Control mice died within 6 to 7 days after infection.

To find out, why DB 75 exhibits anti-plasmodial activity in the *P. berghei* mouse model and why this is not the case when its prodrug is administered, a pharmacokinetic study was performed. It was hoped to find a reasonable explanation for this discrepancy by studying the pharmacokinetics of DB 75 and DB 289 in the mouse.

2. Material and Methods

2. 1. Compounds and chemicals

2.1.1. Compounds and radiolabelled internal standards

DB 75, DB 289 and their stable isotopically labelled counterparts d_8-DB 75 and d_8-DB 289 were synthesised using methods previously described [Das, B.P. (1977)], [Boykin, D.W. (1996)], [Stephens, C.E. (2001)].

2.1.2. Chemicals

Tween 80 was obtained from Fluka (Buchs, Switzerland). Ethanol and NaCl were purchased from Merck (Dietikon, Switzerland), potassium fluoride from Sigma Aldrich (Basel, Switzerland) and heparin from Fresenius (Stans, Switzerland).
HPLC- grade acetonitrile, methanol, water and formic acid were obtained from Fisher Scientific (Chicago, IL, USA), ammonium formate and trifluoroacetic acid (TFA) from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Drug solutions

2.2.1. Drug solutions for treatment of mice

The calculated amount of drug was weighed (5 mg/ml DB 75 or 10 mg/ml DB 289) and dissolved in one tenth of the final volume of 70% Tween 80/30% ethanol, which was then 10-fold diluted with water resulting in 7% Tween 80 and 3% aqueous ethanol.

2.2.2. Standard curves and internal standard

These were prepared as described previously [see "Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice"].

2.3. Animal experiment

2.3.1. Animals

In all experiments female NMRI mice with a bodyweight of 20 to 22 g were used.

2.3.2. Administration of compounds

50 mg/kg of DB 75 subcutaneously, 100 mg/kg DB 289 per os, respectively, was administered to infected and uninfected mice.

2.3.4. Infection and animal care

Mice were infected intravenously on day 0 with the *P. berghei* strain ANKA. Blood from an infected mouse was diluted with physiological saline solution till 10^8 parasitised erythrocytes per ml were obtained. 0.2 ml of this solution was applied. Pharmacokinetic parameters were determined in an early stage of the infection (day 1). A parasitemia of around 1% is expected.

Animal use adhered to the guidelines issued by the Swiss Federal Veterinary Department (BVET) for laboratory animals.

Plasma samples were kindly provided from the MMV group at the Swiss Tropical Institute.

For information about harvesting of blood and plasma preparation, sample preparation, description of instruments and data analysis see "Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice".

3. Results

3.1. Comparison of plasma levels in infected and uninfected mice

3.1.1. Active compounds

DB 75 in plasma of uninfected and *P. berghei*-infected mice after s.c. application (Fig. 1)

Plasma concentrations in infected and uninfected mice were similar during the whole 24 hr observation period. No difference could be observed, neither in the amount of drug available for the organism, nor in the elimination process between infected and uninfected animals (Chapter 10, Appendix E, Table 1).

3.1.2. Prodrugs

DB 289 in plasma of uninfected and *P. berghei*-infected mice after p.o. application (Fig. 2)

Available amount of drug was twice as high in the infected animals compared to their uninfected counterparts. The AUMC even was 4 times higher in infected animals. It took twice the time to eliminate the prodrug from infected animals than from uninfected. C_{max} occurred at the same time point. Plasma levels of uninfected animals declined thereafter steadily. In infected animals a minimum after 6 hr was observed, which was followed by a big rise in the plasma concentration at 8 hr. Plasma levels declined thereafter, but a considerable amount of prodrug still was detectable in the plasma of infected mice after 24 hr (Chapter 10, Appendix E, Table 2).

3.2. Comparison of the conversion of the prodrug to the active drug

Conversion of DB 289 to DB 75 in uninfected (Fig. 3) and *P. berghei*-infected (Fig. 4) mice after orally administered DB 289

In either case, infected and uninfected, only a small amount of around 10% prodrug was activated to DB 75. After 24 hr, the highest plasma concentration of active compound was detected within the whole observation period of 24 hr in infected as well as in uninfected animals (Chapter 10, Appendix E, Table 3).

DB 75 in plasma of infected vs. uninfected mice after treatment with DB 289 p.o. (Fig. 5)

During the first 7 hr, plasma levels of activated compound were more or less the same in infected and uninfected animals. Within this period, plasma concentrations rose steadily. After 7 hr, DB 75 concentration in infected animals increased to a higher extent than in uninfected animals. This increase in infected animals led to the fact that around one fourth more active compound was at disposal over 24 hr than in the uninfected mice.

4. Discussion

4.1. Plasma levels of infected and uninfected mice

4.1.1. Active drugs

Infected animals showed the same plasma concentration course as uninfected animals. Accumulation of DB 75 in plasmodium was observed in vitro and in vivo at different stages of the parasite [Schild, N. (2003)]. Since there is no difference detectable between plasma concentrations in infected and uninfected animals it could be that this accumulation process is rather unimportant in relation to the total amount of available drug. It is also possible that this effect is masked by the increase of drug concentration in the plasma obtained by inhibition of the metabolism. Shertzer reported that a trypanosome infection is accompanied by a significantly impaired capacity of the liver to metabolise drugs via monooxygenation [Shertzer, H.G. (1981)]. The same may apply for plasmodia infected mice.

4.1.2. Prodrugs

Relevant differences between the pharmacokinetic profiles occurred not until the 6^{th} hr. At this time, plasma levels dropped and rose again thereafter in infected animals but not in uninfected. The reason for this behaviour is not known.

The large surplus of drug in infected animals compared to uninfected may be due to the inhibition of metabolism. Since enzymes like hepatic cytochrome P 450 and associated mixed-function oxidases may be inhibited, less of the prodrug would be converted to the active compound and therefore more DB 289 is detectable. Accumulation processes of the prodrug were not reported so far.

4.2. Transformation of the prodrug to the active drug

Activation of the prodrug was performed only to a small extent in either case (infected or uninfected). Comparing the conversion to the active compound, no difference in the amount

of drug available was visible up to 8 hr. After this time, concentration of DB 75 increased in both cases, but stronger in infected animals.
Lower plasma levels in infected animals were expected, because accumulation of DB 75 in the parasite may take place. The consequence of this accumulation process would be, that more prodrug is converted to the active drug, and therefore lower plasma levels would be assessable. As noticeable from the graphs, the opposite applied: higher plasma levels were obtained in infected animals. This indicated that the inhibition of the metabolism may play the more important role.

Conclusion

Plasma concentrations showed a great variability independent from the status of infection. Since a non-serial sampling method was used, this variability was expected. Each value represents an average of the plasma concentration obtained from three individual mice, possessing each a slightly different genetic background. Taking serial probes by cannulating the mice was not possible, because the amount of plasma needed for determination exceeded the total amount of plasma in one mouse.

Various factors change during an infection. One of these is an increase of various plasma proteins. For drug transport within the body, several classes of proteins must be taken into consideration. Generally, albumin is responsible for the transport of acidic drugs, lipoproteins for neutral drugs and alpha-1-acid glycoprotein (α-AGP) for bases.
An increase of plasma proteins like α-AGP would lead to higher plasma concentration of alkaline compounds like diamidines, since bound and free drug are assessed with the determination method of this pharmacokinetic study. Human α-AGP levels are known to be higher in malaria patients. Hochepied (2002) reported, that in mice during an acute phase reaction, like an infection is, concentrations of α-AGP increase 2-5 times from the normal level of 0.2-0.4 mg/ml, leading it to one of the dominant plasma proteins. α-AGP is assumed to bind the cationic DB 75 [Hochepied, T. (2002)]. Protein binding is known to be 70% in the case of DB 75 and 99% in the case of DB 289 [Hall J. E., personal communication].

The pharmacokinetic profile was influenced by the status of infection in two ways:

1. Possible accumulation of compound in the parasite

Accumulation of diamidine compounds in infected erythrocytes is known for several years now. In vitro, pentamidine was concentrated 500 fold by *P. falciparum* infected erythrocytes within 3 hr [Stead, A.M. (2001)]. No accumulation factors are known for DB 75 and DB 289 so far, but fluorescence microscopy indicated that DB 75 is accumulated in *P. falciparum* and *P. berghei* [Schild, N. (2003)]. The suggested mechanism of this uptake is a transport through the new permeability pathway (NPP), that is induced in the host erythrocyte membrane by the intracellular parasite. A pore system such as the NPP allows the equilibration of substrates, but not an accumulation. To concentrate the drug, transporters or receptors, specific and/or unspecific, are needed. One potential drug receptor could be ferriprotoporphyrin (FPIX), that is generated during the digestion of haemoglobin [Francis, S.E. (1997)]. By interacting (diamidine with FPIX), the conversion from FPIX, toxic to the parasite, to non-toxic hemozoin is inhibited. In vitro, pentamidine was shown to bind to FPIX. This binding would be essential for anti-malarial activity as already shown for other antimalarials like chloroquine.

2. Inhibition of metabolism

As discussed previously [“Pharmacokinetic studies of selected DB compounds in trypanosome infected and uninfected mice”], a decrease of metabolic activity in trypanosome infected mice by inhibition of mixed-function oxidases was reported by Shertzer [Shertzer, H.G. (1981)]. In a malaria infection an impaired oxidation, glucuronidation and biliary excretion in the isolated perfused rat liver was shown by Murdoch [Murdoch, R.T. (1991)].

This pharmacokinetic study was performed, to reveal why no cure could be achieved after administration of the prodrug DB 289 in *P. berghei* infected mice, whereas an activity for DB 75 could be demonstrated. It was expected that data obtained in this experiment could help to explain this phenomenon.

Conversion of DB 289 to its active form DB 75 also takes place in infected animals - even to a similar extent as in the uninfected animals. A possible explanation for the insufficient activity against plasmodium could be, that the amount of free drug in the plasma was not long enough available or even is too low to exhibit an anti-protozoal effect. Considering a protein binding of 70% for DB 75 less than one third of the measured amount of DB 75 is free in the

plasma (around 25 nM up to 115 nM in infected animals) and therefore responsible for the activity. IC_{50} values, determined in a time dose assay in vitro for strain K1 (*P. falciparum*) showed that an incubation with at least a concentration of 132.5 nM free compound for 1 hr is needed to be effective against 50% of the parasites [Schild, N. (2003)]. Prolonging the incubation time up to 4 hr, an IC_{50} of 103.4 nM was determined in vitro. To kill 50% of the parasites, when incubating the drug for 24 hr, 20.3 nM would be needed. In general, values 3-5 times higher than the IC_{50} would be needed to eliminate the parasites (minimal inhibitory concentration: MIC), what would be 60 to 100 nM for a period of 24 hr (Chapter 9, Appendix E, Table 4). Similar values were expected for *P. berghei*. Considering a plasma protein binding of 70%, this amount was just achieved. Taking higher plasma protein levels from the type α-AGP, which were proven to increase during a *P. falciparum* infection, into consideration, the amount of free drug in the plasma declines. Therefore the actual concentration of free drug could be below the curative dose.

Allen and co-workers reported that uptake of radiolabelled DB 75 was not observed in *P. berghei*, but in *P. falciparum* [Poster Allen J. (2005), Congress: Medicine and Health in the Tropics, Marseille]. They concluded that this may be the reason why no cure was achieved after application of DB 289 in the mouse.

Fluorescence microscopy revealed blue fluorescent structures in *P. berghei* infected erythrocytes extracted from a mouse treated with 40 mg/kg DB 75. These structures were identical to structures which were obtained when stained with the conventional DNA-dye Hoechst 33258 [Schild, N. (2003)]. Uninfected erythrocytes did not show fluorescence when inspected. The fluorescence indicated an accumulation of DB 75. This observation contradicts the findings of Allen and co-workers.
P. falciparum appeared to exhibit greater fluorescence than *P. berghei* [Schild, N. (2003)]. Probably, a lower accumulation in *P. berghei* infected erythrocytes occurs than in *P. falciparum* infected erythrocytes. This has to be subject for further investigations.

Another observation which indicated the accumulation of DB 75 in *P. berghei* infected erythrocytes is that the in vitro assay with erythrocytes isolated from a *P. berghei* infected mouse showed a similar IC_{50} as *P. falciparum* in vitro did [Wittlin S., personal

communication]. It is possible to keep *P. berghei* infected erythrocytes in culture for 24 hr, therefore the duration of the performed assay was reduced to 24 hr (instead of 72 hr) for both *P. berghei* and *P. falciparum* infected erythrocytes.

Another phenomenon which was observed during in vivo screening and which was surprising, was that the mean survival days varied a lot after DB 289 treatment: from 8 to 25 days after treatment with 4 x 100 mg/kg p.o, whereas the activity in % was more or less constant with around 42%. Such variations in the survival days were not observed after application of standard antimalarials.
Why these differences in the survival days were obtained is not known to date. It is assumed, that since plasma levels insufficient to kill the parasites completely were achieved, the general constitution (status of health and immune system) of the mouse may be relevant for longer survival. The velocity with which drugs act and parasites are killed may be influence the variability in survival days. Survival days do not spread that much when classical antimalarials were tested in the *P. berghei* mouse model. The variation of the survival days after treatment with chloroquine and artemether lies within a range of +/- 10%. Survival days after treatment with 1 x 10 mg/kg ranged in the case of chloroquine from 8 to 10 days (mean: 8.9 days), the ones of artemether from 9 and 11 days (mean 9.7 days) [Wittlin S., personal communication]. Both are known as fast-acting drugs.

Outlook:
In this study, we could demonstrate that the prodrug DB 289 was converted into DB 75 to the same extent in healthy as well as in infected mice. Unfortunately, only a small fraction of prodrug was converted to the active compound. This amount of active compound seemed to be insufficient to kill the parasites. Since prodrug is present in excess and the amount of converted prodrug is more or less constant over time, developing a different galenic formulation to deliver more prodrug is not necessary. An important step to improve the extent of conversion, for instance would be inducing the enzymes which are involved in the activation of the prodrug to the active compound.
Metabolism of DB 289 was elucidated by Zhou (2004), revealing enzymes like cytochrome (CYP) P 450 and cytochrome b_5 to play a key role [Zhou, L. (2004)], [Ansede, J.H. (2004)], [Saulter, J. (2005)]. The first step, the N-demethylation of DB 289 is catalysed by CYP P 450 1A2, 4F2, 4F3B. CYP 450 1A2 is known to be induced by phenobarbital, phenytoin,

rifampicin, ritonavir, polycyclic aromatic hydrocarbons found in cigarette smoke or charcoal broiled foods [Michalets, E.L. (1998)]. Cytochrome b5 reacts relatively insensitive to induction by exogenous drugs and chemicals [Gibson, G.G. (1994)]. In a first step, one has to clarify, which enzymatic reaction within the activation of the prodrug is the limiting step. Once this is known, interaction at the level of this enzyme may be possible and therefore higher levels of active compounds may be achieved in mice.

Conversion of prodrug to active compounds was demonstrated to be more efficient in rats. Therefore, the *P. berghei* mouse model may not be ideal to assess the effectiveness of DB prodrugs in vivo. In humans, the enzymes involved in this activation are even more effective [Hall J. E., personal communication]. This must be the reason for the promising results for DB 289 in the phase II trial in malaria patients, completed 2003 in Bangkok, with a cure rate of 96% [Yeramian, P. (2005)].

It could also be, that uptake of DB 75 in *P. berghei* infected erythrocytes is reduced compared to uptake in *P. falciparum* infected erythrocytes and that therefore insufficient levels of drug in the parasites were obtained.

Fig. 1: DB 75 in plasma of uninfected and *P. berghei*-infected mice after s.c. application

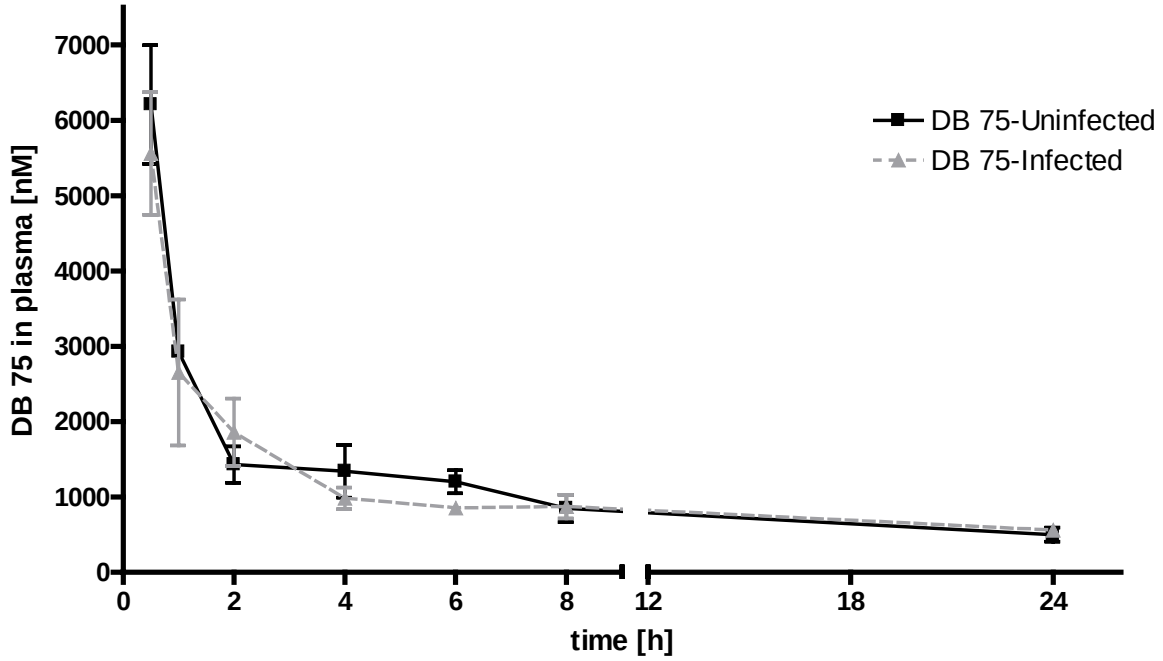


Fig. 2: DB 289 in plasma of uninfected and *P. berghei*-infected mice after p.o. application

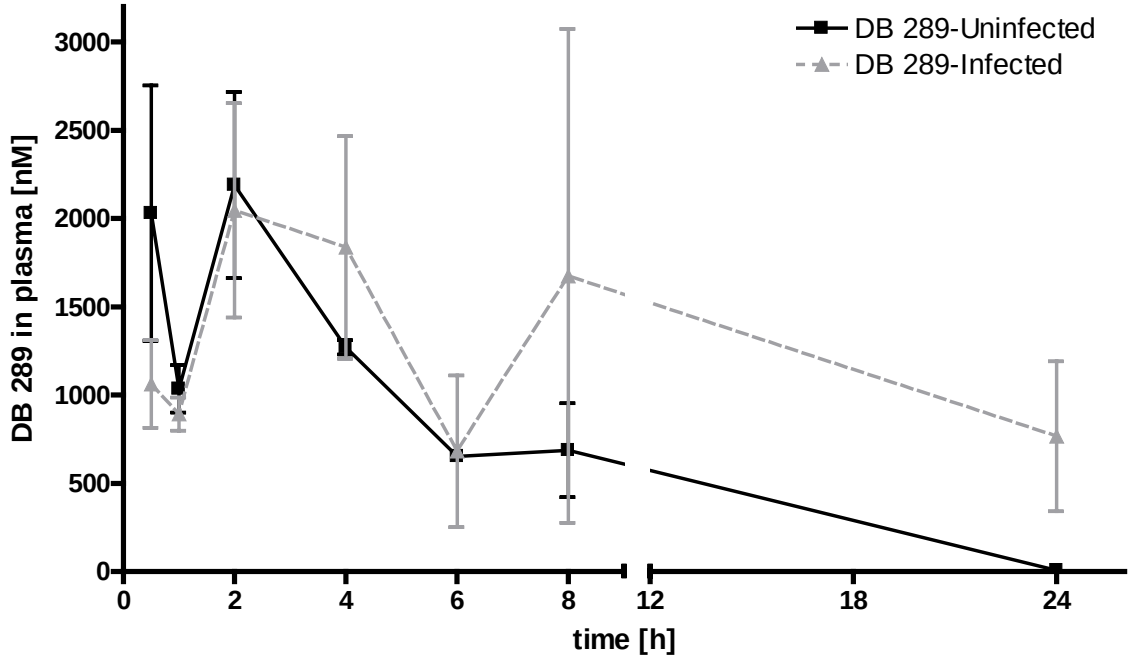


Fig. 3: Conversion of DB 289 to DB 75 in plasma of uninfected mice after orally administered DB 289

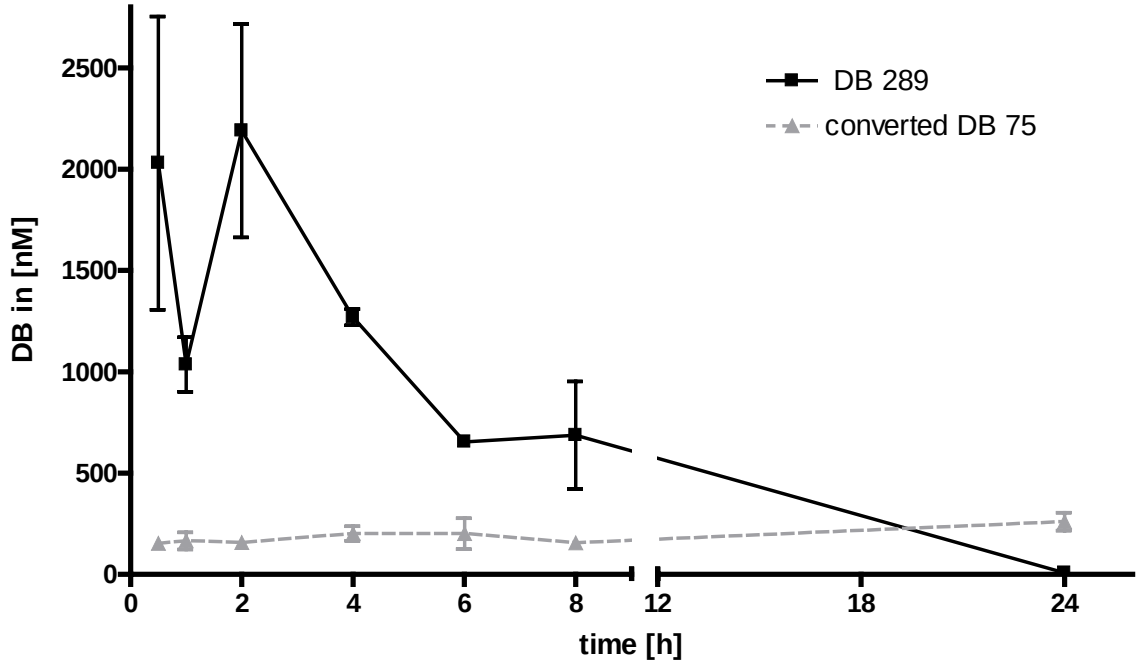


Fig. 4: Conversion of DB 289 to DB 75 in plasma of *P. berghei*-infected mice after orally administered DB 289

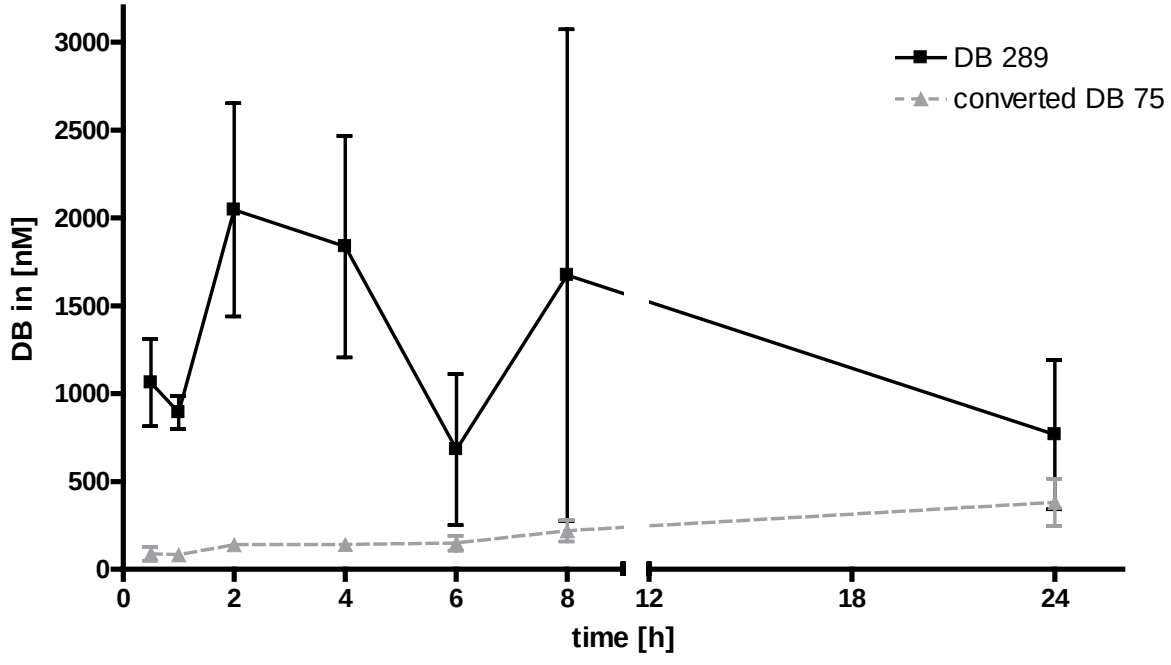
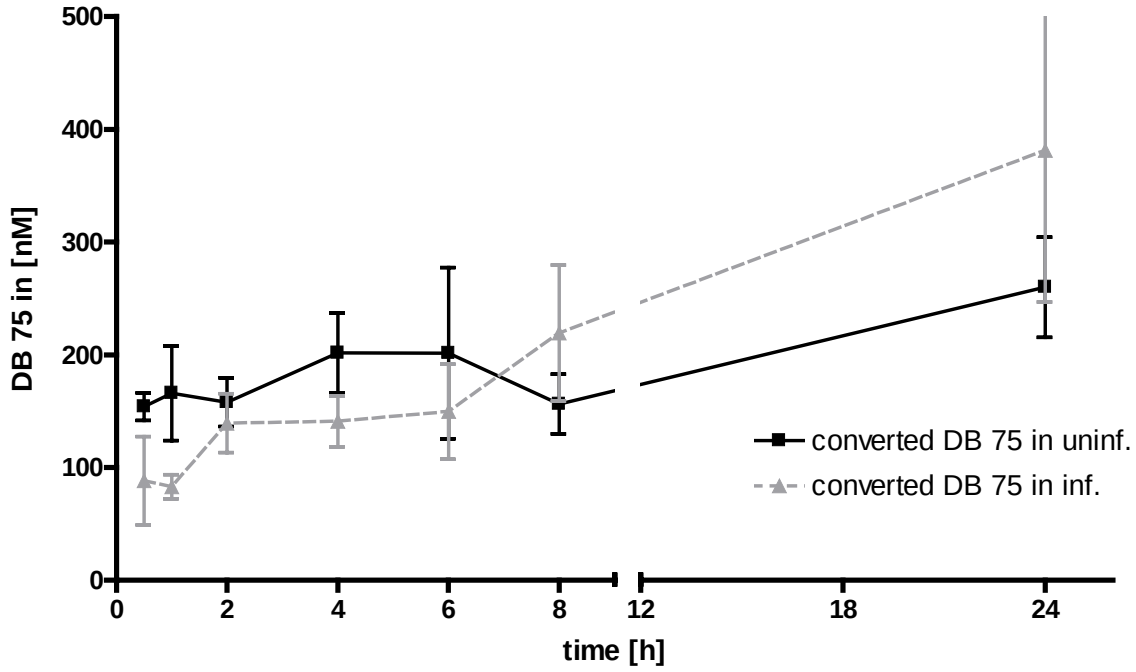


Fig. 5: DB 75 in plasma of uninfected and *P. berghei*-infected mice after treatment with DB 289 p.o.



References:

1. Ansede JH, Anbazhagan M, Brun R, Easterbrook JD, Hall JE, Boykin DW. O-alkoxyamidine prodrugs of furamidine: in vitro transport and microsomal metabolism as indicators of in vivo efficacy in a mouse model of *Trypanosoma brucei rhodesiense* infection. J Med Chem 2004; 47:4335-8.

2. Bell CA, Hall JE, Kyle DE, Grogl M, Ohemeng KA, Allen MA et al. Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. Antimicrob Agents Chemother 1990; 34:1381-6.

3. Boykin DW, Kumar A, Hall JE, Bender BC, Tidwell RR. Anti-Pneumocystis activity of Bis-amidoximes and Bis-O-Alkylamidoximes prodrugs. Bioorg Med Chem Letters 1996; 6:3017-20.

4. Das BP, Boykin DW. Synthesis and antiprotozoal activity of 2,5-bis(4-guanylphenyl)furans. J Med Chem 1977; 20:531-6.

5. Francis SE, Sullivan DJ, Jr., Goldberg DE. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. Annu Rev Microbiol 1997; 51:97-123.

6. Gibson GG, Skett P, . Introduction to Drug Metabolism. Blackie Academic & Professional 1994.

7. Hatz C, Abdulla S, Mull R, Schellenberg D, Gathmann I, Kibatala P et al. Efficacy and safety of CGP 56697 (artemether and benflumetol) compared with chloroquine to treat acute falciparum malaria in Tanzanian children aged 1-5 years. Trop Med Int Health 1998; 3:498-504.

8. Hochepied T, Wullaert A, Berger FG, Baumann H, Brouckaert P, Steidler L et al. Overexpression of alpha(1)-acid glycoprotein in transgenic mice leads to sensitisation to acute colitis. Gut 2002; 51:398-404.

9. Michalets EL. Update: clinically significant cytochrome P-450 drug interactions. Pharmacotherapy 1998; 18:84-112.

10. Murdoch RT, Ghabrial H, Mihaly GW, Morgan DJ, Smallwood RA. Malaria infection impairs glucuronidation and biliary excretion by the isolated perfused rat liver. Xenobiotica 1991; 21:1571-82.

11. Saulter J, Kurian J, Trepanier L, Tidwell R, Bridges A, Boykin D et al. Unusual Dehydroxylation of Antimicrobial Amidoxime Prodrugs by Cytochrome b5 and NADH Cytochrome b5 Reductase. Drug Metab Dispos 2005.

12. Schild, N. DB 75: A Diamidine with Antiplasmodial Activity: Uptake and Activity in *P. falciparum* and *P. berghei*. 2003. Ref Type: Thesis/Dissertation

13. Shertzer HG, Hall JE, Seed JR. Hepatic mixed-function oxidase activity in mice infected with *Trypanosoma brucei gambiense* or treated with trypanocides. Mol Biochem Parasitol 1981; 3:199-204.

14. Stead AM, Bray PG, Edwards IG, DeKoning HP, Elford BC, Stocks PA et al. Diamidine compounds: selective uptake and targeting in *Plasmodium falciparum*. Mol Pharmacol 2001; 59:1298-306.

15. Stephens CE, Patrick DA, Chen H, Tidwell RR, Boykin DW. Synthesis of Deuterium-Labelled 2,5-Bis(4-amidinophenyl)furan, 2,5-Bis[4-(methoxyamidino)phenyl]furan, and 2,7-Diamidinocarbazole. J Labelled Cpd and Radiopharm 2001; 44:197-208.

16. Yeramian P, Meshnick SR, Krudsood S, Chalermrut K, Silachamroon U, Tangpukdee N et al. Efficacy of DB289 in Thai patients with *Plasmodium vivax* or acute, uncomplicated *Plasmodium falciparum* infections. J Infect Dis 2005; 192:319-22.

17. Zhou L, Thakker DR, Voyksner RD, Anbazhagan M, Boykin DW, Hall JE et al. Metabolites of an orally active antimicrobial prodrug, 2,5-bis(4-amidinophenyl)furan-bis-O-methylamidoxime, identified by liquid chromatography/tandem mass spectrometry. J Mass Spectrom 2004; 39:351-60.

General discussion

This Ph. D. thesis was embedded in an international project of a public-private-partnership with the aim to develop new drugs for the treatment of human African trypanosomosis. In this thesis, several facets of the lead compound DB 75 have been under investigation. Data at hand, resulting from various studies, helps to draw a detailed picture of the behaviour of this drug not only in vitro but also in vivo in mice. Additionally, findings for DB 75 and its prodrug DB 289 may be applicable in lead optimisation. Understanding of the lead, facilitates the selection for new compounds when a rational drug design like molecular modelling and/or target research is performed.

This project started with an experiment which helped to understand the complex interplay between mice, trypanosomes and drug treatment. Thereto, a mouse study was performed, where the development of a first infection, of a first and of a second relapse parasitaemia was observed in immunocompetent and immunodeficient mice. Additionally, parasite clearance after drug treatment and duration of the aparasitic period was monitored in both immunocompetent and -deficient mice. Based on the data collected a precise description of the impact of host and parasite factors in a developing parasitaemia was possible. Understanding the kinetics of parasitaemia in trypanosome infected mice without and with DB 75 treatment is a pre-requisite in any study where DB 75 administered to mice is involved. By comparing morphology, size distribution and sensitivity to selected drugs of trypanosomes isolated from each stage like first infection, first relapse or second relapse, changes due to interaction with the host and/or the treatment could be assessed. The obtained results clearly showed no differences between the various trypanosome populations. The conclusion of these results was, that reappearance of trypanosomes after drug treatment with DB 75 was not due to surviving resistant trypanosomes, but rather due to invasion from extravascular sites (niches). Therefore, the next step was the identification of those niches. A method amplifying and detecting mRNA of trypanosomes was established. It was possible to gain first results, but unfortunately, limitations in time did not allow improvements of the evaluated method and therefore prevented the complete identification of those niches. However, it could be shown that the method itself worked. More investments on the

extraction method leading to an increase of the RNA yield and improvement of the sensitivity of the detection method are required.
After having improved this method, it would also be possible to perform studies with the aim to find out at what stage of the infection passage of the blood brain barrier is likely taking place. This could be performed with different trypanosomes strains like the GVR 35 (*T. brucei brucei*) which leads to an chronic infection and is used when compounds were tested for treatment of second stage trypanosomosis, and the STIB 900, leading to an acute infection. Since relapses also occur in the African green monkey model, identification of possible niches may be detected taking results of the mouse model into consideration.

Cross resistance is well documented for drugs used to treat trypanosomosis. Therefore, new drug candidates, especially drugs which were synthesised as analogues to drugs already on the market and from which drug resistance already had been reported, should be tested in an early stage of their development for their cross resistance to existing drugs. Cross resistances which may be monitored in laboratory strains may also occur in the field.
Trypanosomes to which either melarsoprol or pentamidine resistance was induced in vitro were shown to maintain their sensitivity towards DB 75 to a certain extent. This finding is rather surprising, since transport for DB 75 is assumed to happen via the P2 transporter. Alterations of the P2 transporter were shown to be important in melarsoprol and pentamidine resistant strains. The moderate cross resistance to DB 75 may indicate that additional transporter like the P1 transporter, which do not transport pentamidine, may be responsible for drug uptake.
Laboratory induced resistance of STIB 900 trypanosomes to melarsoprol and pentamidine, delivers a new biological tool to check for cross resistance of newly synthesised drugs and may therefore be an important tool in lead optimisation.

In a second approach to understand the interplay between mice, trypanosomes and drug, pharmacokinetics of DB 75 in infected and uninfected animals was studied. To complete the picture, the same study was performed with the corresponding prodrug DB 289 and also with the closely related drug candidate DB 820 and its prodrug DB 844. By performing the pharmacokinetic studies also with DB 820, DB 844 it could be shown that minor changes in structure like the replacement of one phenyl moiety within the spacer region by one pyridyl moiety may result in big differences referring to the pharmacokinetic profile.

Metabolic activation of the prodrugs and reduction of plasma levels of free compound due to host and/or parasite factors were found to play an important role. From the side of the host, plasma protein binding of the drug has to be considered, whereas parasites interact by accumulating active compound and/or by inhibiting metabolism. The drug itself may also induce the inhibition of metabolism.
These studies clearly showed that predictions concerning plasma levels are not easy to make since all mentioned factors had an influence on pharmacokinetic parameters. The extent of the influence of these parameters depended on the host, the parasite and on drug related properties.

In an in vitro study, the extent of a possible accumulation of DB 75 in trypanosomes was assessed. This was done to find out, whether the difference obtained between plasma levels of DB 75 after DB 289 application in infected mice compared to the one in uninfected mice may be explained by accumulation of the active compound. Data obtained in this study showed that an accumulation of DB 75 in vitro up to one million fold is possible what is sufficient to explain the detected differences between infected and uninfected animals. When assessing the dosage of a treatment, possible accumulation in parasites have to be considered, otherwise application of subcurative doses would be the consequence. Not all DB compounds were observed to accumulate to the same extent, therefore influence of infection had to be assessed preferably for each compound.

In another study related to the pharmacokinetic studies, the presence of active metabolites after DB 75, DB 289 and DB 820, DB 844 application was assessed using the bioassay. Anti-trypanosomal activity was determined as equivalent of active drug and compared to the amount of active drug obtained by HPLC/MS/MS method. For each of the compounds applied, indications for the presence of at least one active metabolite were found. Further studies are urgently needed to verify these results and to identify possible metabolites. May be one of those metabolites may serve as new lead. Thereto, this metabolite has to be synthesisable, stable and exhibit a stronger anti-parasitic activity than the parent compound.

Pharmacokinetic studies in *P. berghei* infected mice, were performed to find an explanation why application of the prodrug DB 289 did not lead to a significant reduction (~ 40% reduction) in parasites as the application of the active compound DB 75 (~ 85% reduction)

does. Analysis of the obtained pharmacokinetic profiles revealed that the conversion of the prodrug to the active drug was insufficient. Plasma levels of active drugs converted from prodrugs did not reach the required therapeutical dose as a comparison with an in vitro time dose assay showed. This result questions the *P. berghei* mouse model to be adequate when testing DB prodrugs for their activity. Experiments in rats showed that the enzyme(s) which were responsible for conversion of the prodrug to the active compound act more efficiently. In humans, prodrug is reported to be readily converted to the active drug [Boykin, D.W. (1996)]. This may explain the high cure rate of 96% obtained in an efficacy trail for DB 289 in *P. falciparum* and *P. vivax* infected patients conducted in Thailand [Yeramian, P. (2005)].

At time, DB 289 is undergoing phase III clinical trials for treatment of first stage trypanosomosis at multiple sites in the Democratic Republic of Congo, Sudan, and Angola. During the Phase III trial, it is aimed to submit for the status of the so called Treatment IND (Treatment Investigational New Drugs) to the U.S. FDA for approval which, if granted, would enable DB 289 to be sold for the treatment of trypanosomiasis in desperately ill patients while the clinical trials are being completed [www.immtech-international.com/documents/news_aug_25_2005.pdf].

In the case of malaria, enrolment for clinical trial phase II in Bangkok started in May 2005. The trial conducted by Immtech International in collaboration with the Medicines for Malaria Venture (MMV) will compare the effectiveness of three-day dose regimens of DB 289 given as mono-therapy and in combination with artesunate.

References:

1. Boykin DW, Kumar A, Hall JE, Bender BC, Tidwell RR. Anti-Pneumocystis activity of Bis-amidoximes and Bis-O-Alkylamidoximes prodrugs. Bioorg Med Chem Letters 1996; 6:3017-20.

2. Yeramian P, Meshnick SR, Krudsood S, Chalermrut K, Silachamroon U, Tangpukdee N et al. Efficacy of DB289 in Thai patients with *Plasmodium vivax* or acute, uncomplicated *Plasmodium falciparum* infections. J Infect Dis 2005; 192:319-22.

Table 1: Data hypoxanthine assay group A

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
A NMRI K	900	1	14	50	2.16	4.18
		2	20	58	2.57	5.35
		3	13	44	2.18	5.31
A NMRI KR		1	13	24	3.54	11.33
		2	21	39	3.34	7.61
		3	12	38	2.17	5.73
A NMRI KSt		1	9	16	2.66	8.06
		2	12	37	7.03	5.06
		3	17	56	3.26	4.73
average			**14.56**	**40.22**	**3.21**	**6.37**
stand. dev.			**3.97**	**13.88**	**1.53**	**2.26**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
A SCID K	900	1	13	25	4.44	1.82
		2	15	34	3.14	5.7
		3	12	28	4.01	4.25
A SCID R		1	8	11	1.48	2.58
		2	16	32	3.01	5.74
		3	16	32	4.15	5.95
A SCID KSt		1	19	37	3.57	8.07
		2	15	19	2.4	7.07
		3	13	41	1.81	10.37
average			**14.11**	**28.78**	**3.11**	**5.73**
stand. dev.			**3.1**	**9.3**	**1.04**	**2.65**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
A NMRI R	795	1	4	6	2.7	3.98
		2	10	18	5.23	9.36
		3	6	10	3.92	7.55
A NMRI KR		1	7	9	3.63	5.49
		2	11	19	4.97	9.84
		3	7	14	4.75	8.52
A NMRI KSt		1	3	5	2.1	4.23
		2	9	16	4.61	7.81
		3	6	11	4.51	7.57
average			**7**	**12**	**4.05**	**7.15**
stand. dev.			**2.65**	**5.05**	**1.06**	**2.12**

Table 2: Data hypoxanthine assay group B

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
B NMRI R	900	1	19	50	2.85	6.83
		2	11	25	2.04	4.43
		3	10	39	2.31	9.69
B NMRI St		1	17	50	4.38	7.13
		2	8.54	33.71	2.18	7.43
		3	22	97	3.55	7.06
B NMRI KSt		1	15	50	2.41	6.03
		2	23	98	3.15	4.77
		3	11.61	75	1.7	6.1
average			**15.24**	**57.52**	**2.73**	**6.61**
stand. dev.			**5.32**	**26.55**	**0.84**	**1.56**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
B SCID St	900	1	10	45	3.17	4.86
		2	19	38	2.85	5.94
		3	15	72	2.53	7.33
B SCID KR		1	12	62	3.35	4.68
		2	14	66	2.63	3.88
		3	19	18	3.21	7.94
B SCID KSt		1	25	80	3.12	6.2
		2	10	20	2.67	4.69
		3	7	25	2.78	3.34
average			**14.56**	**47.33**	**2.92**	**5.43**
stand. dev.			**5.64**	**23.55**	**0.29**	**1.54**

Table 3: Data hypoxanthine assay group C

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
C NMRI K	900	1	14	67	4.05	4.96
		2	8	53	3.51	6.25
		3	10.05	28.4	2.15	5.22
C NMRI R		1	8	66	3.58	4.96
		2	18	56.9	3.19	6.25
		3	11	34	4.06	5.22
C NMRI KSt		1	11	41.74	4.56	7.66
		2	13.84	54.6	4.03	10.47
		3	12.65	62.58	3.19	9.21
average			**11.84**	**51.58**	**3.59**	**6.69**
stand. dev.			**3.19**	**13.92**	**0.7**	**2.01**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
C SCID K	900	1	14	33	10.62	1.9
		2	19	41	2.7	4.69
		3	10	16	1.68	2.57
C SCID St		1	23	64	5.61	4.91
		2	12	24	2.17	3.36
		3	16	19	1.89	6.46
C SCID KSt		1	11	21	1.73	3.82
		2	12	46	2.18	3.9
		3	17	64	2.82	3.79
average			**14.89**	**36.44**	**3.49**	**3.93**
stand. dev.			**4.26**	**18.57**	**2.93**	**1.34**

Table 4: Data hypoxanthine assay group D

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
D NMRI K	900	1	6	76	3	4.61
		2	9	30	2.13	6.75
		3	19	62	1.93	4.73
D NMRI St		1	11	88	3.71	5.03
		2	16	66	2.13	9.2
		3	19	84	3.26	6.42
D NMRI KSt		1	10	61	3.98	5.26
		2	20	72	2.57	6.71
		3	18	74	3.04	5.32
average			**14.22**	**68.11**	**2.86**	**6**
stand. dev.			**5.24**	**16.97**	**0.72**	**1.46**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
D SCID K	900	1	15	25	2.53	6.67
		2	10	17	2.39	7.14
		3	20	73	2.24	11.17
D SCID St		1	14	28	3.27	6.6
		2	21	54	2.4	5.95
		3	15	70	2.28	4.62
D SCID KR		1	21	37	1.84	14.25
		2	11	75	2.66	4.52
		3	21	55.24	2.49	8.64
average			**16.44**	**48.25**	**2.46**	**7.73**
stand. dev.			**4.42**	**22.18**	**0.38**	**3.18**

Table 5: Data hypoxanthine assay group E

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
E NMRI K	900	1	9	21	3.62	10.56
		2	12.8	64.7	1.8	7.23
		3	14.2	57.2	2.71	9.02
E NMRI R		1	23	36	2.98	7.78
		2	19	85	2.92	6.85
		3	21	54	1.9	3.73
E NMRI St		1	19	79	4.22	5.6
		2	18.35	64.13	3.06	4.94
		3	11.52	38.86	3.76	3.6
average			**16.43**	**55.54**	**3**	**6.59**
stand. dev.			**4.72**	**20.72**	**0.81**	**2.36**

mouse	tryp. strain	replicate	IC_{50} DB 75	IC_{50} diminazene	IC_{50} melarsoprol	IC_{50} pentamidine
E SCID K	900	1	6	29	2.26	4.63
		2	13	34	3.07	8.49
		3	22	39	2.38	5.13
E SCID KR		1	25	33	3.35	9.08
		2	14	28	2.67	5.03
		3	9	47	1.52	4.18
E SCID		1	21	77	1.87	4.59
		2	7	60	3.34	6.6
		3	8	28	3.72	6.43
average			**13.89**	**41.67**	**2.69**	**6.02**
stand. dev.			**7.15**	**16.91**	**0.74**	**1.77**

RAW DATA

Pharmacokinetic parameters of active compounds:

Table 1: DB 75

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	13090	10397	+ 26 %
t_{max} [h]	0.25	0.25	
AUC	26222	21523	+ 21.8 %
AUMC	158506	108340	+ 46.3 %
MRT [h]	6.04	5.03	+ 19.8 %
$t_{1/2}$ [h]	4.2	3.5	+ 20 %
k_{el} (1/MRT)	0.17	0.2	- 15 %

Table 2: DB 820

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	21420	16607	+ 29 %
t_{max} [h]	0.25	0.25	
AUC	33889	21635	+ 56.6 %
AUMC	109161	90282	+ 20.9 %
MRT [h]	3.22	4.17	- 22.8 %
$t_{1/2}$ [h]	2.2	2.9	- 24.1 %
k_{el} (1/MRT)	0.31	0.24	+ 29.2 %

Pharmacokinetic parameters of prodrugs:

Table 3: DB 289

parameter	infected mice	uninfected mice	inf/uninf.
C_{max} [nM]	981	1963	- 50 %
t_{max} [h]	2	4	!
AUC	8996	16106	- 44 %
AUMC	48143	91889	- 48 %
MRT [h]	5.35	5.71	- 7 %
$t_{1/2}$ [h]	3.7	4.0	- 7.5 %
k_{el} (1/MRT)	0.19	0.18	+ 5.6 %

Table 4: DB 844

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	7950	8823	- 9.9 %
t_{max} [h]	1	1	
AUC	42717	56763	- 24.7 %
AUMC	236473	327675	- 27.8 %
MRT [h]	5.54	5.77	- 4 %
$t_{1/2}$ [h]	3.8	4.0	- 5 %
k_{el} (1/MRT)	0.18	0.17	+ 5.9 %

Pharmacokinetic parameters of active compounds after prodrug application:

Table 5: DB 75 after DB 289

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	255.7	413	- 38.1 %
t_{max} [h]	4	4	
AUC	4565	6681	- 31.7 %
AUMC	48688	67429	- 27.8 %
MRT [h]	10.66	10.09	+ 5.6 %
$t_{1/2}$ [h]	7.4	7	+ 5.7 %
k_{el} (1/MRT)	0.09	0.1	- 10 %

Table 6: DB 820 after DB 844

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	431	371	+ 16.2 %
t_{max} [h]	8	8	
AUC	7254	6224	+ 16.5 %
AUMC	71441	62070	+ 15.1 %
MRT [h]	9.85	9.97	- 0.1 %
$t_{1/2}$ [h]	6.8	6.9	- 1.4 %
k_{el} (1/MRT)	0.10	0.10	

Pharmacokinetic parameters comparing analogues:

Table 7: DB 75 - DB 820 in uninfected mice

parameter	uninfected mice DB 75	uninfected mice DB 820	820/75
C_{max} [nM]	10397	16607	+ 59.7
t_{max} [h]	0.25	0.25	
AUC	21523	21635	+ 5.2 %
AUMC	108340	90282	- 16.7 %
MRT [h]	5.03	4.17	- 17.1 %
$t_{1/2}$ [h]	3.5	2.9	- 17.1 %
k_{el} (1/MRT)	0.20	0.24	+ 20 %

Table 8: DB 75 - DB 820 in infected mice

parameter	infected mice DB 75	infected mice DB 820	820/75
C_{max} [nM]	13090	21420	+ 63.6 %
t_{max} [h]	0.25	0.25	
AUC	26222	33889	+ 29.2 %
AUMC	158506	109161	- 31.1 %
MRT [h]	6.04	3.22	- 46.7 %
$t_{1/2}$ [h]	4.2	2.2	- 47.6 %
k_{el} (1/MRT)	0.17	0.31	+ 82.4 %

Table 9: DB 289 – DB 844 in uninfected mice

parameter	uninfected mice DB 289	uninfected mice DB 844	844/289
C_{max} [nM]	1963	8823	x 4.5
t_{max} [h]	4	1	!
AUC	16106	56763	3.5 x
AUMC	91889	327675	3.5 x
MRT [h]	5.71	5.77	+ 1.1 %
$t_{1/2}$ [h]	4.0	4.0	
k_{el} (1/MRT)	0.18	0.17	- 5.6 %

Table 10: DB 289 – DB 844 in infected mice

parameter	infected mice DB 289	infected mice DB 844	844/289
C_{max} [nM]	981	7950	8 x
t_{max} [h]	2	1	!
AUC	8996	42717	4.7 x
AUMC	48143	236473	4.9 x
MRT [h]	5.35	5.54	+ 3.6 %
$t_{1/2}$ [h]	3.7	3.8	+ 2.7 %
k_{el} (1/MRT)	0.19	0.18	- 5.3 %

Pharmacokinetic parameters of actives compounds after conversion:

Table 11: Converted DB 75 and DB 820 in uninfected mice

parameter	DB 75 uninf.	DB 820 uninf	820/75
C_{max} [nM]	413	371	+ 10.17 %
t_{max} [h]	4	8	!
AUC	6681	6224	6.8 %
AUMC	67429	62070	7.9 %
MRT [h]	10.09	9.97	- 1.2 %
$t_{1/2}$ [h]	7	6.9	- 1.4 %
k_{el} (1/MRT)	0.1	0.10	

Table 12: Converted DB 75 and DB 820 in infected mice

parameter	DB 75 inf.	DB 820 inf.	820/75
C_{max} [nM]	255.7	431	1.7 x
t_{max} [h]	4	8	!
AUC	4565	7254	1.6 x
AUMC	48688	71441	1.5 x
MRT [h]	10.66	9.85	- 7.6 %
$t_{1/2}$ [h]	7.4	6.8	- 8.1 %
k_{el} (1/MRT)	0.09	0.10	+ 11.1 %

Pharmacokinetic parameters comparing application routes:

Table 13: DB 75 i.p. vs. s.c.

parameter	uninfected mice i.p.	uninfected mice s.c.	s.c./i.p.
C_{max} [nM]	10397	13667	+ 31.5 %
t_{max} [h]	0.25	0.25	
AUC	21523	58390	2.7 x
AUMC	108340	312666	2.9 x
MRT [h]	5.03	5.35	+ 6.4 %
$t_{1/2}$ [h]	3.5	3.7	+ 5.7 %
k_{el} (1/MRT)	0.2	0.19	- 5 %

Table 14: DB 820 i.p. vs. s.c.

parameter	uninfected mice i.p.	uninfected mice s.c.	s.c./i.p.
C_{max} [nM]	16607	36533	2.2 x
t_{max} [h]	0.25	0.25	
AUC	21635	94971	4.4 x
AUMC	90282	449591	5 x
MRT [h]	4.17	4.73	+ 13.4 %
$t_{1/2}$ [h]	2.9	3.3	+ 13.8 %
k_{el} (1/MRT)	0.24	0.21	- 12.5 %

Table 15: Pharmacokinetic parameters: DB 75 s.c. vs. DB 820 s.c.

parameter	uninfected mice DB 75 s.c.	uninfected mice DB 820 s.c.	820/75
C_{max} [nM]	13667	36533	2.7 x
t_{max} [h]	0.25	0.25	
AUC	58390	94971	+ 63 %
AUMC	312666	449591	+ 44 %
MRT [h]	5.35	4.73	- 11.6 %
$t_{1/2}$ [h]	3.7	3.3	- 10.8 %
k_{el} (1/MRT)	0.19	0.21	+ 10.1 %

Table 1: Average amount of DB 75 in supernatant after various incubation periods

Average amount of DB 75 in ng/ml, measured in the supernatant of trypanosomes solutions containing various densities of trypanosomes after 1 hour incubation with DB 75:

DB 75 in ng/ml	DB 75 in ng/ml in supernatant after incubation with 1*10e4 tryps/well	DB 75 in ng/ml in supernatant after incubation with 2*10e5 tryps/well	DB 75 in ng/ml in supernatant after incubation with 1*10e6 tryps/ well
250	87.11	81.15	105.64
500	232.66	219.41	286.75
1000	807.80	556.88	890.43
2500	1280.71	1181.23	1624.16
5000	2799.84	2571.28	3574.72
10000	6084.25	5718.80	7006.90

Average amount of DB 75 in ng/ml, measured in the supernatant of trypanosomes solutions containing various densities of trypanosomes after 2 hours incubation with DB 75:

DB 75 in ng/ml	DB 75 in ng/ml in supernatant after incubation with 1*10e4 tryps/well	DB 75 in ng/ml in supernatant after incubation with 2*10e5 tryps/well	DB 75 in ng/ml in supernatant after incubation with 1*10e6 tryps/ well
250	91.01	85.00	101.21
500	229.31	215.13	244.98
1000	727.75	693.26	695.83
2500	1219.77	1145.21	1370.34
5000	2690.38	2429.93	2937.86
10000	5725.07	5327.30	6096.31

Average amount of DB 75 in ng/ml, measured in the supernatant of trypanosomes solutions containing various densities of trypanosomes after 4 hours incubation with DB 75:

DB 75 in ng/ml	DB 75 in ng/ml in supernatant after incubation with 1*10e4 tryps/well	DB 75 in ng/ml in supernatant after incubation with 2*10e5 tryps/well	DB 75 in ng/ml in supernatant after incubation with 1*10e6 tryps/ well
250	128.97	116.13	104.23
500	315.88	262.93	257.27
1000	1064.96	708.40	788.54
2500	1601.36	1349.09	1444.48
5000	3089.27	3050.45	3111.59
10000	6469.27	6474.27	6461.84

RAW DATA BIOASSAY I

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.25	WU01	9190	13904.55	30.0	1.5
0.25	WU02	10620	30969.23	30.0	2.9
0.25	WU03	11380	26545.05	30.0	2.3
0.5	WU04	7720	16011.30	20.0	2.1
0.5	WU05	4560	5688.23	10.0	1.2
0.5	WU06	3600	2067	10.0	0.6
1	WU07	1400	954	10.0	0.7
1	WU08	1760	1391.25	5.0	0.8
1	WU09	2850	3686.81	5.0	1.3
2	WU10	689	429.3	3.0	0.6
2	WU11	786	906.3	1.0	1.2
2	WU12	1560	2544	5.0	1.6
4	WU13	482	620.1	1.0	1.3
4	WU14	329	341.85		1.0
4	WU15	447	333.9	1.0	0.7
8	WU16	277	318		1.1
8	WU17	273	413.4		1.5
8	WU18	242	389.55		1.6
24	WU19	297	294.15		1.0
24	WU20	257	532.65		2.1
24	WU21				

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.25	WI01	16420	26966.4	30.0	1.6
0.25	WI02	13240	40449.6	30.0	3.1
0.25	WI03	9610	30969.23	30.0	3.2
0.5	WI04	5090	12640.5	20.0	2.5
0.5	WI05	5120	11587.13	20.0	2.3
0.5	WI06	6520	10533.75	20.0	1.6
1	WI07	1580	1311.75	1.0	0.8
1	WI08	1800	2424.75	5.0	1.3
1	WI09	1930	1208.4	4.0	0.6
2	WI10	1850	7695.6	4.0	4.2
2	WI11	803	1073.25	3.0	1.3
2	WI12	1960	3458.25	5.0	1.8
4	WI13	442	1351.5	1.0	3.1
4	WI14	455	882.45		1.9
4	WI15	403	771.15		1.9
8	WI16	340	1256.1	1.0	3.7
8	WI17	294	969.9		3.3
8	WI18	826	1892.1	1.0	2.3
24	WI19	318	604.2		1.9
24	WI20	387	286.2	1.0	0.7
24	WI21				

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.5	XU22				
0.5	XU23	83.1	166.95	1.5	2.0
0.5	XU24	135	1081.2		8.0
1	XU25	207	437.25		2.1
1	XU26	165	198.75		1.2
1	XU27	618	190.8		0.3
2	XU28	519	1637.16	1.0	3.2
2	XU29	234	302.1		1.3
2	XU30	263	127.2		0.5
4	XU31	477	818.85		1.7
4	XU32	441	159	1.0	0.4
4	XU33	322	198.75		0.6
6	XU34	221	190.8		0.9
6	XU35	3970	2067	10.0	0.5
6	XU36	191	143.1		0.7
8	XU37	383	739.35		1.9
8	XU38	322	214.65		0.7
8	XU39	223	477		2.1
24	XU40	202	437.25		2.2
24	XU41	214	262.35		1.2
24	XU42	150	405.45	1.5	2.7

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.5	XI22	64.3	198.75		3.1
0.5	XI23	19.6	262.35		13.4
0.5	XI24				
1	XI25	148	321.98	1.5	2.2
1	XI26	140	206.7		1.5
1	XI27	191	182.85		1.0
2	XI28	174	198.75		1.1
2	XI29	228	381.6		1.7
2	XI30	207	206.7		1.0
4	XI31	229	771.15		3.4
4	XI32	217	516.75		2.4
4	XI33	321	278.25		0.9
6	XI34	189	890.4		4.7
6	XI35	198	270.3		1.4
6	XI36	243	341.85		1.4
8	XI37	211	500.85		2.4
8	XI38	275	842.7		3.1
8	XI39	199	691.65	1.5	3.5
24	XI40	177	174.9		1.0
24	XI41	89.9	333.9		3.7
24	XI42	154	166.95		1.1

sample codes:

W= treatment with DB 75 **X= treatment with DB 289** **U= uninfected mice**

Y= treatment with DB 820 **Z= treatment with DB 844** **I= infected mice**

ratios bigger than a factor of 2 are highlighted

RAW DATA BIOASSAY II

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.25	YU01	17480	20165.93	30.0	1.2
0.25	YU02	16000	30246.90	30.0	1.9
0.25	YU03	11380	17551.83	30.0	1.5
0.5	YU04	6600	29502.01	30.0	4.5
0.5	YU05	4740	3983.39	20.0	0.8
0.5	YU06	3600	4232.36	10.0	1.2
1	YU07	23.2	336.11	20.0	14.5
1	YU08	2070	8555.54	5.0	4.1
1	YU09	2050	1271.09	10.0	0.6
2	YU10	864	501.1	5.0	0.6
2	YU11	719	549.99	1.0	0.8
2	YU12	650	757.76	3.0	1.2
4	YU13	301	110	1.0	0.4
4	YU14	351	281.11		0.8
4	YU15	244	391.1		1.6
8	YU16	277	342.22		1.2
8	YU17	256	110		0.4
8	YU18	187	488.88		2.6
24	YU19	168	452.21		2.7
24	YU20	197	220		1.1
24	YU21	182	1307.75	1.0	7.2

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.25	YI01	19940	22406.59	30.0	1.1
0.25	YI02	12920	35477.10	30.0	2.7
0.25	YI03	31400	22406.59	100.0	0.7
0.5	YI04	13920	8215.75	30.0	0.6
0.5	YI05	9220	5499.9	20.0	0.6
0.5	YI06	7930	10829.85	30.0	1.4
1	YI07	6500	3734.43	30.0	0.6
1	YI08	3200	1649.97	10.0	0.5
1	YI09	5500	2138.85	10.0	0.4
2	YI10	606	1161.09	1.0	1.9
2	YI11	643	439.99	3.0	0.7
2	YI12	1210	1081.65	3.0	0.9
4	YI13	469	403.33		0.9
4	YI14	290	1002.2		3.5
4	YI15	293	311.66	1.0	1.1
8	YI16	209	507.21		2.4
8	YI17	254	556.1		2.2
8	YI18	229	482.77		2.1
24	YI19	183	577.49	1.5	3.2
24	YI20	216	262.77		1.2
24	YI21	173	409.44		2.4

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.5	ZU22	139	275		2.0
0.5	ZU23	152	531.66		3.5
0.5	ZU24				
1	ZU25	162	97.78	3.0	0.6
1	ZU26	176	525.55		3.0
1	ZU27	242	299.44		1.2
2	ZU28	225	226.11		1.0
2	ZU29	235	323.88		1.4
2	ZU30	233	275		1.2
4	ZU31	264	293.33		1.1
4	ZU32	292	598.88		2.1
4	ZU33	263	226.11		0.9
6	ZU34	356	562.21	1.0	1.6
6	ZU35	319	470.55		1.5
6	ZU36	178	806.65		4.5
8	ZU37	458	745.54	1.0	1.6
8	ZU38	334	708.88		2.1
8	ZU39	322	207.77	1.0	0.6
24	ZU40	160	244.44		1.5
24	ZU41	132	531.66		4.0
24	ZU42	159	146.66		0.9

time [h]	sample code	HPLC/ MS/MS	bioassay	dil. factor	ratio
0.5	ZI22	86.5	256.66		3.0
0.5	ZI23	85.5	430.83	1.5	5.0
0.5	ZI24	152	482.77		3.2
1	ZI25	506	366.66	1.0	0.7
1	ZI26	248	409.44		1.7
1	ZI27	248	262.77		1.1
2	ZI28	290	354.44		1.2
2	ZI29	261	378.88		1.5
2	ZI30	232	250.55		1.1
4	ZI31	286	696.65		2.4
4	ZI32	313	232.22	1.0	0.7
4	ZI33	409	329.99		0.8
6	ZI34	379	983.87		2.6
6	ZI35	335	403.33		1.2
6	ZI36	249	623.32		2.5
8	ZI37	536	525.55		1.0
8	ZI38	323	537.77	1.0	1.7
8	ZI39	433	1209.98		2.8
24	ZI40	172	238.33		1.4
24	ZI41	172	110		0.6
24	ZI42	169	531.66		3.1

sample codes:

W= treatment with DB 75 **X= treatment with DB 289** **U= uninfected mice**

Y= treatment with DB 820 **Z= treatment with DB 844** **I= infected mice**

ratios bigger than a factor of 2 are highlighted

RAW DATA

MALARIA

Pharmacokinetic parameters of active compound:

Table 1: DB 75:

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	5980	6210	- 10.5 %
t_{max} [h]	0.5	0.5	
AUC	30307	33766	- 10.2 %
AUMC	268153	290582	- 7.7 %
MRT	8.85	8.61	+ 2.8 %
$t_{1/2}$	6.1	6.0	+ 1.7%
k_{el} (1/MRT)	0.11	0.12	- 2.6 %

Pharmacokinetic parameters of prodrug:

Table 2: DB 289:

parameter	infected mice	uninfected mice	inf/uninf.
C_{max} [nM]	2046.7	2190	- 6.5 %
t_{max} [h]	2	2	
AUC	30516	15170	2 x
AUMC	297792	76764	3.9 x
MRT	9.76	5.06	1.9 x
$t_{1/2}$	6.8	3.5	1.9 x
k_{el} (1/MRT)	0.10	0.20	- 48,5%

Pharmacokinetic parameters:

Table 3: DB 75 after DB 289:

parameter	infected mice	uninfected mice	inf./uninf.
C_{max} [nM]	381	260	+ 46.7 %
t_{max} [h]	24	24	
AUC	5921	4732	+ 25.1 %
AUMC	92434	65842	+ 40.4 %
MRT	15.61	13.32	+ 12.1 %
$t_{1/2}$	10.8	9.6	+ 12.5 %
k_{el} (1/MRT)	0.06	0.07	- 14.3 %

Table 4: Calculations of free plasma levels and required dose to kill malaria parasites

time in h	DB 75 [nM] in uninfected	free DB 75 plasma level [nM] with protein binding of 75%	70%
0.5	154.00	38.50	46.20
1.00	166.00	41.50	49.80
2.00	158.00	39.50	47.40
4.00	201.67	50.42	60.50
6.00	201.33	50.33	60.40
8.00	156.33	39.08	46.90
24.00	260.00	65.00	78.00

time in h	DB 75 [nM] in infected	free DB 75 plasma level [nM] with protein binding of 75%	70%
0.5	88.40	22.10	26.52
1.00	82.87	20.72	24.86
2.00	139.33	34.83	41.80
4.00	141.00	35.25	42.30
6.00	149.67	37.42	44.90
8.00	219.33	54.83	65.80
24.00	381.33	95.33	114.40

P. falciparum in vitro IC_{50} for DB 75 [Schild N. (2003)]:

time in h	IC_{50}	IC_{50}	IC_{50}	average in [ng/ml]	average in [nM]
1	23	98	29	50.0	132.5
2	19	96	19	44.7	118.4
4	22	67	28	39.0	103.4
8	15	33	14	20.7	54.8
24	4	11	8	7.7	20.3

required dose of DB 75 to kill parasites (MIC= 3-5x IC_{50}):

time in h	3x IC_{50} in [nM]	5x IC_{50} in [nM]
1	397.5	662.5
2	355.1	591.8
4	310.05	516.8
8	164.3	273.8
24	60.95	101.6

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Nov. 1993 – Nov. 1999	Studies of Pharmaceutical Sciences at the University of Basel: Swiss Federal Diploma in pharmacy

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Conferences

2005	16th International Congress for Tropical Medicine and Malaria, Marseille 11.-15.09.05
2004	21st Swiss Annual Trypanosomatid Meeting, Leysin 29.-31.01.04
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	1st Student Symposium at the Swiss Tropical Institute, Basel 13.02.03
2002	Symposium on Clinical Significance of Drug Transporting Proteins, Basel 22.03.02
	19th Swiss Annual Trypanosomatid Meeting, Leysin 31.01.-2.02.02

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