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**NEW THERAPEUTIC STRATEGIES AGAINST
TRYPANOSOMIASIS AND LEISHMANIASIS**

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Abstract:

Leishmaniasis and African Trypanosomiasis are diseases caused by the Kinetoplastida parasites of *Leishmania sp.* and *Trypanosoma sp.* respectively. Control and management of these diseases, which affect a significant number of people in the tropics and subtropical areas of the world, is beset with numerous problems such as drug toxicity, affordability and the emergence and spread of parasites resistance to most of the routinely used drugs. This situation calls for an urgent search for new drugs that would address these concerns. Based on report of excellent antimicrobial activities against other parasites and the possession of other known good values, analogues of choline and curcumin were thoroughly assessed in this study for their potential as antitrypanosomal and antileishmanial drugs.

Standard methods such as the Alamar Blue, propidium iodide and direct microscopy methods were used to determine the susceptibility of the parasites to the different analogues. Toxicity tests were performed to determine the effect of these compounds on Human Embryonic Kidney (HEK) cells. The presence of mediated transport of these compounds across the parasite plasma membrane was investigated using the classical uptake technique. In order to investigate the possible mechanism of antiparasitic action of the compounds, this study employed flow cytometry to assess the mitochondrial membrane potential Ψ_m , as well as parameters such as production of reactive oxygen species (ROS), the permeability of the plasma membrane and any effects of the test compounds on the parasite's cell cycle.

Five out of 7 choline compounds tested in this study had EC_{50} values of 0.13-1.8 μM against *T. brucei*, 0.14-6.9 μM against *L. major*, *L. mexicana* promastigotes and 1.69-12.9 μM against *L. mexicana* amastigotes. With regard to the curcuminoid compounds, 35 out of 98 tested were observed to exhibit trypanocidal activity better than the original curcumin with EC_{50} values between 0.05 and 1 μM . Against Leishmania, most of the compounds displayed higher antiparasitic activity than curcumin but lower than observed against trypanosomes. The activity of choline analogues was very similar against *L. mexicana* and *L. major* promastigotes ($P > 0.05$), and much higher than against *L. mexicana* amastigotes. Interestingly, some of the compounds displayed EC_{50} values below that of pentamidine, the routinely used drug.

Assessment of parasite growth pattern in the presence of choline analogues showed that two of the compounds, T1 and MS1, are fast acting, killing the population of BSF *T. b.*

brucei within 8 h with the onset of cell death at 2-4 hours of treatment. In contrast, the other three choline compounds observed to have antiparasitic activities acted more slowly, completely killing the trypanosome population after more than 30 hours of incubation. However, all the choline compounds appeared to rapidly inhibit trypanosome proliferation.

The choline compounds exhibited low toxic effects against HEK cell line T29, with the selectivity index (S.I.) being high for some of the compounds. The curcumin compounds, too, were observed to have generally similar or lower toxicity against the human cells than the parent curcumin compound (AS-HK001), which in itself is not considered toxic and routinely used in food. Investigations on the toxicological and pharmacological effects of the curcumin compounds on the survival and the glutathione and protein content of primary murine hepatocytes showed no significant difference between hepatocyte cells treated with curcuminoid compounds AS-HK001, AS-HK009, and AS-HK014 compared with controls.

We also investigated how choline and its analogues enter the trypanosome. Evidence gathered in this study strongly suggests that unlike in *Leishmania* species and *Plasmodium*, choline transporters are not expressed in the bloodstream form of *T. b. brucei*. It was also conclusively shown that the P2, high affinity pentamidine transporter (HAPT) and low affinity pentamidine transporter (LAPT) do not play any significant role in the uptake of this compound. Lacking radiolabeled forms of the choline analogues, this study could not identify a definitive route of uptake of this class of compounds into the parasite.

Analysis of cell cycle progression, by flow cytometry, showed trypanosomes in the G1, S, and G2/M stages. Curcuminoids do not appear to cause any important changes in the proportion of cells in G1, S or G2/M phase of the cell cycle. Cells exposed to various concentrations of some curcumin compounds, such as AS-HK014 and AS-HK096, showed a rapid increase in cell permeability, reaching between 80% and 90% in 4 hours. The permeability was observed to increase with increasing drug concentration and/or the incubation time. Investigations of cell membrane permeability also showed that choline analogues caused plasma membrane defects which could probably lead to cell death.

With regard to the effect of the compound on mitochondrial membrane potential Ψ_m the dicationic choline compounds, including M38, G25, T4 and MS1, were observed to have pronounced effects on Ψ_m with an onset as early as 8 h of contact and we believe the mitochondria could be the main target of these compounds rather than indicating the induction of apoptosis, as the action of the test compounds was not associated with the

production of reactive oxygen species. Indeed, both choline and curcumin analogues reduced the production of reactive oxygen species in *T. b. brucei* cultures. Furthermore, there were no major defects in choline phospholipid metabolism upon treatment with the choline compounds, suggesting that phospholipid metabolism is not the target of the anti-trypanocidal activity of these compounds.

Preliminary results with infected ICR mice infected with *T. b. brucei* did not reveal significant *in vivo* activity of the three curcumin compounds on blood parasitemia when they were injected intra-peritoneally with two doses of 50 mg/kg body weight.

With reference to evidence obtained in this study, it can firmly be concluded that analogues of choline and curcumin display highly promising antiparasitic activities and are generally non-toxic to human cells. Information provided in this thesis could therefore assist in the further development of these classes of compounds as lead compounds against kinetoplastid diseases. We strongly recommend that further investigation be carried out to understand the full mechanism of action of these compounds in order to facilitate this strategy.

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Dedication

This thesis is dedicated to:

my mum and late father

my brothers and sisters

my lovely wife; *Sana*, my children; *Mohamed* and *Aala*.

Hasan Mohamed Saleh Ibrahim

DECLARATION

I declare that the results presented in this thesis are my own work and that, to the best of my knowledge, it contains no material previously substantially overlapping with material submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Hasan Mohamed Saleh Ibrahim

List of Abbreviations

AAT	Animal African trypanosomiasis
AAT	acyl-CoA acyltransferase
AB	assay buffer
ACS	acyl-CoA synthetase
Ado	adenosine
AIDS	acquired immune deficiency syndrome
AK	adenosine kinase
apoL1	apolipoprotein L1
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BBB	blood brain barrier
BMB	Bone marrow aspirate
BSF	bloodstream form
BW	Body weight
CATT	Card Agglutination Test for Trypanosomiasis
CBSS	Carter's balanced salt solution
CIATT	Indirect Agglutination Test for Trypanosomiasis
CL	cutaneous leishmaniasis
CNS	central nervous system
COX-2	cyclooxygenase-2
CQ-R	chloroquine resistant
CQ-S	chloroquine sensitive
DAT	direct agglutination test
DCF	dichlorofluorescein
DCL	diffuse cutaneous leishmaniasis

DDT	Dichlorodiphenyltrichloroethane
DFMO	difluoromethylornithine
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC ₅₀	50% effective concentration
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme-linked immunosorbence assay
ER	endoplasmic reticulum
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
GI	growth inhibitory
Gro-P-Cho	glycerophosphocholine
Gro-P-Etn	glycerophosphoethanolamine
GPIno	glycosylphosphatidylinositol
GSH	glutathione
HAPT1	high affinity pentamidine transporter 1
HAT	human african trypanosomiasis
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HI-FCS	heat-inactivated fetal calf serum
HIV	human immunodeficiency virus
h	hour
IC ₅₀	50% inhibitory concentration
IFAT	immunofluorescence test
im	Intramuscular
INS	Insoluble
ip	intraperitoneally

iv	Intravenous
KH	Krepes Hepes buffer
K_i	Inhibition constant
K_m	Michaelis-Menten constant
KO	knockout
LAPT1	low affinity pentamidine transporter 1
LDL	low density lipoprotein
lyso-PtdCho	lyso-phosphatidylcholine
μg	microgram
μl	microlitre
μM	micromolar
MCL	mucocutaneous leishmaniasis
mol	mole
MOPS	3-(N-Morpholino)propanesulfonic acid
MT	metacyclic trypomastigotes
mV	millivolt
n	number
NADH	nicotinamide adenine dinucleotide dehydrogenase
ND	not determined
NE	no effect
nM	nanomolar
NNN	Novy-MacNeal-Nicolle
No.	number
NS	not significant
ODC	ornithine decarboxylase
OPT	O-phthaldehyde
ORF	open reading frame

PAO	Phenyl Arsine Oxide
PC	Phosphatidylcholine
PCR	polymerase chain reaction
PE	Phosphatidylethanolamine
PI	propidium iodide
PL	phospholipid
PLA ₁	phospholipase A ₁
pmol	picomol
PS	Phosphatidylserine
PSG	phosphate buffered saline plus glucose
PT	procyclic trypomastigotes
PTRE	post-treatment reactive encephalopathy
ROS	reactive oxygen species
rpm	Revolutions per minutes
s	second
SAR	structure activity relationship
SDM	schneider's drosophila medium
SDS	sodium dodecylsulphate
SE	standard error
SS	short stumpy
TbAT1	<i>trypanosome brucei</i> adenosine transporter 1
TCA	trichloro acetic acid
THC	tetrahydrocurcumim
TI	therapeutic index
TLF	trypanosome lytic factor
TMRE	Tetramethylrhodamine ethyl ester
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate

U	unit
UV	ultraviolet
V	volts
VL	visceral leishmaniasis
Vmax	maximum velocity
VSG	variant surface glycoprotein
WHO	World Health Organization
WT	wild type

Chapter one

1 General introduction.

1.1 African Trypanosomiasis

1.1.1 Disease

Human African trypanosomiasis (HAT), or sleeping sickness, is one of the world's major killers and is resurgent in Sub-Saharan Africa (WHO, 2006a). This disease is caused by two closely related parasites, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. African trypanosomes are flagellated protozoa belong into the subphylum kinetoplasta, family trypanosomatidae, order kinetoplastida, genus *Trypanosoma* and species *Trypanosoma brucei*. Based on the characteristics of the disease, *Trypanosoma brucei* is generally divided into three sub species; first, *Trypanosoma brucei gambiense*, which causes a chronic sleeping sickness and is found in West Central Africa; second, *Trypanosoma brucei rhodesiense*, which causes acute sleeping sickness and is found in Angola, Zambia, Tanzania, and Zimbabwe; third, *Trypanosoma brucei brucei*, which is restricted to wild animals and cattle (Jeffrey and Leach, 1991; Liew and Cox, 1998). *Trypanosoma brucei brucei* can not infect the human because it is killed by a haptoglobin called trypanosome lytic factor (TLF) found in the human serum (Smith *et al*, 1995). This factor is identified as apolipoprotein L1 (apoL1) and is associated with human high-density-lipoprotein that described by presence of Haptoglobin-related protein (Vanhamme *et al*, 2003; Pays *et al*, 2006). The latter protein leads to convert the function of trypanosome haptoglobin-hemoglobin receptor in order to educe the innate human immunity against the trypanosome (Vanhollebeke *et al*, 2008).

The two forms of Human African trypanosomiasis are transmitted by several species of tsetse flies (*Glossina* sp.). Therefore, the distribution of these diseases is connected with the distribution of the vector (Ehrhardt *et al.*, 2006). The most important vector species that carry the parasites that are responsible for chronic disease are *Glossina palpalis* and *G. tachinoides* in West Africa, and *G. fuscipes* in Central and East Africa. On the other hand, *G. morstans*, *G. swynnertoni* and *G. pallidipes* are the most important species involved in the transmission of acute disease (Service, 1996).

According to a WHO report, sleeping sickness exists in more than 36 countries in sub-Saharan Africa (WHO, 2006c). The number of people infected with the parasite was reported to be rising every year. Incidence of the disease in 1996 was 25,000 (Service, 1996) whilst 40,000 cases were recorded in 2004 (Gibson, 2004).

In 1999, there were 300,000 to 500,000 reported cases, and about 60 million people are at risk (Barrett, 1999) Figure 1.1. However, the actual prevalence of infected cases in 2006 was estimated to be 50,000 to 70,000 (WHO, 2006a). Recent control strategies have started to bring the prevalence down significantly from its peak in the late 1990s (Simarro *et al*, 2008)

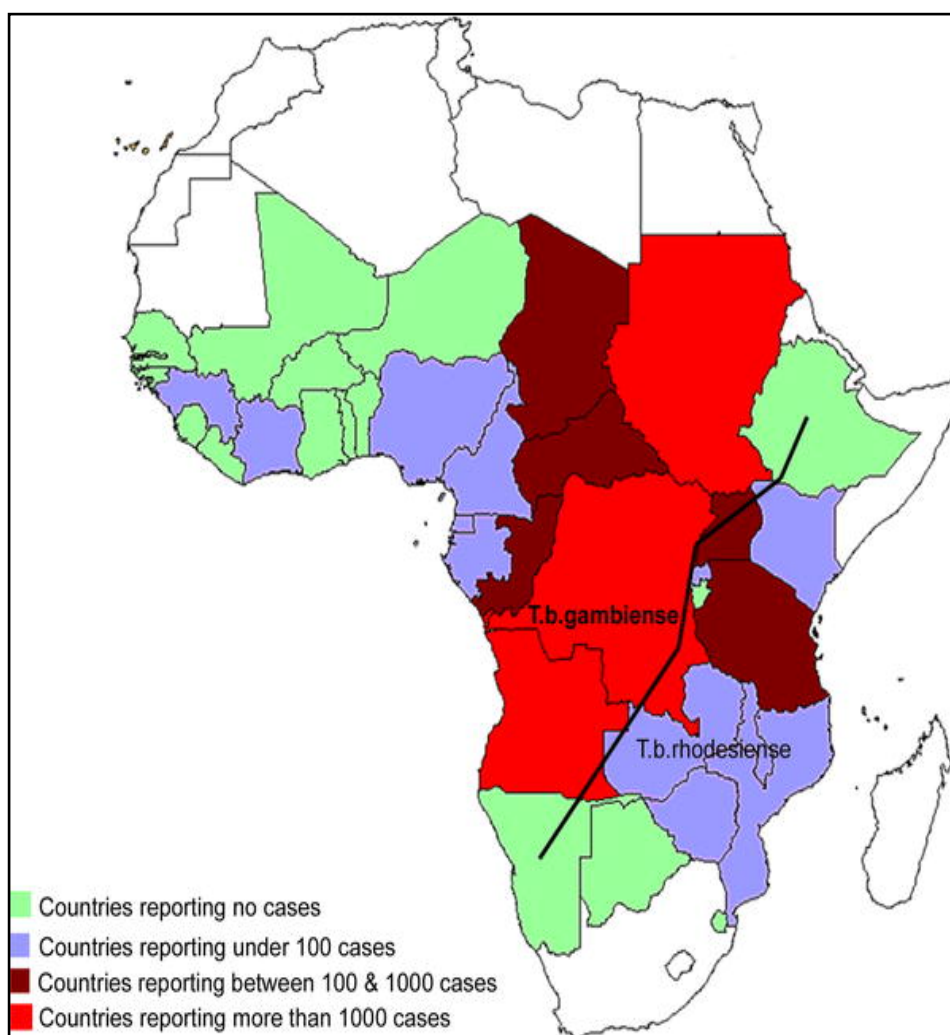


Figure 1.1. The geographical distribution of sleeping sickness in African countries. Reproduced from (Simarro *et al*, 2008).

1.1.2 Morphology and life cycle

Even though trypanosomes are morphologically indistinguishable and have similar life cycles, they have different clinical symptoms and different epidemiologies. They also share many biochemical features with other pathogenic organisms such as *Leishmania sp.* and *Trypanosoma cruzi*. Since it is difficult to morphologically distinguish between the three sub species of *Trypanosoma brucei*, molecular and biochemical methods are the only reliable means for differentiation (Hide & Tilley, 2001; Garcia, 2007).

T. brucei has two main hosts: a vertebrate host (human, or domestic and wild animals) and an invertebrate host (Tsetse fly). The life cycle of the parasite, as illustrated in Figure 1.2, starts when the metacyclic trypomastigotes are injected by tsetse flies during feeding on the host's blood. These forms multiply and modify into trypomastigotes (14-33 μm long and 1.5-3.5 μm wide) and are related to as slender blood stream forms (figure 1.3). This form has a long flagellum multiplies by longitudinal binary fission in the blood and lymph nodes. After many divisions, it transforms into a stumpy form. The stumpy form without free flagellum ($\sim 15 \mu\text{m}$ long), is shorter and thicker than the slender bloodstream form. This form cannot divide in the bloodstream and is pre-adapted to infect the tsetse fly vector.

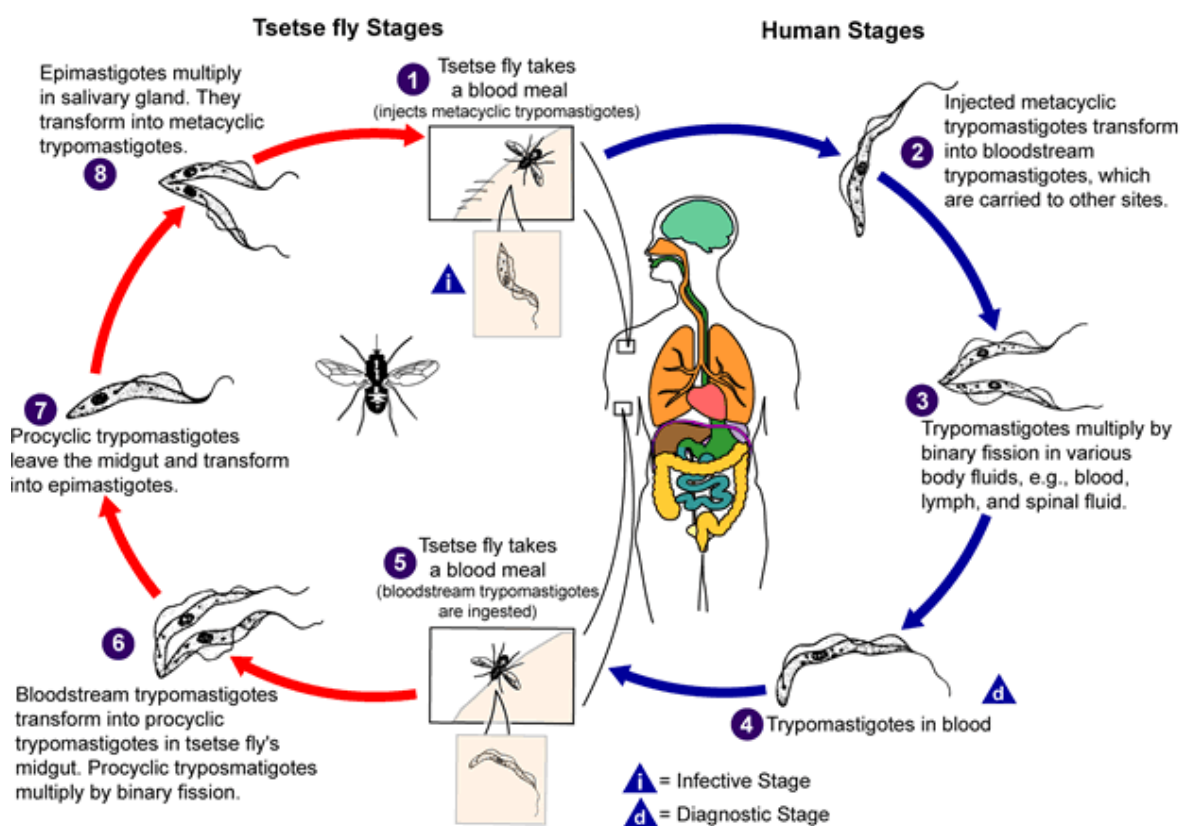


Figure 1.2. shows the life cycle of *T. brucei* in the two hosts: human and tsetse fly. Reproduced from The Centres for Disease Control (<http://pathmicro.med. sc.edu>).

The infective forms are taken with the blood meal by male and female tsetse flies (*Glossina* sp.), and they pass through the oesophagus to reach the mid-gut. The differentiation into long procyclic trypomastigotes occurs in this place. The new forms multiply and penetrate the membrane. They then migrate to the esophagus, pharynx, and

later to the salivary glands. In the salivary glands, the procyclic trypomastigotes metamorphose to epimastigotes and then to metacyclic trypomastigotes (infective stage) which is injected into the host blood during the next blood feeds. The period from the infected hosts to the infective metacyclic trypomastigotes in the salivary glands is 18-34 days. (Jeffrey & Leach, 1991; Service, 1996; Garcia, 2007).

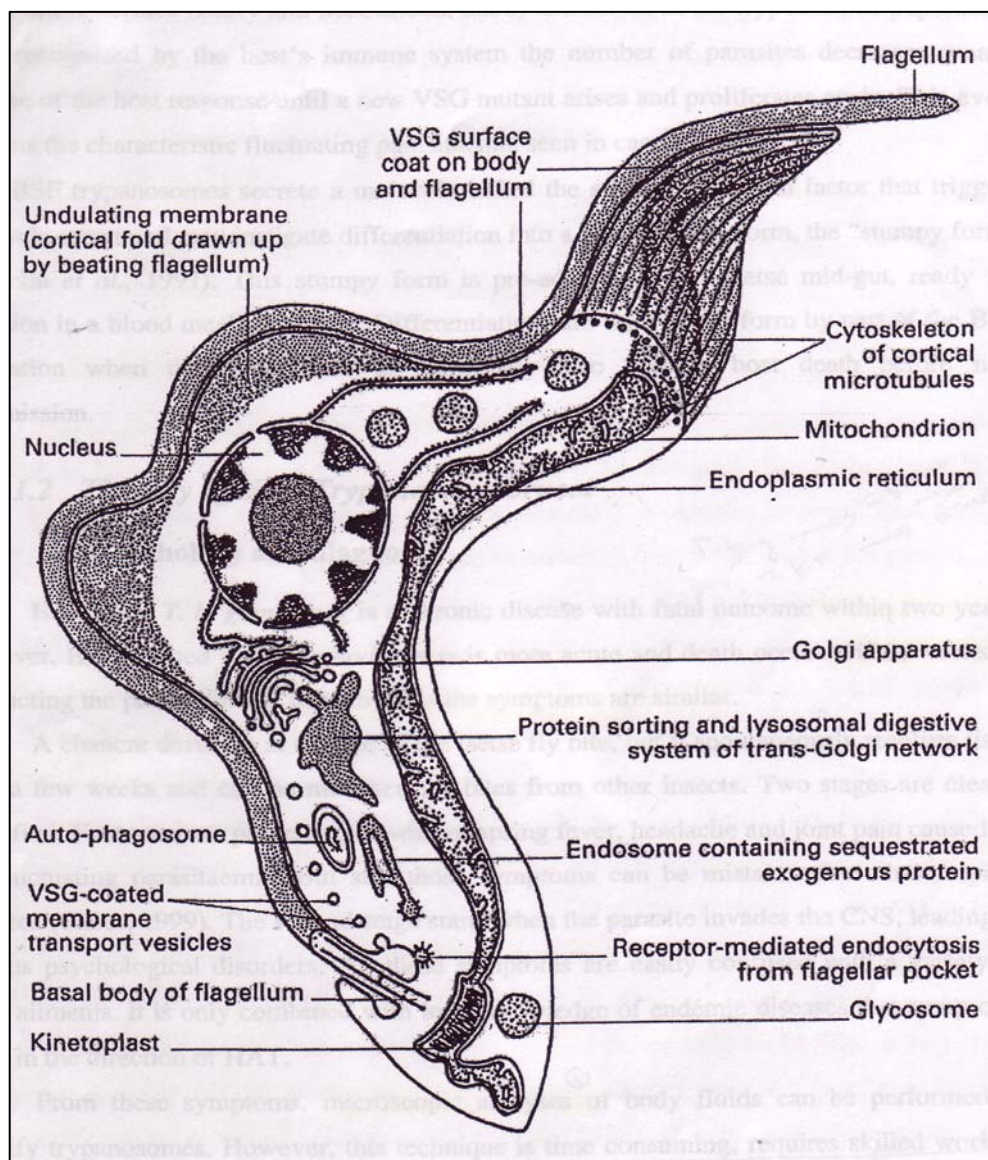


Figure 1.3. Diagram shows the general structure of the bloodstream form trypomastigotes of *Trypanosoma brucei*. Reproduced from (Vickerman *et al*, 1993).

In Gambian sleeping sickness, it is traditionally believed that there is no animal reservoir and that humans are the only hosts, although in West Africa some wild animals and domestic pigs may harbor this kind of parasites. On the other hand, the Rhodesian disease is a zoonosis and the animals do not play an important role as reservoir hosts (Service, 1996).

1.1.3 Clinical manifestation and pathology

The clinical manifestations of sleeping sickness are similar and depend on the type of disease. Two types of disease are known, chronic disease which is caused by *T. b. gambiense*, and acute disease resulting from an infection with *T. b. rhodesiense*. The former is called Gambian or West African sleeping sickness, and it has a long incubation period; from several weeks to months, sometimes several years. The latter infection is called Rhodesian or East African sleeping sickness; the incubation period is shorter than the first and clinical signs will typically develop within two to three weeks. Death usually occurs after several years of the chronic disease, and within a year in the case of the acute disease (Vickerman *et al*, 1993; Markell E. *et al*, 1999).

The symptoms of sleeping sickness start after few days of infection with a trypanosomal chancre appears within days of a bite by an infected tsetse fly at the site of inoculation. Two stages of the disease are distinguishable; the early stage which does not involve the Central Nervous System (CNS), and the late stage (Jeffrey and Leach, 1991). During the early stage of infection is characterized by irregular episodes of fever as a result of high and low levels of parasites. The main reason for the variable parasitaemia is the antigenic variation of these organisms. Lymphadenopathy, the swelling of lymph nodes, is one of the main signs of sleeping sickness. Additionally, the spleen and liver are also affected and become enlarged. Backache, headache, tachycardia and encephalitis are associated with chronic sleeping sickness caused by *T. b. gambiense*; whereas headache, vomiting, shivering, bone pain and encephalitis with rapid development of coma are caused by *T. b. rhodesiense*. The invasion of the CNS occurs within a few weeks in Rhodesian sleeping sickness, and between months to some years in the Gambian species (Markell E. *et al*, 1999; Jeffrey & Leach, 1991; Garcia, 2007).

1.1.4 Diagnosis

Clinical history is very important in the diagnosis of the disease. Diagnosis of the early stage of trypanosomiasis is usually done by identifying the organisms in the blood, lymph nodes, and sometimes bone marrow. This is achieved through the preparation of peripheral blood films stained with Giemsa's stain. However, this method is not useful in some cases because of the very low and irregular parasitemia levels, especially in infections with *T. b. gambiense* (Kreier and Baker, 1987; Chappuis *et al*, 2005). Furthermore, it is time consuming and needs skilled workers.

The microhaematocrit centrifugation technique is another method used to identify the presence of the parasites (particular *T. b. gambiense*). In this technique, the trypanosomes are concentrated by centrifugation before microscopic examination. Inoculation of a patient's blood to laboratory animals (mice and rats) is also used as a method of diagnosis (Kreier and Baker, 1987). However, this method is not successful in some species like *T. b. gambiense* (Garcia, 2007), as this species grows poorly in laboratory rodents.

There are several Immunodiagnostic methods which are used to diagnose African trypanosomes, for example, enzyme-linked immunosorbence assay (ELISA), indirect immunofluorescence, and agglutination. The Card Agglutination Test for Trypanosomiasis (CATT) is a test for anti- *T. b. gambiense* antibodies in serum, and this test is simple in use and it can be easily used in the field. Furthermore, there is a new type of this test which is called Card Indirect Agglutination Test for Trypanosomiasis (CIATT) that can be used in the diagnosis of the disease (Kreier and Baker, 1987; Barrett, 2000; Chappuis, 2005).

1.1.5 Chemotherapy and control:

Despite the spread of trypanosomiasis in Sub-Saharan African countries, the World Health Organization (WHO) and the Governments of these countries were unaware of the growing incidence of sleeping sickness throughout the 1980's and early 1990's. This situation has led to an increase in mortality rate of trypanosomiasis in these countries. A major factor militating against the successful treatment of the disease is the emergence and spread of parasites resistant to the commonly used anti-trypanosoma drugs. There is therefore an urgent need for novel anti-trypanosoma drugs.

Another factor is the antigenic variation of trypanosomes, which enables them to change their cell surface and trick the host's immune system. Trypanosoma have a typical membrane, which is covered by surface coat (Seed & Hall, 1992). Indeed the metacyclic and bloodstream form trypanosomes are uniformly coated with a glycoprotein called Variant Surface Glycoprotein or VSG (Liew & Cox, 1998). Consequently, the oscillating parasitaemia is connected to the ability of this parasite to alter its VSG and evade the immune system (Herbert, 2001). Moreover, poverty in developing countries has deprived nations of funds in order to deal with the problem of trypanosomiasis. Moreover, the movements of tourists and migrant workers have contributed to the spread of the disease.

There are many control programmes in the endemic areas that aim to eradicate sleeping sickness from Africa. Vector control using DDT is one of the measures employed to reduce the incidence and spread of the disease. This compound remains effective in killing the flies for at least 2–3 months (Service, 1996). Chemotherapy is another control measure and is usually effective when the drug is administered before parasites invade the CNS. Surveillance, health service and health education are also very important in attempts to eradicate sleeping sickness. WHO (2002) has identified surveillance as “an essential step towards the elimination of sleeping sickness”. It also suggested that the weak surveillance and high proportion of sleeping sickness cases at the late stage may be due to either patient environment or health system. Problems include the financial status of patients and the diagnostic techniques used. For example, in Uganda surveillance of sleeping sickness is unacceptably poor and half the cases are detected when they are in the late stage (Odiit *et al*, 2004).

Four drugs are currently licensed for the treatment of human African trypanosomiasis. These are pentamidine, suramin, melarsoprol and eflornithine (DFMO).

1.1.5.1 Pentamidine

Pentamidine is an aromatic diamidine drug (Figure 1.4A) used against HAT (Table 1). Another diamidine drug, Berenil® (diaminazene aceturate) is licensed for veterinary trypanosome infections. Pentamidine was introduced in 1941 to treat the early stage of *T. b. gambiense* infections. This drug is given as a course of seven to ten daily intramuscular (im) injections of 4 mg/kg (Jannin and Cattand, 2004). The side effects of this drug are vomiting, abdominal pain, hypotension and hypoglycaemia (Legros *et al*, 2002; Croft *et al*, 1997).

The mode of action of pentamidine is still not fully understood even though it has been in use for many years. This drug, which is reported to be taken up by three different transporters, the P2 adenosine transporter; high affinity pentamidine transporter (HAPT1) and the low affinity pentamidine transporter (LAPT1), accumulates inside the cells to millimolar concentrations (De Koning, 2001b). The suggested targets of this drug include the inhibition of the enzymes S-adenosylmethionine decarboxylase (Fairlamb, 2003), topoisomerase II (Shapiro & Englund, 1990) and the Ca²⁺ ATPase in the plasma membrane (Benaim *et al*, 1993). Considering the very high level to which pentamidine accumulates within the trypanosome, it is likely that it affects multiple processes.

1.1.5.2 Suramin

Suramin is a sulfonated naphthylamine polyanionic compound, and has been used to treat the early stage of *T. b. rhodesiense* since the early 1920s. Being highly charged, this drug can not traverse biomembranes or the blood brain barrier (BBB) (Fairlamb, 2003) and it is therefore used to treat only the early stage of the disease. A typical recommended dose of suramin is 20 mg/kg a day for five days, and not more than 1 g per injection (Jannin and Cattand, 2004). Some reported side effects associated with the drug are: nausea, vomiting, pruritus, renal failure, anaphylactic shocks, haemolytic anaemia, jaundice, severe diarrhoea, neurotoxic signs, severe cutaneous reactions and hypoesthesia (Legros *et al*, 2002; Fairlamb, 2003). The mode of action of suramin is still unclear. The drug is taken up and accumulated in the cells by low density lipoprotein (LDL) receptor mediated endocytosis (Vansterkenburg *et al*, 1993). It has also been reported that suramin inhibits several enzymes and receptors (Wierenga *et al*, 1987).

1.1.5.3 Melarsoprol

Melarsoprol was introduced for the first time by Friedheim in 1949 to treat the late stage of both of *T. b. gambiense* and *T. b. rhodesiense* infections. The general structure of this drug is shown in Figure 1.4C. It is also effective against the early stage of both trypanosome species, but because of its high risk of serious side effects during treatment it is not recommended for use at this stage. The drug is given in one dose for ten consecutive daily intravenous (iv) injection of 2.2 mg/kg (Legros *et al*, 2002; Jannin & Cattand, 2004). Melarsoprol is a prodrug which is directly converted in patients' plasma to melarsen oxide within 1 hour of administration. It has a half-life of 3.5 h (Fairlamb, 2003; Barrett *et al*, 2007). Both melarsoprol and melarsen oxide are capable of crossing the BBB and accumulate in the CNS to about 1-2 % of maximum plasma level (Burri *et al*, 1993). However, melarsoprol is toxic and has several side effects such as a reactive encephalopathy, also known as a post-treatment reactive encephalopathy (PTRE). This effect is observed in 5-15% of cases, and death occurs in up to 50% of these patients (Pepin & Milord, 1994; Jannin & Cattand, 2004).

The mode of action of melarsoprol and its metabolite, melarsen oxide, is still unclear. One of the suggestions is that melarsen oxide interferes with the glycolytic pathway and inhibits its enzymes which are responsible for the production of ATP. This action leads to quick cell lysis before the ATP reaches a critically low level (Van Schaftingen *et al*, 1987; Denise & Barrett, 2001). Trypanothione, a molecule found in trypanosomatids and not in

mammalian cells (Fairlamb *et al*, 1985) is another potential target for these arsenical compounds, (Fairlamb *et al*, 1989). Melarsen oxide interacts with this thiol, yielding melarsoprol-trypanothione adduct (MeIT), which inhibits trypanothione reductase.

1.1.5.4 Eflornithine

Eflornithine or α -difluoromethylornithine (DFMO) is an analogue of the amino-acid ornithine (Figure 1.4D). This drug is used to treat the late stage of *T. b. gambiense* infections, and it was registered for use in the beginning of the 1990s. Treatment is achieved with a dose of 100 mg/kg every 6 hours for two weeks, by slow intravenous (iv) infusion (Barrett *et al*, 2007). Side effects of this drug include pancytopenia, diarrhoea, convulsions and hallucinations (Legros *et al*, 2002; Jannin and Cattand, 2004).

DMFO has a similar affinity for both mammalian and trypanosomal enzymes (Phillips *et al*, 1988; Denise & Barrett, 2001). However, enzyme ornithine decarboxylase (ODC), a key enzyme in the polyamine pathway, is the main target for this drug (Bacchi *et al*, 1980; Barrett *et al*, 1999). The very rapid turnover rate of enzyme ODC and its short half-life in human (10-20 min, (Tabor & Tabor, 1984), and in *T. b. rhodesiense* (~4 h, (Iten *et al*, 1997) made it ineffective as anti-tumour and Rhodesian trypanosomiasis drugs, respectively (Delespaux & De Koning, 2007). In contrast in *T. b. gambiense*, this enzyme is very effective and has a long half-life (18-19 h) (Iten *et al*, 1997).

1.1.5.5 Nifurtimox

Nifurtimox is a 5-nitrofurantoin developed in the 1960s to treat Chagas' disease, which is caused by the American trypanosome, *Trypanosoma cruzi*. This drug (Figure 1.4E) is not licensed for use to treat African trypanosomes, however, it has been found to be effective against the late stage of *T. b. gambiense* especially in those patients who do not respond to treatment with melarsoprol (Garcia, 2007). The most promising results were obtained by the study performed by Checchi and colleagues (Checchi *et al*, 2007) who reported that a combination of Nifurtimox and Eflornithine is effective in the treatment of the late stage of sleeping sickness.

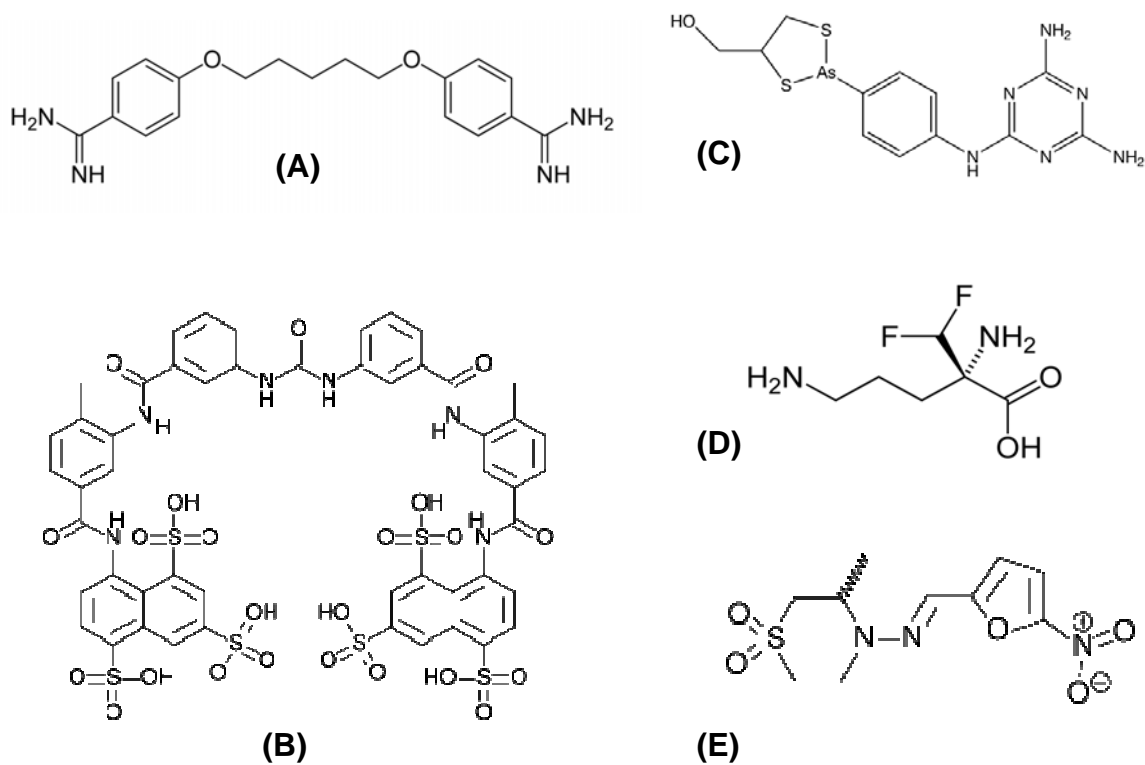


Figure 1.4.General structures of the trypanocidal copounds licensed for use against Human African trypanosomiasis (HAT).

Pentamidine (A); suramin (B); melarsoprol (C); eflornithine (D) and nifurtimox (E).

Table 1.1: Overview of trypanocidal drugs.
Compiled from (Legros *et al*, 2002; Jannin & Cattand, 2004).

drug	species	Stage & start use	dosage	route	Half life	Side effects
Pentamidine isethionate	<i>T. b. gambiense</i>	Early stage 1941	Intramuscular (im)	7 injections 4mg/kg per day	9.4 h (im) 6.4 h (iv)	Hypotension, hypoglycaemia
Suramin	<i>T. b. rhodesiense</i>	Early stage 1920s	Slow intravenous (iv) infusion	Start with test dose followed by 5 doses of 20mg/kg in 5-7 days	50 days	Anaphylactic shocks, severe cutaneous reaction, neurotoxic signs, renal failure
Melarsoprol	<i>T. b. gambiense</i> & <i>T. b. rhodesiense</i>	Late stage 1949	Intravenous (iv) injection	2.2mg/kg/day for 10 consecutive days	35 h	Encephalopathic Syndrome
Eflornithine	<i>T. b. gambiense</i>	Late stage 1981	Intravenous (iv) infusion	100 mg/kg every 6 h for 14 days	3 h	Pancytopenia, diarrhoea, convulsions Hallucination
Nifurtimox	<i>T. b. gambiense</i>	Late stage 1977	orally	3 doses for 2 weeks. 15 mg/kg for children 20 mg/kg for adults	3 – 5 h	Anorexia and neurological complications

1.1.6 Drug resistance

Report of resistance of *T. brucei* to most of the available anti-trypanosome drugs have a major public health problem, which could hinder the control and treatment of the disease. The incidence of drug resistance by parasites seems to be rising (Peregrine, 1994), and the resistance to at least one of the trypanocidal drugs in use has been reported in some countries in sub-Saharan Africa (Afewerk *et al*, 2000). Donald (1994) reported that the resistance of trypanosome parasites leads to reduce the production efficacy, increasing costs and exhausts the effective control devices.

Generally, the suggested mechanism of the parasite's resistance to drug includes the loss of one or more transport functions in the plasma membrane of the parasite (Figure 1.5).

Another mechanism may be due to inactivation, excretion or modification of the drug within the parasite's cytoplasm (Borst & Ouellette, 1995; Borst, 1991). As suggested in the figure, there could also be an increase in repair mechanisms by the cells damaged by the drug.

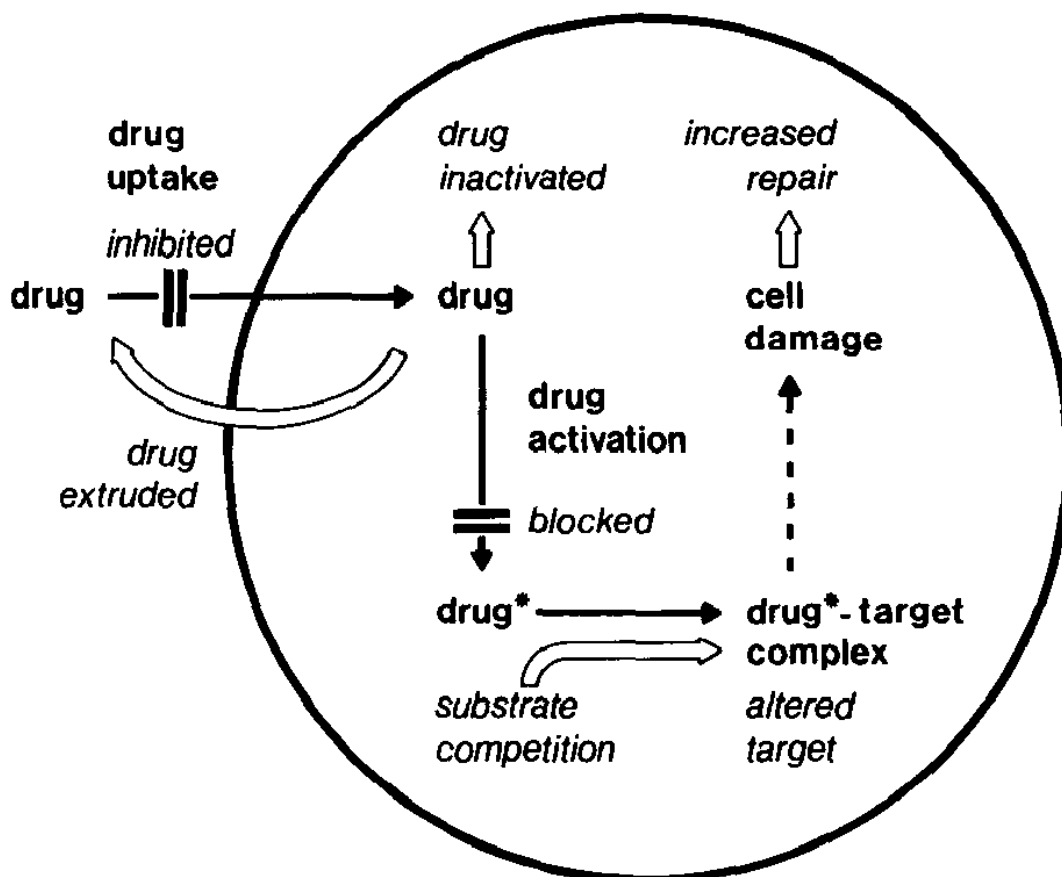


Figure 1.5. The mechanism of drug resistance.

(Adapted from Borst and Ouellette, 1995).

It has been reported that trypanosome resistant to melarsoprol are cross-resistant to diamidine (Bacchi, 1993; Fairlamb *et al*, 1992). These observations suggest that the two compounds are taken up into the parasite through a common transporter. It has been demonstrated that loss of function the P2 aminopurine transporter, which is responsible for melarsoprol and diamidine uptake into *T. brucei* results in parasites resistant to these drugs (Carter & Fairlamb, 1993; Carter *et al*, 1995; De Koning & Jarvis, 1999; Barrett *et al*, 1995). It was therefore concluded that a decrease in drug uptake because of the loss of the transporter resulted in resistance to arsenicals and diamidines in African trypanosomes. However it must be emphasized that P2 transporters are involved in the uptake only of an

estimated 50% of pentamidine, with the rest being transported by a low-capacity high-affinity pentamidine transporter (HAPT) and a high-capacity lowaffinity pentamidine transporter (LAPT) (De Koning, 2001b). In contrast, diminazene aceturate has been shown to be solely transported by the P2 transporter (De Koning *et al*, 2004). Deletion of the gene encoding P2 thus results in a high level of diaminazene resistance, but barely changes sensitivity to pentamidine (Matovu *et al*, 2003).

It is likely that this differences contributes to the observation that while resistance to diminazene aceturate widely reported (Anene *et al*, 2006; Ainanshe *et al*, 1992; Geerts *et al*, 2001). There are no credible reports of pentamidine resistance in the field, after more than 60 years of use (Delespaux & De Koning, 2007). They recent characterisation of the *T. b. brucei* clone B48, which has lost both P2 and HAPT function reveals that resistance to both pentamidine and melarsoprol dramatically increases when HAPT is lost from a P2⁻ background (Bridges *et al*, 2007).

1.2 Leishmaniasis

1.2.1 Disease

Leishmaniasis is a group of diseases caused by several species of protozoan parasites called *Leishmania*. This genus of parasites was first described in 1903 by Leishman and Donovan and classified under the kinetoplastidae family (Olliaro *et al*, 2002). *Leishmania* genus was divided in two sub genera: *Leishmania* and *Viannia* (Garcia, 2007), and it has an organelle which is called a kinetoplast. They belong to the class Zoomastigophora, order Kinetoplastida (Kreier & Baker, 1987). Several species, subspecies, and strains of *Leishmania* parasites cause three different types of Leishmaniasis: cutaneous (CL), mucocutaneous (MCL), and visceral (VL) leishmaniasis (Service, 1996).

More than 12 million people in at least 88 countries, especially developing countries, are infected by leishmaniasis, and annually there are 2 million new cases (WHO, 2006b). About 75% of infected people suffer from cutaneous leishmaniasis (Figure 1.6A) and 25% from visceral leishmaniasis (Figure 1.6B) (Matlashewski, 2001; TDR, 2004a). There are also about 350 million people at risk (TDR, 2004a). About 90% of the cases caused by CL are concentrated in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria; whereas, 90% of that by VL occur in five countries: Bangladesh, India, Nepal, Sudan and Brazil (TDR, 2004a; TDR, 2004b; TDR, 2005). In Kabul alone, it is estimated that 67,500 people are infected with cutaneous leishmaniasis (Reithinger & Coleman, 2007). Leishmaniasis and HIV co-infection has been reported in more than 35 countries in all the world continents except Australia and North America (Harms & Feldmeier, 2005). The co-existence of this disease with HIV plays an important role in increasing the number of infected people, for example: in Afghanistan the numbers have increased from 14,200 cases in 1994 to 67,500 in 2002; in Aleppo, Syria from 3,900 cases in 1998 to 6,275 in 2002; and from 21,800 cases in 1998 to 60,000 in 2003 in Brazil (TDR, 2004a).

The epidemiology of leishmaniasis has been studied for many years, and the distribution of this disease is connected with the prevalence of the vector. The disease is transmitted from reservoir animals to man and animals by phlebotomine sandflies, which include four genus: *Phlebotomus*, *Lutzomyia*, *Psychodopygus* and *Sergentomyia*. The first two genera are the most important in leishmania transmission, and they play a significant role in increasing the prevalence of leishmaniasis. The genus *Phlebotomus* is only found in the Old World such as the Mediterranean region, semi-arid savannah areas and tropical Africa, especially in Central and south Africa (Service, 1996). In contrast, the species of *Lutzomyia* are only

found in the New World, and transfer the same disease in the forested areas of Central and South America (Service, 1996; WHO, 2000).

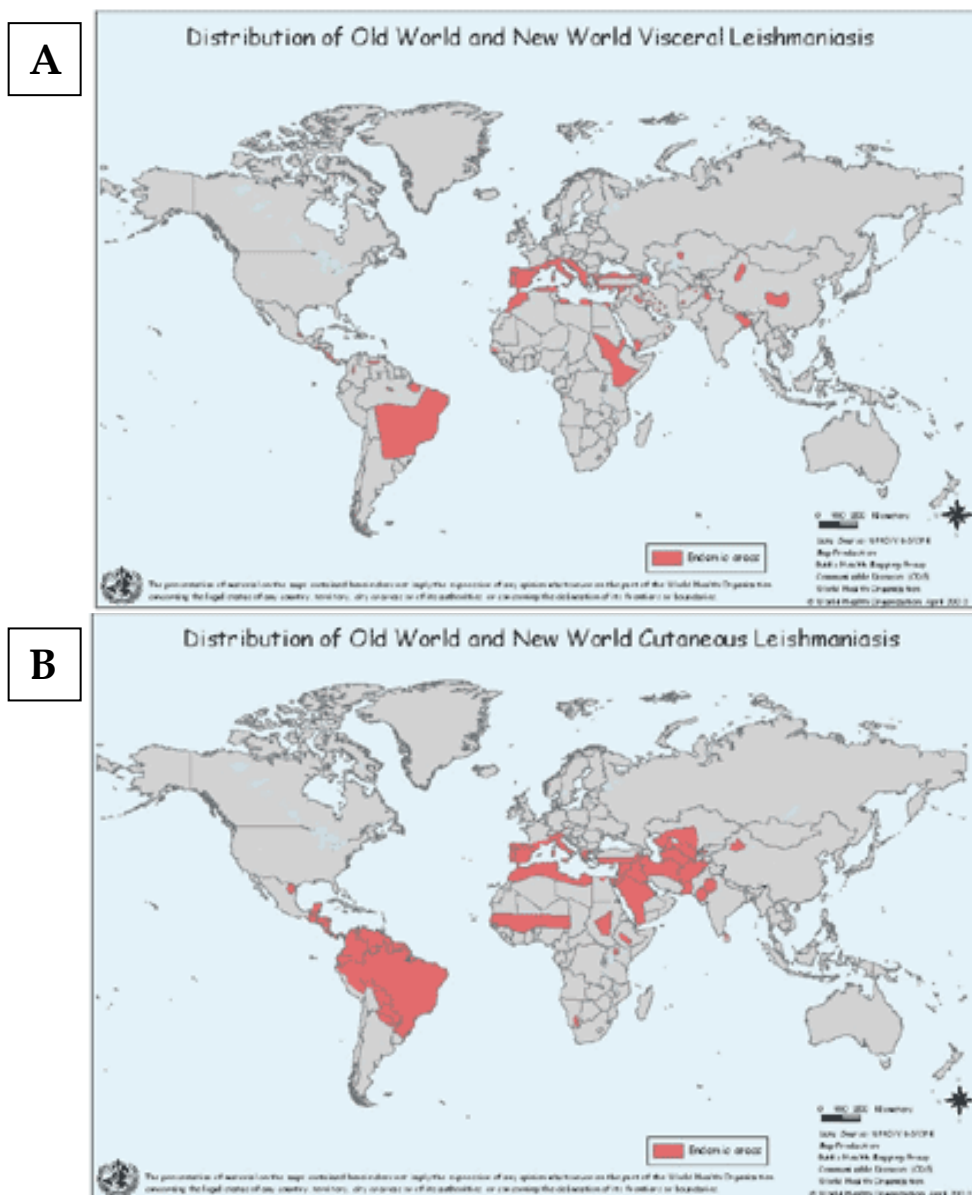


Figure 1.6. Distribution of visceral (A) and cutaneous Leishmaniasis (B) in the Old and New Worlds.

Obtained from WHO, 2000.

Most types of leishmaniasis are zoonoses (transmitted to human from animals), and the most important species of sand flies include: *Phlebotomus papatasi*, *P. sergenti*, *P. argentipes*, *P. ariasi*, *P. perniciosus*, *lutzomyia longipalpis* and species of *lutzomyia flaviscutellata* complex. The reservoir hosts are infected human, wild animals such as rodents, and domestic animals such as dogs (Service, 1996).

1.2.2 Morphology and Life cycle

Leishmania parasites are morphologically represented in two forms: amastigote and promastigote. Amastigote forms are rounded in shape, nonflagellate, 3 - 4 μm in diameter and live within macrophages in vertebrate hosts. These forms are ingested by female sandflies when the insects are feeding on the blood of infected hosts. These flies normally feed on plant juices, but sometimes the females need blood-meal in order to develop eggs. The blood can be taken from humans or many animals like farm livestock, dogs, birds, wild rodents, snakes and lizards. After emerging from the macrophages, amastigotes multiply by binary fission in the midgut, and develop flagellum and became elongated. After further development, they transform into non-infective procyclic promastigotes that rapidly modify into infective metacyclic promastigotes. The latter migrate to the oesophagus and multiply again. Infective forms will be found in the mouthparts and the transmission to vertebrate hosts occurs when the infected females feed on blood of another host (Figure 1.7).

The promastigote forms are spindle-shaped, elongated, 14–20 μm in length, and 1.5-3.5 μm in width, with free terminal flagellum (15-28 μm long). The infective flies inject the promastigotes into the bloodstream where they are quickly ingested by macrophages and transform to amastigotes. The latter start to divide by binary fission and when the infected macrophages die the amastigotes will be ingested by other macrophages (Kreier and Baker, 1987; Service, 1996; Matlashewski, 2001).

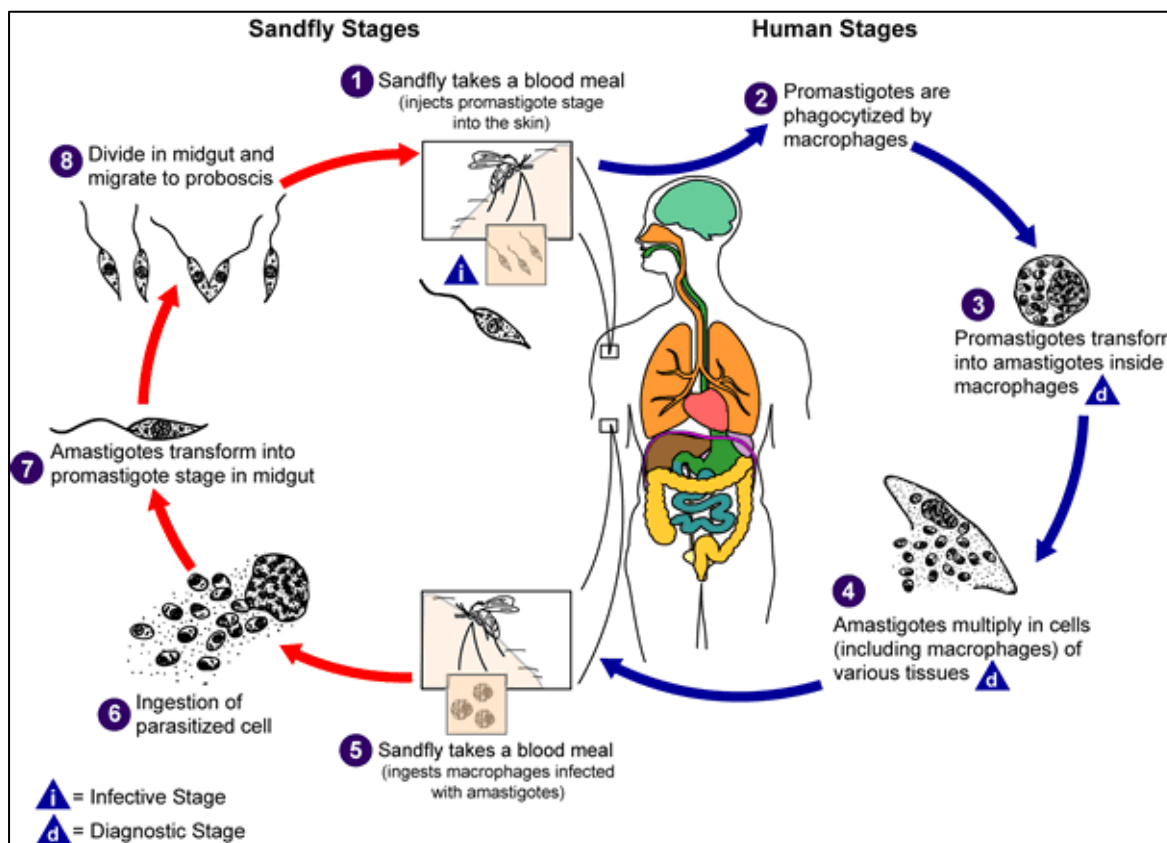


Figure 1.7. Diagram showing the life cycle of *Leishmania* species.

Reproduced from <http://pathmicro.med.sc.edu>

1.2.3 Clinical manifestation and pathology

Cutaneous leishmaniasis (CL) is a disease known in the Old World as Oriental sore, and occurs in the Middle East, India, central Asia, North Africa, and west and southern Africa. Several local names of CL in the Old World such as Baghdad boil, Delhi boil, Biskra button and Aleppo evil are associated with the geographical distributions of disease. Many species of genus *Leishmania* such as *Leishmania tropica*, *L. major*, *L. aethiopica* and rarely *L. infantum* are responsible for causing this sort of leishmaniasis. This type of disease is also found in the New World, especially in the forests from Mexico to northern Argentina. The common names of this disease are sometimes associated with the places where they are found, these include; chiclero ulcer (Mexico), dicera de Baurid (Brazil), uta (Peru), and pain bois (Guyana). *L. mexicana* complex, *L. braziliensis* complex, and *L. guyanensis* complex cause this type of disease.

The incubation period of CL depends on the parasite species, and it takes from two to eight weeks, and sometimes can be long as three years. The disease starts as lesions, which are

erythematous papules on the part of the body that received the sandfly bite. The papule grows and increases in size to 2 cm or more (Figure 1.8). In *L. major* the papule is covered by serious exudates and the ulceration occurs early, whereas it is dry in *Leishmania tropica* and *L. aethiopica* and ulcerates after several months (Markell E. *et al*, 1999). Multiple lesions may occur depending on the number of bites and the exposure area of the body.



Figure 1.8. Image of cutaneous leishmaniasis showing a 2-cm lesion located at the exposed area on the arm.

Taken from: <http://www.emedicine.com/med/byname/Leishmaniasis.htm>

Amastigote forms of *L. aethiopica* and few subspecies of *L. mexicana* (*L. m. pifanoi* and maybe *L. m. amazonensis*) diffuse across the skin and cause a severe condition called diffuse cutaneous leishmaniasis (DCL). This kind of disease occurs when the anergic patients are incapable of mounting a response to the infection. It starts as a regular lesion but then develops to affect many places as nonulcerative nodules on the body. In the Old World, the disease caused by *L. major* and *L. tropica* has been described as crusted ulcers, and in the New World (caused by *L. mexicana*) as tiny closed lesions (Balana-Fouce *et al*, 1998; Chang *et al*, 1985; Garcia, 2007; Jeffrey & Leach, 1991; Kreier & Baker, 1987; Service, 1996).

Mucocutaneous leishmaniasis (MCL) is an American disease found in Central and South America from Mexico to Argentina. The common name of this disease is Espundia. Three

species of genus *Leishmania*, *Leishmania panamensis*, *L. braziliensis* and *L. guyanensis*, are responsible for this disease. The main target organs that are usually infected by this disease are nose, mouth, ear, larynx and pharynx (Kreier & Baker, 1987; Jeffrey & Leach, 1991; Service, 1996). Other symptoms such as weight loss, fever and anaemia are also present. The mucosal lesions by *L. braziliensis* are common in South America, and 5% of patients with cutaneous leishmaniasis develop this disease after several months to 30 years from the initial infection (Chang *et al*, 1985; Matlashewski, 2001). In over 80% of cases, this disease infects the nose, and it may move to the mouth and throat in 25% of patients (Chang *et al*, 1985).

Visceral leishmaniasis (VL) is the most severe form of the disease. It is commonly known as Kala-azar (Figure 1.9). The description of it was given by British doctor, Dr. Leishman, in 1900, and the development of a diagnosis of this disease was by Dr. Donovan (Matlashewski, 2001). VL is caused by *Leishmania donovani donovani* in Africa and India; and by *L. d. infantum* in Asia and the Near East; whereas in South America it is caused by *L. d. chagasi*. Human, jakals and dogs infected by visceral leishmaniasis will die if untreated (Chang *et al*, 1985). The inner organs, including liver, spleen, lymph nodes and bone marrow are infected by large numbers of macrophages filled with amastigotes.

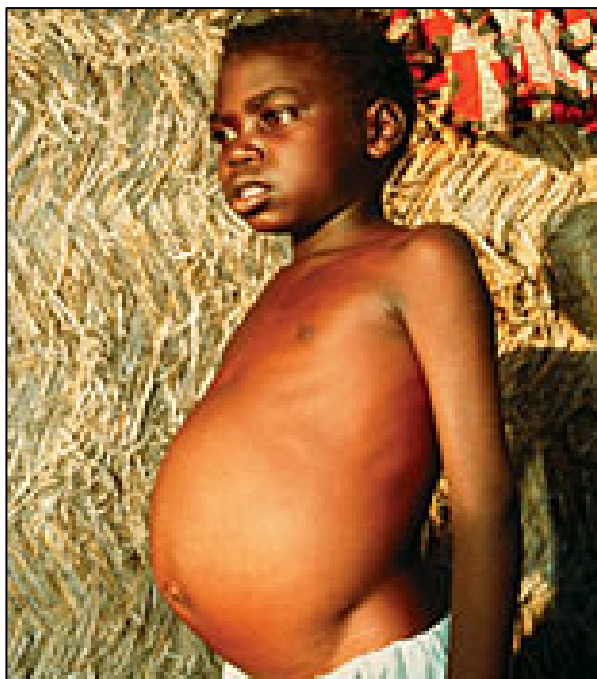


Figure 1.9. Picture showing a patient infected with visceral leishmaniasis.

Image obtained from: (<http://pubs.acs.org/cen/80th/images/>).

The main symptoms of VL are irregular fever, organized granuloma, enlargements of lymph nodes, liver (hepatomegaly) and spleen (splenomegaly). Furthermore, a sharp rise of nephrosis, anemia, leucopenia typical ashiness of skin color may occur. Transmission of VL from infected human to others may also occur through blood transfusion, kidney transplantation, and sharing of needles (Chang *et al*, 1985; WHO, 2000). The mortality rate of VL reaches 100% if untreated, and it is characterized by substantial loss of weight (WHO, 2000; TDR, 2004b).

1.2.4 Diagnosis

Because there are no direct tools to detect the presence of the parasites, the diagnosis of this disease is difficult. One of the important problems in diagnosis of leishmaniasis is the co-infection with HIV. The dependency on the clinical manifestations to diagnose the leishmaniasis is not always effective, because these signs (fever, weight loss, enlargement of liver and spleen, inflammation of lymph nodes) are not always present. On the other hand, the diagnosis of leishmaniasis, especially visceral leishmaniasis, may be confused by the symptoms of the related diseases such as cryptosporidium, disseminated cryptococcosis, mycobacterial infection and cytomegalovirusinfection (WHO, 2000). These signs may also assimilate that of typhoid, malaria, shistosomiasis and tuberculosis (Schallig *et al*, 2001). The diagnosis of cutaneous leishmaniasis is usually performed in the endemic areas by aspiration of the fluid from the ulcer area to demonstrate parasites (Markell *et al*, 1999). In visceral leishmaniasis, diagnosis can be performed by aspirations from lymph nodes, bone marrow, spleen, liver or skin. Parasites taken from venous blood or specimens of the organs can be cultured on an appropriate medium under laboratory conditions (Jhingran *et al*, 2008). There are three mediums that could be used for this technique: Schneider's *Drosophila* medium supplemented with 10% (v/v) fetal calf serum (FCS), Novy-MacNeal-Nicolle (NNN) medium supplemented with 20-30% rabbit blood (Jhingran *et al*, 2008) or RPMI medium 1640 (Markell *et al*, 1999). Bone marrow aspirate (BMB) is considered to be the most secure and most sensitive technique. In addition, liver biopsy and spleen aspirate are also used in this diagnosis (WHO, 2000).

The most important diagnosis tools in the field are the direct agglutination test (DAT) and the immunofluorescence test (IFAT). These serological tests are used to detect the antibodies, and the consolidation DAT-PCR may lead to detection of the parasites in the patients (Schallig *et al*, 2001; TDR, 2005). The Latex agglutination test (KAtex) is also

used to detect the presence of antigens in the urine of patients infected with Kala-azar (Jhingran *et al*, 2008; TDR, 2005), rK39 is another test based on re-combinant leishmanial antigen (Jhingran *et al*, 2008; TDR, 2005). However, the most popular, sensitive and specific assay is ELISA (enzyme-linked immunosorbent assay). This technique can easily differentiate between visceral and cutaneous leishmaniasis due to presence or absence of gp63 antigen which is found in the former and not in the latter (Shreffler *et al*, 1993).

1.2.5 Chemotherapy

Public health attention is drawn more towards Visceral leishmaniasis (VL) because of its severity and prevalence (Olliaro *et al*, 2002). In most cases of cutaneous leishmaniasis (CL), the granuloma heals in a few months after the infection; therefore, treatment is sometimes not necessary. However, in some cases, the lesions multiply and are present for more than 6 months (Chang *et al*, 1985).

1.2.5.1 Antimonial therapy

The first line of treatment of the disease is the use of Antimonial. These drugs have been used since 1947 for treating both Visceral and cutaneous leishmaniasis. Two different preparations for antimonial therapy are available: Pentostam ((Sodium Stibogluconate-Wellcome) Figure 1.10A) and Glucantime (Meglumine Antimoniate-Rhodia, Specia). Malaise, vomiting and anorexia are early signs of toxicity of pentavalent antimonials (Chang and Bray, 1985; Kreier and Baker, 1987). The first drug is distributed in English-speaking countries, while the second is found in French and Spanish-speaking countries (Balana-Fouce *et al*, 1998). Visceral leishmaniasis usually responds to antimony therapy. Relapse is possible and retreatment of the disease is sometimes with amphotericin B or pentamidine (Olliaro *et al*, 2002). Approximately 50% of cases have suffered from VL relapses (Ganguly, 2002). The main idea of pentavalent antimonials is based on the amount of antimony (Sb) absorbed per body weight. The optimal dosage which was recommended in 1982 by WHO for treating leishmaniasis is 20 mg per kg per day and this dosage should not be increased to more than 850 mg of Sb per day for 28 days (Balana-Fouce *et al*, 1998).

The mode of action of pentavalent antimonials in amastigotes forms of different species of *Leishmania* is that these drugs interfere with glycolysis and the β -oxidation of fatty acids, leading to a depletion in the level of intracellular ATP (Van Voorhis, 1990). Using a

high dose of these drugs leads to many side effects including headache, anorexia, nausea, cardiotoxicity, phlebitis (Navin *et al*, 1992), myalgia, malaise, hepatotoxicity (Hepburn *et al*, 1993), fever, skin rash, cough, gastrointestinal irritation (Wylter & Marsden, 1984), and pancreatitis (Gasser *et al*, 1994). Parasite resistance against pentavalent antimonials leads to treatment failure: relapses are very common in patients and occur in 10-25% of cases (Opperdoes & Michels, 2008). The preferred second-line drugs include: Pentamidine, Amphotericin B and Amphotericin B encapsulated in liposomes (WHO, 2000).

1.2.5.2 Pentamidine

Pentamidine (Figure 1.10B) is an aromatic drug used as a second line of treatment in the case of an antimony-relapsed visceral leishmaniasis. This drug is highly effective, expensive, and toxic. The therapeutic dose of pentamidine is intramuscular injections of 2-4 mg/kg body weight three times a week for 3-5 weeks (Balana-Fouce *et al*, 1998). The side effects of this drug have been noted in 30-50% of treated cases, and these effects including hyperglycemia, hypoglycemia, sudden shock, sterile injection abscess (Sundar, 2001b), skin rashes, anemia, nephrotoxicity, induction of hepatic enzymes (Balana-Fouce *et al*, 1998; Sundar, 2001b) and diabetes mellitus in 10% of pentamidine treated cases (Ganguly, 2002).

This drug is taken up and concentrated inside the cells by an energy dependent mechanism and competes with the arginine and polyamine transport systems (Basselin *et al*, 1996). The mechanism of pentamidine action is not clear right now but one of the possibilities is that this drug affects the parasite's mitochondria by reducing the membrane potential of this organelle (Basselin *et al*, 2002; Mukherjee *et al*, 2006).

1.2.5.3 Amphotericin B

Amphotericin B is a second line drug used for the treatment of all types of leishmaniasis. It has been reported to be active against antimony relapses of visceral leishmaniasis (Olliaro & Bryceson, 1993). This drug is expensive and also toxic. However, the lipid formulations, disteoylphosphatidyl glycerol amphotericin B complex (AmBisome), cholesterol sulfate-amphotericin B liposomes (Amphocil) and Abeleet are less toxic and more active against VL and ML (Yardley & Croft, 2000). Amphotericin B (Figure 1.10C) and amphotericin B liposomes are effective against VL and CL experimental models (Croft *et al*, 2005). The use of this drug in Bihar (India) and Nepal, for example, became wide spread, and extended treatment is necessary if the parasites are still in marrow or splenic smears.

The recommended dose of amphotericin B is from 0.5 to 1 mg/kg body weight i.v. on alternative days. However, at the higher doses, some unusual toxicities are possible including hypokalemia, hypomagnesemia, hepatic toxicity, renal dysfunction, fever, bone marrow suppression, myocytitis and sudden death (Balana-Fouce *et al*, 1998; Sundar, 2001b).

The mode of action of amphotericin B in *Leishmania* and fungi is based on sterol metabolism, and the main sterol synthesized in the membranes of these organisms is 24-ergosterol. Parasite can be killed due to an increase in membrane permeability by the action of amphotericin B which has a high affinity for sterols and changes the cell membrane construction (Balana-Fouce *et al*, 1998; Singh *et al*, 2006).

1.2.5.4 Miltefosine

Miltefosine is an alkyl phospholipid (Figure 1.10D) which was originally developed as an anti-cancer agent but later found to have excellent activity against leishmaniasis. This drug is the first oral remedy for VL (TDR, 2005; Fischer *et al*, 2001) and is also effective against CL (Croft *et al*, 2005). Miltefosine has been tested against VL in Bihar (India) and found to be very effective with more than 94% cure rate (Singh *et al*, 2006). Furthermore, several studies have reported that this agent is clinically effective against CL in Africa and Colombia (Opperdoes & Michels, 2008). The optimal therapeutic dose of miltefosine is 2.5 mg/kg per day for four weeks (Jha *et al*, 1999; Sundar *et al*, 2002). Miltefosine has minor gastrointestinal effects like vomiting in 40% of patients and diarrhea in 20% of cases (Singh *et al*, 2006). On the other hand, miltefosine has a long half-life of 154 h, which might lead to the development of drug resistance if this drug was used alone (Singh *et al*, 2006). A study was performed *in vitro* and *in vivo* by (Seifert & Croft, 2006) on the combination of miltefosine with other drugs such as amphotericin B, Pentostam, paromomycin and sitamaquine. The *in vivo* result of that study revealed that the best combination of miltefosine was with amphotericin B or paromomycin.

This drug is potential teratogenicity, therefore, the use of it is very limited (Olliero *et al*, 2002) and can not be used in pregnant women (TDR, 2005). Furthermore, after healing, the drug leaves a skin eruption.

1.2.5.5 Paromomycin

Paromomycin (Figure 1.10B) is used to treat bacterial and intestinal parasitic infections (such as amoebiasis) that are associated with VL. It is particularly used for the treatment of

highly antimony resistant VL. This drug is cheap, effective and authorized (Sundar, 2001b; Olliaro *et al*, 2002). Paromomycin is safe and used as an alternative to amphotericin B which is toxic and expensive, it has also been used in oral and tropical formulations for the treatment of enteric protozoa and Old World CL respectively (TDR, 2005). The standard dose of this drug is 14-16 mg/kg/day for three weeks with a maximum daily dose of 1 g. Toxicity of this drug includes nerve toxicity and nephrotoxicity (Sundar, 2001b). Paromomycin ointment was assessed in central Tunisia for its suitability for treatment of *Leishmania major*, which causes zoonotic CL. The results imply that this drug is not effective (Ben Salah *et al*, 1995). Furthermore, the combinations between paromomycin and antimonials have been extensively studied to treat the New (Soto *et al*, 1995) and Old leishmaniasis (Jha *et al*, 1998; Thakur *et al*, 2000).

Table 1.2 showing the differentiation between the antileishmanial drugs were used to treat the cutaneous and visceral leishmaniasis throughout 20 years (1985-2005).

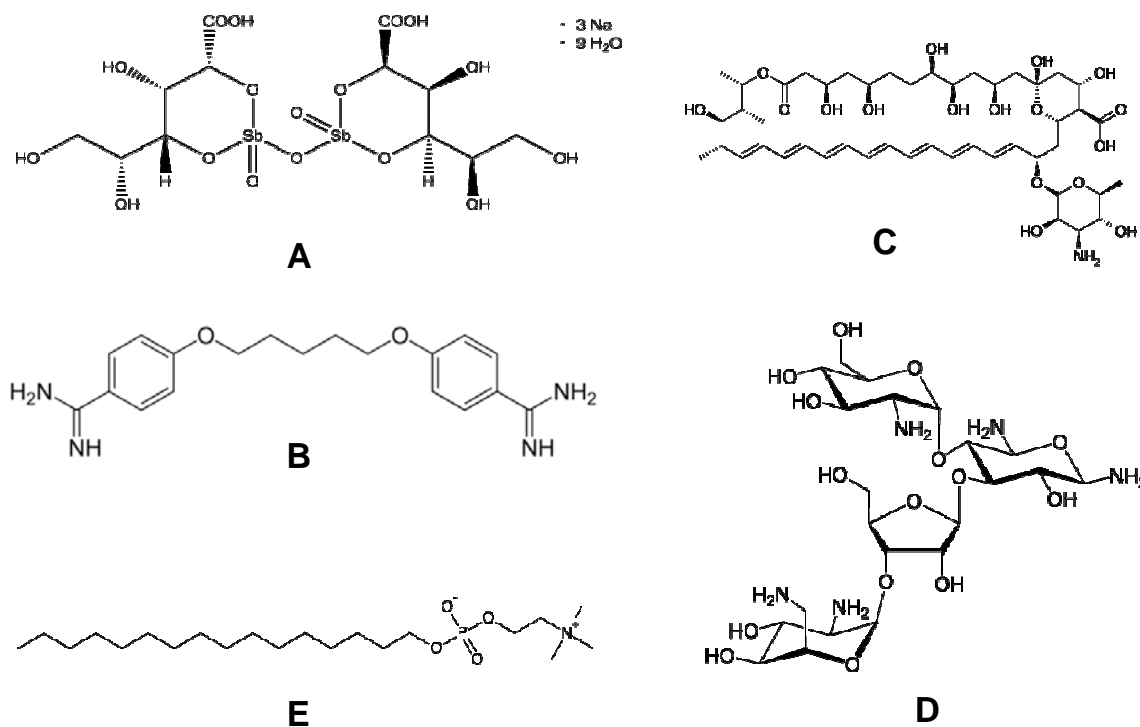


Figure 1.10. Structural formulas of drugs used against Leishmaniasis.

Pentostam (A); Pentamidine (B); Amphotericin B (C); Miltefosine (D) and Paromomycin (E).

Table 1.2 Drugs in use and on trial in 1985 and 2005.**(Adapted from Croft *et al*, 2005).**

Visceral leishmaniasis	1985	2005
First-line drugs	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)	Sodium stibogluconate (Pentostam, generic sodium stibogluconate); meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)	Amphotericin B (Fungizone)
		Liposomal amphotericin B (AmBisome)
	Pentamidine	Pentamidine
Clinical trials	Allopurinol (Phase II)	Miltefosine (oral, Phase IV, registered in India)
		Paromomycin (Phase III)
		Sitamaquine (oral, Phase II)
		Other amphotericin B formulations
Drugs in preclinical development	–	–
Cutaneous leishmaniasis	1985	2005
First-line drugs	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)	Sodium stibogluconate (Pentostam); meglumine antimoniate (Glucantime)
	Amphotericin B (Fungizone)	Amphotericin B (Fungizone)
	Pentamidine	Pentamidine
		Paromomycin (topical formulations)
Clinical trials	Paromomycin (topical formulation, Phase II)	Miltefosine (oral, Phase III)
	Allopurinol riboside (Phase II)	Paromomycin (other topicals, Phase II)
		Imiquimod (topical immunomodulator, Phase II)
Drugs in preclinical development	–	–

1.2.6 Drug resistance

The spread of resistance to antileishmanial agents has become a serious problem in efforts to control the various diseases caused by *Leishmania* parasites. There are few drugs available against leishmaniasis.

Pentavalent antimonials have been used as antileishmanial agents for a long time. The resistance to these drugs is now found in many regions in the world such as: North East India, especially in north Bihar, which has a treatment failure of between 50-65% (Sundar *et al*, 2000; Sundar, 2001a), in Iran (Hadighi *et al*, 2006) and in South America (Rojas *et*

al, 2006). The optimal dose of this drug, which was used for more than half a century, is 10 mg/kg body weight (maximum 600 mg/day) for 6-10 days. The resistance to this drug started in early 1980, therefore, WHO changed the dosage and the duration to 20 mg/kg body weight (maximum 850 mg/day) for 20 days (WHO, 1984). There are colleagues reports that this dosage cures 81% of patients in 20 days, and in 40 days the cure rate is 97% (Thakur *et al*, 1988).

The use of amphotericin B has been limited due to the cost and toxicity. At the moment, no resistance to amphotericin B has been reported. The use of this drug in lipid formulations, which have a long half-life, has become popular in the last few years (Croft *et al*, 2006a). Pentamidine has also been used as a second-line drug for CL, DCL and VL. This drug is limited in use, hence development of resistance to this drug against CL is not a serious problem (Croft *et al*, 2006). However, resistance to pentamidine has been reported for visceral leishmaniasis in different countries in the New and Old Worlds. For instance, a high proportion of patients with this disease did not respond to treatment with pentamidine in India (Jha, 1983; Jha *et al*, 1991; Giri, 1994). Miltefosine is the first oral drug introduced to treat visceral leishmaniasis in India, with a cure rate of 95% (Jha *et al*, 1999). Even though the use of miltefosine in clinical use started several years ago, no resistance has been yet reported to this drug (Choudhury *et al*, 2008). However, the long half-life of this drug in plasma, and the *in vitro* selected miltefosine-resistance cell line suggest that the development of drug resistance may be quite possible (Seifert *et al*, 2003; Sundar *et al*, 2006). Parasite resistance to paromomycin has not yet been reported. This is possibly due to the fact that it has limited use for the treatment of visceral leishmaniasis (Croft *et al*, 2006).

1.3 Natural products derivatised for treatment of protozoal infections

There are a limited number of drugs licensed and used as antiparasitic agents for humans. Because of the current upsurge in drug resistance, many of these drugs have become inactive or less active against the parasites. Furthermore, problems like the toxicity, costs and availability are associated with these drugs especially in the disease endemic and poor regions of the world. Consequently, sources of natural products, such as plants; microorganisms (bacteria, fungi), marine organisms, protozoa, sponges and invertebrates have been examined to ascertain their antiprotozoal activities. Some are already being used to treat diseases (Kayser *et al*, 2003). There are more than 20,000 species of plants that are used in the world as traditional medical remedies to confront a huge number of diseases (Phillipson, 1994). It was estimated that 6.1 billion people in the world (66%) rely on traditional medical drugs because of the limitation of availability and/or the rising costs of pharmaceutical medicines (Tagboto & Townson, 2001). However, a high percentage of these people are using these drugs with poor or no knowledge about their toxicity. Hence the use of some of these compounds leads to fatal consequences.

The first three traditional herbal remedies used as natural products derived from plants are foxglove, ephedra, and morphine. These three drugs are widely used for the treatment of heart disease as respiratory disorders, and as a potent analgesic, respectively (Tagboto & Townson, 2001). On the other hand, most antiprotozoal plant-extracted compounds have been utilized as starting points for the synthesis of new drugs with stronger activity and less toxicity.

A number of natural antiparasitics are going to be discussed in more detail below. Two of these compounds, quinine and artemisinin, are in use to treat malaria, whereas the third, curcumin, has been proposed for use to confront several parasitic and non-parasitic diseases.

1.3.1 Quinine

Quinine is a natural white crystalline alkaloid compound (Figure 1.11A) extracted from *Cinchona* species. The first use of this compound was in South America to treat tropical

fevers. This compound has been used as a treatment for malaria caused by *P. falciparum*. This drug is also used for its analgesic and anti-inflammatory properties.

There are five forms of quinine base: quinine dihydrochloride, quinine hydrochloride, quinine sulfate and quinine gluconate. All these forms of quinine can be given orally or by intravenous injection in doses of 8 mg/kg of quinine base three times a day. However, Barennes and colleagues reported that it can also be safely given rectally or intramuscularly every 12 hours in doses of 20 mg per kg or 12.5 mg/kg of quinine base, respectively (Barennes *et al*, 2006).

Though the mode of action of quinine against the malaria parasites is not well understood, it has been postulated to be similar to that of chloroquine; causing cytotoxicity by inhibiting the polymerisation of haeme into haemozoin, with the consequent built up of free haeme, which is toxic to the parasite (Yakuob *et al*, 1995). It has also been proposed that the drug forms a complex with double-stranded DNA to prevent strand separation or that it blocks DNA replication and transcription and thus prevents proliferation of the malaria parasites.

Quinine has been found to exhibit some side effects including cinchonism, heart attack, stroke, cardiac arrhythmias, thrombocytopenia, liver failure or damage, kidney failure or damage, hearing loss, cardiovascular problems, allergic reactions to quinine and erectile dysfunction among others (<http://www.resource4thepeople.com/defectivedrugs/quinine.html>). Several studies have investigated the use of quinine to treat people infected with malaria. However, very few studies have been conducted to test the effect of this compound on trypanosomes *in vitro*. One of these studies was carried out by Merschjohann and co-workers, which measured the efficacy of this drug together with 33 other alkaloids on two species of *Trypanosoma*: *T. brucei brucei* and *T. congolense* (Merschjohann *et al*, 2001). The IC₅₀ value of quinine on *T. b. brucei* was determined as 4.9 μM in this study.

1.3.2 Artemisinin

Artemisinin (Figure 1.11B) is a compound derived from a herb, *Artemisia annua* (Ferreira & Janick, 1996). It has been identified as the active ingredient of the natural herbs to treat skin diseases, malaria and another parasitic infections such as *Schistosoma mansoni*, *S.*

japonicum and *Clonorchis sinensis* (Hien & White, 1993). It is also used to protect against some types of cancer, especially leukemia and colon cancer (<http://www.antiaging-systems.com/a2z/artemisinin.htm>). Due to its ability to regulate hormones, it is also used in the treatment and prevention of breast cancer (Rowen, 2002). This compound was discovered in the 1960s by Chinese biologist Tu Youyou and was named Qinghaosu in Chinese. In 1970s, the compound was classified as a multi-drug resistance drug for the treatment of *P. falciparum* malaria (Arnold *et al*, 1990).

Derivatives of artemisinin are artemether, artesunate, arteether and artelinate. These compounds are easily converted to their active plasma metabolite, dihydroartemisinin, which is reported to be the active compound that exhibits the anti-malarial activity (Wilairatana *et al*, 1998). The optimum dose of artemisinin for treatment of the disease is 3 g administrated over 3-5 days (about 50 mg per kg). Due to the short half-life of the derivatives, it is recommended that they are combined with other antimalarial drugs of longer half-life (White, 2004).

Although the specific mechanism of action of artemisinin is not clear, a study in 2005 using a yeast model demonstrated that the 'drug acts on the electron transport chain, generates local reactive oxygen species, and causes the depolarization of the mitochondrial membrane' (Li *et al*, 2005).

No side effects have been reported even at a high dosage of 70 mg/kg body weight per day (Rowen, 2002). However a report indicated that the concentrations of serum aspartate aminotransferase (AST) were elevated in a volunteer receiving a high dose (120 mg/kg) of artemisinin over 3 days (Hien & White, 1993). This increase of AST is an indication of damage to acute or mild hepatocytes toxicity.

1.3.3 Curcumin

Curcumin (diferuloyl methane, Figure 1.11C), a phenolic compound, is the predominant compound, and a natural yellow dye, extracted from the roots and rhizomes of different species of *Curcuma* plants, which is known as Zingiberaceae (Perez-Arriaga *et al*, 2006). *Curcuma longa* Linn (turmeric) is a major medicinal plant in this group, and the powder of this herb is widely used to give color, spice and flavour to foods (Pan *et al*, 1999; Iqbal *et al*, 2003; Sharma *et al*, 2005). The active ingredients of *Curcuma* spp. are divided into

three parts: the first is essential oils including zingiberene, turmerones and atlantones, the second is turmerin (a water soluble peptide) and the last is curcuminoids such as curcumin (Sharma *et al.*, 2005).

Curcumin dissolves in acetone, ethanol and dimethylsulphoxide, but is comparatively insoluble in water (Sharma *et al.*, 2005). Wang *et al.*, (1997) show that curcumin is unstable at neutral and basic pH as it is broken down to feruloyl-methane and ferulic acid. Using spectrophotometry, Oetari *et al.*, (1996) also observed that curcumin, which is light sensitive is unstable in phosphate buffer at pH 7.4. In another study by Pan and colleagues it was found that above 90% of curcumin decays in neutral-basic pH buffer (Pan *et al.* 1999). However, the presence of fetal calf serum and some anti-oxidants such as ascorbic acid, glutathione and *N*-acetylcysteine in the culture medium blocks this degradation (Wang *et al.*, 1997). Studies by the groups of Pan and Maheshwari showed that the main curcumin metabolites are dihydrocurcumin and tetrahydrocurcumin, which are further metabolised to monoglucuronide conjugates (Pan *et al.*, 1999) (Maheshwari *et al.*, 2006b). Holder and co-workers have also demonstrated that the main metabolites of curcumin are glucuronide conjugates of tetrahydrocurcumin (THC) and hexahydrocurcumin. (Holder *et al.*, 1978).

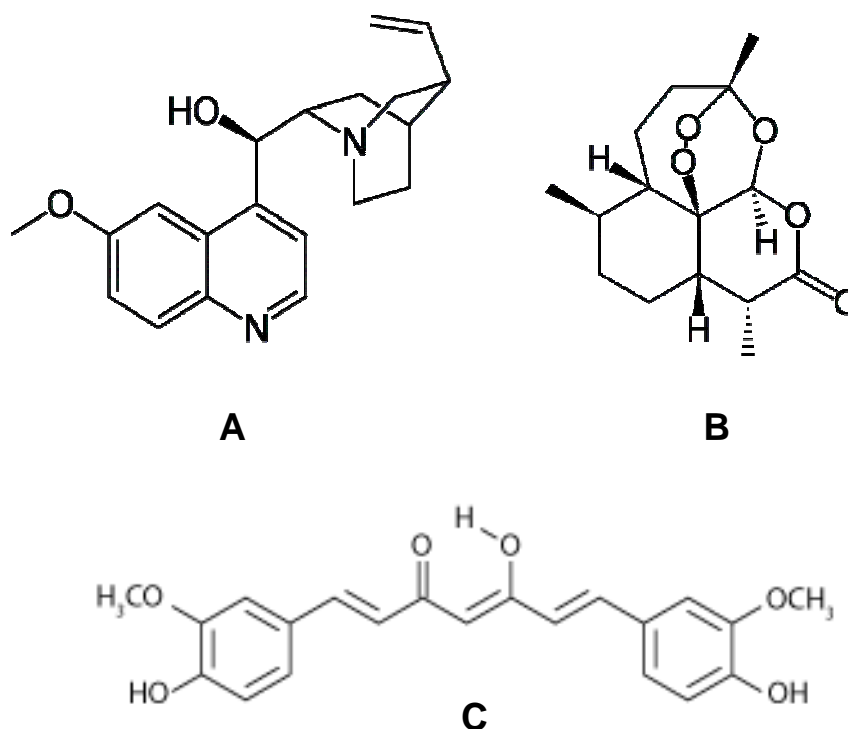


Figure 1.11. Chemical structures of some natural antiprotozoal agents. Quinine (A); artemisinin (B) and curcumin (C).

1.4 Medicinal applications of curcumin

Curcumin has been used to confront two of the main diseases of the developed world, namely cancer and cardiovascular disease. It has also been reported to have anti-oxidant, anti-inflammatory and anti-carcinogenic (tumor-promoting) activities (Sharma *et al*, 2005; Iqbal *et al*, 2003; Koide *et al*, 2002). Curcumin also exhibited many interesting biological activities against microorganisms, for instance, antiprotozoal activity (Araujo *et al*, 1999; Reddy *et al*, 2005), antibacterial activity (Dahl *et al*, 1989; Dahl *et al*, 1994), antifungal activity (Apisariyakul *et al*, 1995) and anti-HIV (Mazumder *et al*, 1995).

Oxidative stress plays a significant role in the pathogenesis of many diseases such as myocardial ischemia, cerebral ischemia-reperfusion injury, neuronal cell injury, cancer and hypoxia (Maheshwari *et al*, 2006). This kind of stress is influenced by the presence of reactive oxygen compounds such as hydrogen peroxide (H₂O₂), superoxide, lipid peroxides and hypochlorous acid (Balasubramanyam *et al*, 2003). Curcumin plays a major role in the protection of different cells from oxidative stress. As an example, it protects against the injury of kidney cells (LLC-PK₁) by blocking lipid peroxidation, lipid degradation and cytolysis (Cohly *et al*, 1998).

Inflammation is an important contributor to chronic neurodegenerative diseases, and cyclooxygenase-2 (COX-2) is reported to play a crucial role in this process (Kang *et al*, 2004). In various cells, the transcription factor NF-kappa B also plays a significant role in the transcriptional regulation of pro-inflammatory gene expression (Jobin *et al*, 1999). Curcumin has been reported to be a promising anti-inflammatory agent due to its ability to block the activity of both COX-2 (Cho *et al*, 2005) and NF-kappa B (Jobin *et al*, 1999; Weber *et al*, 2006). On the other hand, inflammation is one of the three processes of wound healing, granulation and tissue remodeling, and tissue repair (Maheshwari *et al*, 2006). It has been reported that, after curcumin treatment, the treated wound biopsies exhibit an abundance of infiltrating cells including macrophage, fibroblasts and neutrophils (Sidhu *et al*, 1998).

Several studies have demonstrated the use of curcumin as an anti-cancer agent. The compound has been found to be a dose dependent chemopreventive agent against many animal tumor bio-assays such as oral, oesophageal, stomach, colon and duodenal carcinogenesis (Maheshwari *et al*, 2006). Other epidemiological and animal model studies reported further support that the natural curcuminoids have anti-inflammatory or anti-

oxidant activities and can be used as an inhibitor of carcinogenesis (Viaje *et al*, 1977; Kozumbo *et al*, 1983; Nakadate *et al*, 1984; Smart *et al*, 1987; Huang *et al*, 1997). Among the more significant findings in the demonstration that curcumin strongly prevents 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced tumor promotion in mouse skin (Huang *et al*, 1988; Lu *et al*, 1994; Limtrakul *et al*, 1997; Huang *et al*, 1997).

As an anti-parasitic agent, curcumin and its analogues have been shown to possess very good activity against some intestinal protozoal infections. One of these studies reported that this natural agent a potently inhibits the growth of axenic *Giardia lamblia*, with morphological changes and DNA fragmentation (Perez-Arriaga *et al*, 2006). On the other hand, numerous studies using this compound have been performed on kinetoplastid protozoa, especially *Leishmania* species (Koide *et al*, 2002; Araujo *et al*, 1999; Gomes *et al*, 2002a; Gomes *et al*, 2002b; Alves *et al*, 2003; Saleheen *et al*, 2002) and *T. brucei* (Nose *et al*, 1998) with a moderate-to-low activity (see chapter 5, section 5.1).

Curcumin inhibits the growth of both chloroquine sensitive (CQ-S) and resistant (CQ-R) *Plasmodium falciparum* *in vitro* with an IC₅₀ of 3.25 μM and 4.21 μM, respectively (Mishra *et al*, 2008). Furthermore, twelve analogues were tested on the same two strains, and the results showed that the activities of some of these analogues against the parasites are 10 times stronger than the activity of curcumin itself. A study by Reddy and coworkers also showed that curcumin displays dose dependent activity against CQ-R *P. falciparum* with an IC₅₀ of ~5 μM (Reddy *et al*, 2005). In the same study it was also found that oral curcumin fed to mice infected with *Plasmodium berghei* decreased parasitemia by 80-90%. In recent years, curcumin has been used in combination with artemisinin, another natural compound, to treat *P. berghei* with very promising results (Nandakumar *et al*, 2006). Moreover, curcumin-treated *P. falciparum* lead to generation of reactive oxygen species (ROS) and degradation of the nuclear and mitochondrial DNA (Cui *et al*, 2007a).

1.5 Choline metabolism in kinetoplastids as a therapeutic target

Choline performs many essential functions in eukaryotes. Among other things, it is an important substrate required for the structural integrity and signalling functions of cell membranes, acetylcholine synthesis and methyl group metabolism. In addition, a metabolite of choline, betaine, methylates homocysteine to form methionine (Finkelstein, 2000). Methionine then becomes the precursor for *S*-adenosylmethionine (AdoMet), which is the physiological methyl group donor for protein, RNA and DNA methylation (Jeltsch, 2002).

The surface of kinetoplastid protozoa (*T. brucei*, *T. cruzi* and *Leishmania* spp) are morphologically divided into two distinct parts: the flagellar pocket and the plasma membrane (Vial *et al*, 2003). The plasma membrane of these parasites comprises a dense coat of glycoprotein and/or glycolipid. Generally, phospholipids are synthesized by esterification of an alcohol to a phosphatidic acid called 1,2-diacylglycerol 3-phosphate. The most common phospholipids in *T. brucei* are phosphatidylcholine (PC, Figure 1.12 A), phosphatidylethanolamine (PE, Figure 1.12 B) and phosphatidylserine (PS, Figure 1.12 C) which constitute about 80% of the total trypanosomal lipids (Patnaik *et al*, 1993). These phospholipids play an important structural role to the membrane and, in addition, determine membrane fluidity and cell-surface charge (Gibellini *et al*, 2008).

The fatty acid composition of the various phospholipids has been described (Patnaik *et al*, 1993) as has the acyl donor specificity of the *T. b. brucei* lyso-phosphatidylcholine:acyl CoA acyltransferase (Samad *et al*, 1988). The most important saturated fatty acids are myristate (14:0), palmitate (16:0) and stearate (18:0), in addition to unsaturated fatty acids such as oleolate (18:1), linoleate (18:2) and arachidonate (20:4). Of particular significance is myristate, an essential component of glycosyl phosphatidylinositol (GPI_{no}) anchors (Werbovetz *et al*, 1996; Werbovetz & Englund, 1996). GPI_{no} anchors attach the trypanosome's Variant Surface Glycoprotein (VSG) to the plasma membrane (Englund, 1993), which is essential for survival and pathogenesis within the host (Ferguson, 1999). VSG is initially synthesized in the endoplasmic reticulum (ER) and it is immediately taken up to the cell surface by the Golgi apparatus (Ferguson *et al*, 1986; Duszenko *et al*, 1988).

Sphingolipids, such as sphingomyelin (SM), also play an important role in the normal cell membrane of all eukaryotic cells including kinetoplastid protozoa (Fridberg *et al*, 2008). Bloodstream *T. b. brucei* contain sphingomyelin (choline phosphorylceramide) and

ethanolamine phosphorylceramide but no inositol phosphorylceramide (IPC) (Sutterwala *et al.*, 2008), although procyclics do contain IPC (Fridberg *et al.*, 2008; Sutterwala *et al.*, 2008). Ceramide, a fundamental structural unit in all sphingolipids, is synthesized in the ER and then transported to the Golgi apparatus to bind with the polar head groups and produce sphingomyelin (Sutterwala *et al.*, 2007). Unlike *L. major* promastigotes, sphingolipid biosynthesis is essential for *T. brucei* viability (Sutterwala *et al.*, 2007; Zhang *et al.*, 2007).

Phosphatidylcholine can be synthesized in various organisms by three different metabolic ways: the first is called de novo or Kennedy pathway, by which the conversion of choline into phosphatidylcholine occurs in mammalian cells, bacteria and fungi (Carman & Henry, 1989; Lykidis & Jackowski, 2001; Sohlenkamp *et al.*, 2003). The second is CDP-diacylglycerol pathway in mammalian liver cells and yeast. This route starts by the formation of phosphatidylserine from serine and CDP-diacylglycerol, followed by decarboxylation of phosphatidylserine to phosphatidylethanolamine (PE). The latter is then converted to PC by addition of methyl groups donated by S-adenosylmethionine (Kanipes & Henry, 1997; Vance *et al.*, 1997; Sohlenkamp *et al.*, 2003). The last pathway occurs in plants (Mudd & Datko, 1986; Datko & Mudd, 1988) and *Plasmodium falciparum* (Pessi *et al.*, 2004; Pessi *et al.*, 2005). The main idea of this route is the methylation of phosphoethanolamine to phosphocholine followed by synthesis of phosphatidylcholine through the Kennedy pathway.

In *Trypanosoma brucei*, PC accounts for about fifty percent of all phospholipids in both life-cycle stages (procyclic and bloodstream forms), whereas PE accounts for 16 to 21% (Patnaik *et al.*, 1993). Synthesis pathways of PE and PC are very similar, and begin with the generation of phosphoethanolamine and phosphocholine by ethanolamine and choline kinases (Gibellini *et al.*, 2008) through the Kennedy pathway (Figure 1.13). However, choline is not believed to be directly taken up by bloodstream forms of *Trypanosoma brucei*, as the species lacks a choline transporter (Rifkin *et al.*, 1995), and the mammalian bloodstream contains only low levels of free choline to be salvaged (Zeisel, 1981). In contrast, ethanolamine is taken up very efficiently by bloodstream *T. b. brucei*, with a K_m value of 3.7 μM – close to the plasma concentration of this nutrient – and it is estimated that *T. b. brucei* can satisfy its ethanolamine requirements via this transporter (Rifkin & Fairlamb, 1985a). In contrast to the situation in other species including *Saccharomyces cerevisiae* (Nikawa *et al.*, 1986), the ethanolamine transporter does not share affinity with

choline: a 500-fold excess of choline did not inhibit ethanolamine transport in *T. b. brucei* (Rifkin *et al*, 1995).

Thus *T. b. brucei* cannot salvage choline directly from its environment (Rifkin *et al*, 1995) and while *Plasmodium falciparum* synthesises PC by methylation of PE (Pessi *et al*, 2005), this pathway is clearly absent in *T. b. brucei* as [³H]ethanolamine does not label PC (Rifkin & Fairlamb, 1985; Menon *et al*, 1993; Signorell *et al*, 2008). Nor can *T. brucei* salvage PC directly (Samad *et al*, 1988; Mellors & Samad, 1989) and it thus relies entirely on salvage of lyso-phosphatidylcholine (lyso-PC), which attains levels of some 250 µM in the human bloodstream (Zeisel, 1981), as the source of choline.

In leishmania, choline is taken up by a highly selective carrier with a K_m value of 2.5 µM that is not inhibited by ethanolamine (Zufferey & Mamoun, 2002). Instead, ethanolamine may be taken up by a serine transporter in *L. major*, as it is a potent inhibitor of this carrier (dos Santos *et al*, 2009), and structurally similar. Thus, Leishmania is not thought to rely on lyso-phospholipids to the same extent as *T. b. brucei*. The dependence of some trypanosomatids on lyso-phospholipid salvage for phospholipid biosynthesis can be exploited for chemotherapy. For example, miltefosine is a lyso-phospholipid analogue used as a potent drug against trypanosomatid parasites, *Leishmania sp* and *T. cruzi*, and its mode of action by inhibition of phosphatidylcholine biosynthesis (Urbina, 2006).

The utilization of lyso-phosphatidylcholine (lyso-PC) by the parasite is dependent on three enzymes: phospholipase A₁ (PLA₁), acyl-CoA synthetase (ACS) and acyl-CoA acyltransferase (AAT) (Bowes *et al*, 1993). Lyso-PC and lyso-PE are available to *T. brucei*, with concentrations of 250 and 10 µM, respectively (Bell & Coleman, 1980; NYE *et al*, 1961). The biosynthesis of phospholipids PC and PE by the Kennedy pathway (see Figure 1.13) starts when the parasites are exposed to a significant level of lyso-PC (Kennedy, 1956; Bowes *et al*, 1993). Lyso-PC is very toxic to cells and increases the membrane permeability (Gallo *et al*, 1984), it is rapidly metabolized by enzyme PLA₁, associated with plasma membrane, to free fatty acids and glycerophosphocholine (GPC) (Sage *et al*, 1981; Bowes *et al*, 1993). The latter is converted to choline by glycerophosphodiester phosphodiesterase (GDPE) (Smith T., unpublished observation). Choline is immediately converted by choline/ethanolamine kinase2 (CEK2) to choline phosphate (ChoP) (Gibellini *et al*, 2008), which is subsequently activated to CDP-choline, a high energy donor, by CTP:phosphocholine cytidyltransferase (CCT) (Signorell *et al*, 2008). The activated phosphocholine is then finally coupled to 1,2-diacylglycerol by the

enzyme diacylglycerol: CDP-cholinephosphotransferase (CPT), to form PC (Signorell *et al*, 2008; Signorell *et al*, 2009). On the other hand, the free fatty acids generated by PLA₁ are converted by acyl-CoA synthetase (ACS) into acyl-CoA and used by acyl-CoA acyltransferase (AAT) to produce additional amounts of phosphatidylcholine (Bowes *et al*, 1993; Werbovetz & Englund, 1996).

Being crucial to the survival of the parasite, the processes of choline salvage and metabolism constitute potential therapeutic drug targets. The identification and selection of appropriate inhibitors and cytotoxic compounds as anti-kinetoplastid agents will therefore depend on the detailed knowledge of the kinetic characteristics of the choline uptake mechanisms and metabolism in these parasites. Additionally, the plasma membrane of these organisms can itself become a drug target since it is the first point of interaction between the cell and the external environment including phospholipid analogues (Croft *et al*, 2003). The identification and characterization of choline transporters in *Trypanosoma* and *Leishmania* spp in order to facilitate a choline-based chemotherapy of these organisms therefore forms a major objective of the current study. The other objectives are stated below.

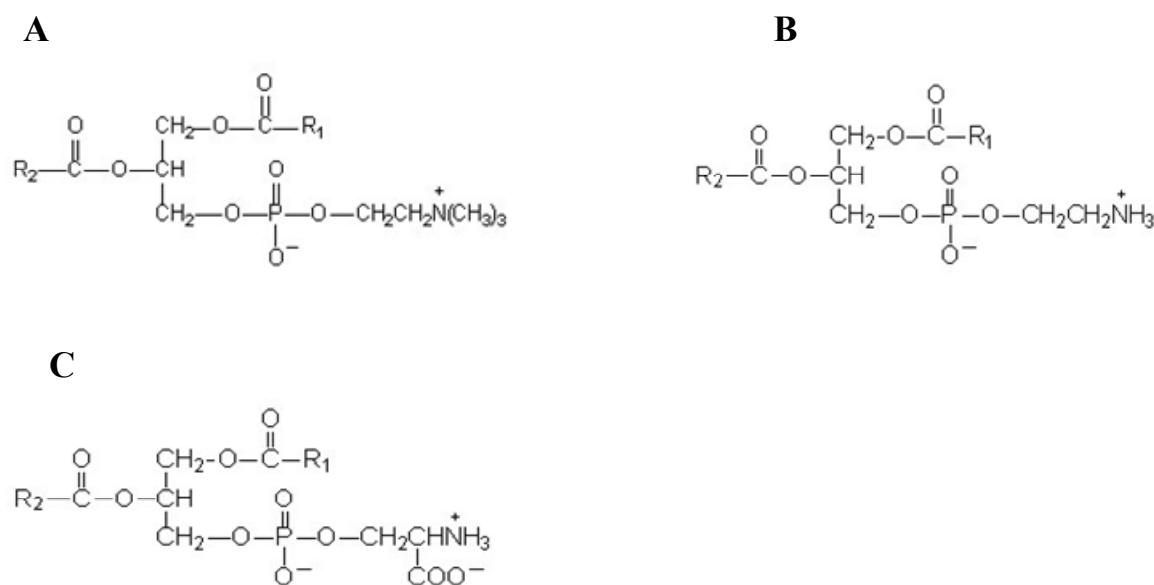


Figure 1.12. The chemical structures of the major phosphlipids. Phosphatidyl-choline (A); Phosphatidylethanolamine (B) and Phosphatidylserine (C).

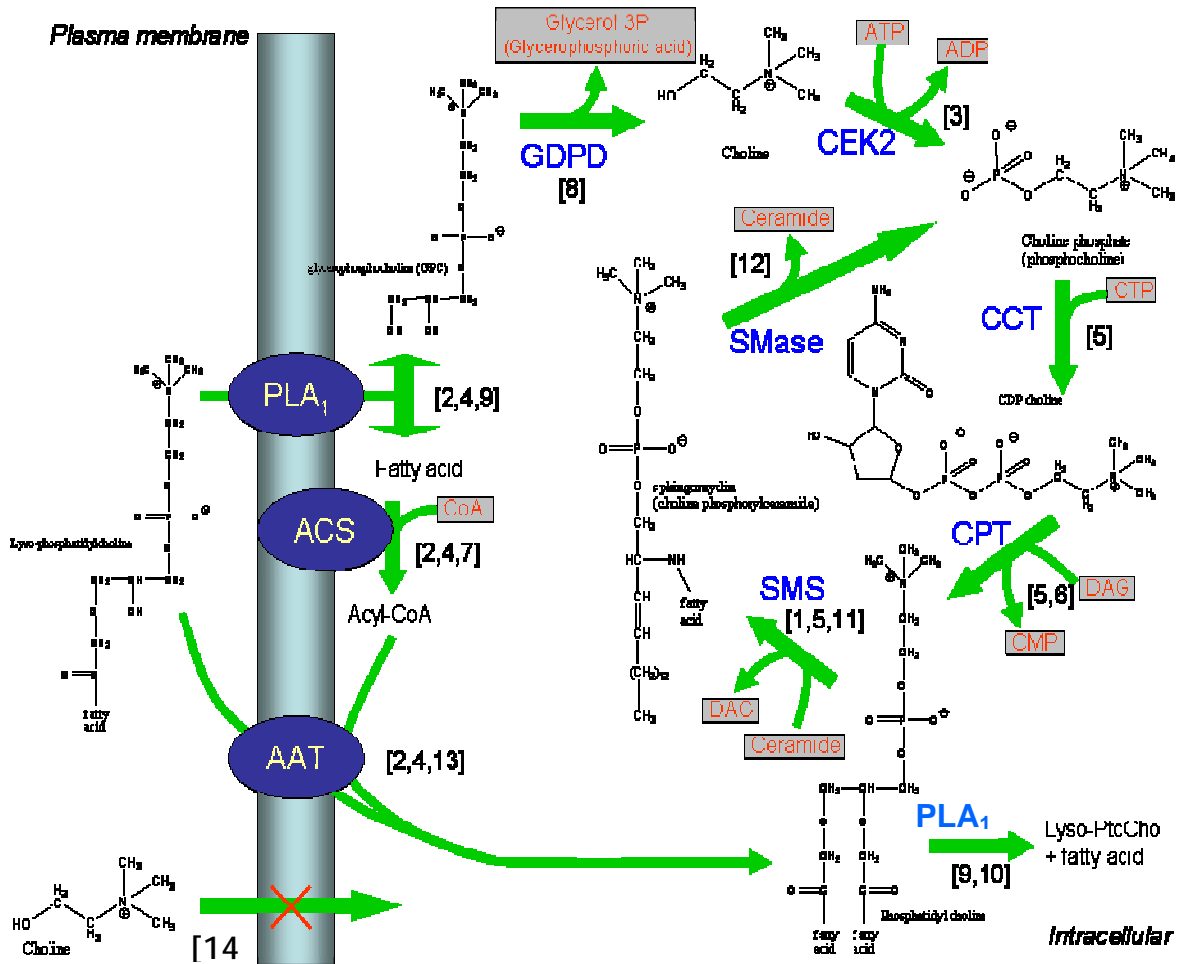


Figure 1.13. Choline metabolism pathway in kinetoplastid parasites.

(PLA1) Phospholipase A1; (GDPD) Glycerophosphodiester phosphodiesterase; (PLA2) Phospholipase A2; (CEK2) Choline/Ethanolamine kinase 2; (AAT) Acyl CoA acyltransferase; (CPT) diacylglycerol:CDP-cholinephosphotransferase; (CCT) CTP:phosphocholine cytidyltransferase; (CMP) cytidylmonophosphate; (SMS) Sphingomyelin Synthetase (TbSLS4, sphingolipid synthase 4); (SMase), Sphingomyelinase; (ACS) Acyl CoA synthase; (DAG) Diacylglycerol; (CoA) Coenzyme A; (1) Sutterwala et al (2008); (2) Bowes et al (1993); (3) Gibellini et al (2008); (4) Werbovetz and Englund (1996); (5) Signorell et al (2008); (6) Signorell et al (2009); (7) Jiang et al (2004); (8) Smith T., unpublished; (9) Sage, L. et al (1981); (10) Ridgley and Ruben (2001); (11) Sutterwala et al (2007); (12) Coppens I. et al (1995); (13) Samad et al. (1988); (14) Rifkin et al. (1995).

1.6 Aims

Specifically, this study will aim to investigate these points:

- To identify new curcumin and choline analogues with activity against African trypanosomes and *Leishmania* spp *in vitro* and *in vivo*.
- To identify structural determinants or motifs responsible for anti-protozoal activity.
- To study the mode of action of the most promising anti-protozoal metabolites identified in the screening.
- To determine the mode of uptake of these potential drugs.
- To study the toxicity of the anti-protozoal metabolites by *in vivo* observation in mice and incubations with hepatocytes.

Chapter two

2 Materials and Methods

2.1 Parasites, cell lines and cultures:

2.1.1 *Trypanosoma brucei* blood stream forms *in-vitro*:

Three clonal lines of trypanosomes were used in this study: *Trypanosoma brucei brucei* strain 427 wild type (WT); TbAT1 knock out (KO) derived from WT, which lacks the TbAT1/P2 aminopurine transporter and as a result is resistant to several important trypanocides (Matovu *et al*, 2003); and the pentamidine-adapted clonal line TbAT1-KO-B48 (Bridges *et al*, 2007). The standard culture medium for bloodstream trypanosomes of *Trypanosoma brucei* was HMI-9 medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (Hirurni and Hirurni, 1989). About 14 µl of β-mercaptoethanol per litre of medium were added before the sterilization of medium (filtration with a Millipore Stericup, capacity 500 ml, pore size 0.22 µm inside a flow cabinet). The parasites were passaged three times per week in this medium (pH 7.4) and incubated in a 37 °C and a 5% CO₂ atmosphere.

2.1.2 *Trypanosoma brucei* blood stream forms *in-vivo*:

Bloodstream forms of *Trypanosoma brucei brucei* strain 427 WT were intraperitoneally injected in adult female rats (Wistar strain) or ICR mice. Parasitaemia was daily monitored by tail venepuncture and examined utilizing a light microscope. The number of parasites in each field was estimated according to the method of Herbert & Lumsden (1976). At peak parasitaemia, the blood was collected from the infected animals by cardiac puncture under terminal anaesthesia using CO₂. The blood was collected into 15 ml Falcon tubes containing 5 ml of heparin in Carter's balanced salt solution (CBSS) 500 unit/ml (Appendix I), and centrifuged at 2500 x g for 15 minutes at 4 °C. Separating the blood into three different layers: a plasma layer, a red blood cell layer, and in the middle a parasites with buffy coat layer. The last layer was gently collected using a plastic Pasteur pipette and loaded onto a DE52 anion-exchange column at pH 8.0 as described by Lanham and Godfrey (1970). The cells were then washed from the column by 200 ml of phosphate saline glucose (PSG) solution pH 8.0 (Appendix I), and washed twice in assay buffer at 2200 x g for 10 minutes at 4 °C. The cell pellet was re-suspended in the required volume (~10⁸ cells/ml), and left at room temperature for 15-20 minutes to adapt to the experimental conditions.

2.1.3 *Leishmania major* & *Leishmania mexicana* promastigotes:

Promastigote forms of *Leishmania major* (Friedlin strain) and *Leishmania mexicana* (MNYC/BZ/62/M379 strain) were propagated at 25 °C in plastic flasks for tissue culture. These parasites were passaged three times a week in 10 ml of HOMEM medium (pH 7.4) with 10% Fetal Calf Serum (FCS). The medium was sterilized by filtration prior to use and stored at 4 °C.

2.1.4 *Leishmania mexicana* amastigotes:

Axenic *L. mexicana* amastigotes were maintained in 80% Schneider's *Drosophila* medium, supplemented with 20% heat-inactivated Fetal Calf Serum (HIFCS), 0.3% gentamicin per ml of medium, and 10 µl of 1.0 M hydrochloric acid per 1 ml of medium (pH 5.5). The parasites were passaged twice a week in 10 ml of the mentioned medium, and the appropriate conditions of this culture are 32 °C in a 5% CO₂ incubator.

2.1.5 Human embryonic kidney (HEK) cells (strain: 293 T):

HEK cells were cultured two or three times a week in a vented culture flasks at 37 °C and 5 -10% CO₂. In order to keep the cells in mid-log phase, the cells were usually passaged at 80-85% confluence. The standard culture medium for these cells is Dulbecco's Modified Eagle's Medium supplemented with New-born Calf Serum and 1% of both L-Glutamax (200 mM) and Penicillin (10,000 units/ml)/Streptomycin (10,000 µg/ml) solution (GIBCO).

2.2 Materials:

2.2.1 Media and growth chemicals:

Alamar Blue (Resazurin sodium salt), Dulbecco's Modified Eagle's Medium, 0.25% Trypsin-EDTA solution, Gentamicin, Diminazene aceturate, Digitonin, Heparin, collagenase, Trypan blue and *O*-phthaldehyde (OPT) and choline chloride were all obtained from Sigma. New-borne Calf Serum, L-Glutamax (200 mM), Penicillin/Streptomycin, Gentamicin, New-borne Calf Serum, HOMEM medium and Schneider's *Drosophila* medium were purchased from GIBCO. Heat-inactivated Fetal Calf Serum (FCS) was purchased from Biosera. Propidium iodide, dichlorofluorescein (DCF) and curcumin were purchased from Fluka. Troglitazone was from Biomol. Alamar BlueTM was obtained from Trek Diagnostics (UK).

2.2.2 Animals

The adult female mice (ICR strain) and adult male rats were purchased from Harlan UK Ltd, Bicester, Oxford Shire, UK.

2.2.3 Radiolabeled compounds

[³H] pentamidine (88 Ci/mmol), and [³H] choline chloride (83 Ci/mmol) were purchased from Amersham, and [³H] curcumin (10 Ci/mmol) was obtained from Moravek. These compounds were prepared from tritium gas and purified by high performance liquid chromatography.

2.2.4 Tested compounds:

2.2.4.1 Choline compounds:

This group consist of seven compounds namely: G25 (Bisquaternary), T3 and T4 (Bisthiazolium), T1 (Mono), M38, MS1 and M53 (Quaternary ammonium/ Alkylamidine). The compounds referred to as choline analogues in this thesis are not closely related to choline, as they are derived from the choline template after several iterations. A more accurate description would be choline derivatives. These compounds were tested in collaboration with Henri Vial, University of Montpellier, France. All these compounds were dissolved completely in DMSO at 20 mM, except M53, which dissolved in DMSO at 0.5 mM due to limitations of its solubility. The stock solutions are stored in the freezer at -20 °C for the *in vitro* assays. The structures of the choline compounds are shown in section 3.1.

2.2.4.2 Curcumin analogues:

This group includes 158 analogues, which are labelled as AS-HK 001 to 158. All these analogues were dissolved in DMSO at 20 mM, and the stock solutions were stored at -20 °C. These compounds were synthesised by Apichart Suksamrarn, Ramkhamhaeng University, Department of Chemistry, Bangkok, Thailand. The structures of curcumin compound and its analogues are shown in the appendix II.

2.3 In-vitro drug sensitivity using Alamar Blue dye:

The drug susceptibility of the different forms of the mentioned parasites (*T. brucei* WT, KO and KO-B48 bloodstream forms, *L. major* promastigotes and *L. mexicana* promastigotes and amastigotes) was assessed using a fluorescence method Alamar Blue. This technique was performed according to Raz *et al* (1997), Mikus & Steverding (2000) and Fumarola *et al* (2004) with minor modifications. Alamar Blue (Resazurin sodium salt)

was prepared by the addition of 12.5 mg of Resazurin (Sigma) in 100 ml of Phosphate Buffered Saline (PBS) at pH 7.4 and filter-sterilized for use in the experiments. This material can be stored in the dark at 4 °C for up to 20 months or frozen at -70 °C (Raz *et al.*, 1997). All the drug dilutions were freshly prepared in the respective medium on the same day of the assay, and the final concentration of DMSO did not exceed 1%, which had no effect on the growth of parasites.

2.3.1 Alamar Blue assay in *T. b. brucei* bloodstream forms:

Setting up of the experiments in the presence of anti-trypanosomal drugs was performed as follows:

- 1- The stocks of the drugs were prepared by dilution in HMI-9 medium at a concentration of 200 μ M (i.e. 2 x highest concentration required in the assay).
- 2- Into each well in the first column of the first (A), third (C), fifth (E) and seventh (G) rows of the 96-well plates, 200 μ l of different drugs which would be tested were pipetted. Two rows were used for each drug and each plate was used for four drugs. Diminazene aceturate was used as an internal control for each experiment.
- 3- Into all remaining wells, 100 μ l of HMI-9 medium was pipetted, and 23 serial doubling dilutions of drugs were prepared with a multi-channel pipette by transferring 100 μ l from the first column and mixing with the medium in the wells of column number 2, then another 100 μ l from number 2 to the number 3 and so on. 100 μ l from the 23rd dilution was discarded (second column of the B, D, F and H rows). The last wells of every two rows (the wells B,D,F and H in the first column) do not received any drug solution and remain free of drug as a control.
- 4- A cell count of trypanosome culture was performed and a culture with an adjusted density of 2×10^5 cells per ml was prepared. Then 100 μ l of this prepared culture was added to all the plate wells, and the final density became 1×10^5 cells per ml.
- 5- The plates were incubated for 48 hours at 37 °C with 5% CO₂ atmosphere, after which 20 μ l of Alamar Blue solution was added to each well and the plates were incubated as before for a further 24 hours and after 72 hours incubation the absorbance of the plates was measured using a FLUOstar OPTIMA plate reader at 544 nm excitation and 590 nm emission.

2.3.2 Alamar Blue assay in *L. major* and *L. mexicana* promastigotes:

The sensitivity of anti-leishmanial drugs was performed in the same way as in *T. brucei* as mentioned in section 2.3.1 with the following modifications:

- 1- The medium in this case was HOMEM 90% + 10% FBS.
- 2- The control drug for *L. major* promastigotes was pentamidine.
- 3- The primary cell culture density of the promastigotes was set at 2×10^6 cells per ml, and the final density was 1×10^6 cells per ml.
- 4- The 96-well plates were incubated at $27\text{ }^\circ\text{C} + 5\% \text{CO}_2$ for 72 hours before 20 μl of Alamar Blue (Resazurin sodium salt) was added to each well and the plates were incubated for a further 48 hours.

2.3.3 Alamar Blue assay in *L. mexicana* amastigotes:

All steps were as those in *L. major* promastigotes (section 2.3.2) with two exceptions:

- 1- The medium for amastigotes was 80% Schneider's Drosophila Medium (SDM), with 20% HI-FCS and 0.3% Gentamicin.
- 2- The 96-well plates were incubated at $32\text{ }^\circ\text{C}$ in the presence of 5% CO_2 for the same period as *L. major* promastigotes.

2.4 In-vitro drug sensitivity using propidium iodide dye:

This assay was developed in our lab (Gould *et. al.*, 2008), and it was set up similar as Alamar Blue assay with one exception: the cells were incubated for 72 h in trypanosomes and Leishmania, after which 20 μl of a mixture of 90 μM propidium iodide and 200 μM digitonin was added to each well and followed by a 1-hour incubation in the same conditions. The plates were then read in a FLUOstar OPTIMA fluorimeter and the fluorescence measured at 544 nm excitation and 620 nm emission.

2.5 In-vitro toxicity assay using human embryonic kidney cells:

This technique was performed using an adaptation of the above Alamar Blue protocol. In this assay, the culture flasks of HEK cells (strain 293) were taken from the incubator at the mid-log phase and about 2 ml of 0.25% Trypsin- 0.02% EDTA in Hanks' Balanced Salt Solution was added to each flask after removing the medium. After 5 minutes, 10 ml of

medium, pre-warmed at 37 °C, was added and the suspension was transferred to a 50 ml falcon tube. The cells were centrifuged at 1200 x g for 5 min, after which the supernatant was decanted and the cells were re-suspended in 10 ml of fresh medium.

In standardised protocol, the cells were counted at 2 pm and 100 µl of 3×10^5 cells/ml was added to each well of a 96-well plate. The plates were left for 3 hours. Using other 96-well plates, serial dilutions of tested drugs were prepared at two times the highest concentration to be used as follows: 260 µl of drug stock was added into the first column of plates, and 130 µl of DMEM medium was added into the remaining wells; 130 µl was taken from the first column by the multichannel pipette and mixed with the medium in the wells in the second column, etc. The last column was left as drug free control.

At 5:00 pm on the same day and using a multi-channel pipette, 100 µl from each well of the plates containing the drug solutions was added to the equivalent well containing the HEK cells. At 9 am the next day, 20 µl of Resazurin solution was added to each well, and the plates were read after a further 24 hours, using a Perkin-Elmer fluorescence LS55B plate reader at 530 nm excitation and 590 nm emission. Phenyl Arsine Oxide (PAO) was used as a positive control at 50 µM and all other compounds were tested at 400 µM (1% DMSO).

2.6 Monitoring in-vitro cell growth:

2.6.1 Using cell count:

In this method, the cell count was taken in duplicate at six time points for each drug: 2, 4, 6, 8, 10, and 24 hours. 24 well plates were used, and 1 ml is the total volume for each well. The final concentration of drugs was 20 µM, solutions were prepared at 10 times the final concentration and the plates were incubated at 37 °C and 5% CO₂. At each time point, 10 µl of culture was loaded onto a haemocytometer in order to take the cell count for the growth curve and the rest of the culture was immediately transferred to sterile Eppendorf tubes, spun at 2500 rpm for 10 min and washed twice in fresh medium. The suspension was transferred onto new 24-well plates and placed again in the incubator. A final cell count was taken at 24 hours. This procedure assessed whether the effect of the drug was reversible.

2.6.2 Using a spectrophotometer assay:

This lysis assay was performed exactly as described previously (Bridges *et al*, 2007) with minor differences. The inhibition curves of trypanosome parasites were determined using a UV/Visible spectrophotometer (HP-8453, Hewlett Packard). Bloodstream forms of Tb 427 strain were used in this real-time assay, at a minimum cell density of 1×10^7 cells/ml. In order to get this concentration, 200 ml (100 ml in 450 ml flask) of culture were seeded with trypanosomes two days before. On the day of the experiment, the test compounds were prepared at 20 times the final concentration, and the culture was centrifuged for 10 min at 2200 rpm and washed twice in HMI-9 medium, re-suspended in the appropriate volume of HMI-9 to obtain the designed cell density, and placed in a water bath for 15 min.

One ml of medium was added in the first cuvette and the spectrophotometer was blanked at 750 nm wavelength. 950 μ l of cell suspension was added to all cuvettes and a reading was taken every 30 seconds. After 15 min, 50 μ l of each test compound was added and mixed to the appropriate cuvette, leaving the first and second cuvettes as negative (drug free) and positive (PAO) controls, respectively.

For the reversibility curves, at a specific incubation time (30, 60, 180 min), the samples were spun at 2500 rpm for 10 min and washed twice in fresh medium, returned to restart the instrument and incubated further in the presence and absence of the same concentration of test compound.

2.6.3 Propidium iodide assay:

Propidium iodide (PI) assays were performed using 96-well plates. 100 μ l of an appropriate (HMI-9 for bloodstream forms and HOMEM for promastigotes) was added to wells, leaving the first column empty. 200 μ l of different test compounds, at twice the final concentration, was added to the wells in the first and second rows in the plate and so on (4 drugs in each plate). Doubling dilutions were performed leaving the last column drug free as a negative control. 100 μ l of PI solution (18 μ M) was added to all the wells in the first row of each test compound as controls. The same volume of cells at 1×10^7 cells/ml in PI solution was added to the second rows of drugs. The plates were then incubated in a FLUOstar OPTIMA fluorimeter at 37 °C with 5% CO₂ atmosphere for bloodstream forms, and at 25 °C for promastigotes, and the fluorescence was monitored over time at 544 nm excitation and 620 nm emissions.

2.7 In-vivo assessment of drug action:

2.7.1 Assessment of *in vivo* toxicity:

In order to determine whether a test compound displays any acute toxicity, the most promising curcumin analogues (AS-HK 09, AS-HK 14 and AS-HK 27) were selected for in-vivo activity. Adult female mice (ICR strain; weights between 25.0 and 33.0 g) were used. Three mice were used in each procedure. The drugs were prepared at 50 mM in DMSO, and mice were intra-peritoneally injected once with 150 µl solvent' DMSO administering initially 1 mg/kg. If no adverse effects are observed a different mouse would be injected with 10 mg/kg, and finally 50 mg/kg BW. The period of observation after each procedure was two weeks and the mice were monitored twice daily.

2.7.2 *In vivo* efficacy of curcumin analogues:

The second step of the experiment was to assess the effect of candidate drugs against trypanosomes using 5 groups (5 mice in each group), the first three groups for the three tested compounds and the 4th and 5th groups as a positive (diminazene at 7 mg/kg twice) and negative (150 µl DMSO) controls, respectively. The animals were weighted and the drug doses were calculated at 50 mg/kg BW. The T. b s427 culture was diluted in a sterile HMI-9 medium to 1×10^5 parasite/ml and 100 µl of this solution was injected intra-peritoneally into each mouse, giving each mouse a dose of 1×10^4 parasites per mouse. Two drug doses were given to each mouse, the first after 6 hours from infection, and the second 24 hours after the first administration. The animals were checked daily and the parasitaemia was monitored under the light microscope (X400 magnification) by taking a blood drop from the mouse's tail and making a wet film on a glass slide. The level of parasitaemia was calculated according to the method of Herbert & Lumsden (1976).

2.8 Pharmacological and toxicological experiments:

2.8.1 Isolation of hepatocytes:

Stock (Hank's Buffer (10x) and Krebs-Henseleit Buffer (2x)) and Perfusion (Hank I, Hank II, Krebs-Albumin Buffer, and Krebs-HEPES Buffer) solutions were prepared as shown in the appendix I, and filtered and stored at 4 °C.

2.8.1.1 Perfusion apparatus:

This apparatus was situated in a laminar flow cabinet and consisted of a peristaltic pump, a water bath, three plastic 200 ml beakers with lids, a reservoir and rubber tubes for connecting the apparatus.

2.8.1.2 Procedure:

Male Spragus-Dawley rats (180 – 250 g) were used in this procedure. The rats were anaesthetised by intra-peritoneal injection of 60 mg/kg (100 µl/100 g) sodium pentobarbitone (60 mg/ml). The peritoneal cavity was subsequently opened by mid-transversal incision. Heparin (500 unit in 0.1ml) was injected in the inferior vena cava. The hepatic portal vein was cannulated by a steel cannula that has an internal diameter of 1.75 mm and a 2.50 mm external diameter. The cannula was fixed in place with a clip. The liver was initially perfused with Hank I buffer after which it was dissected. The flow rate of perfusion was adjusted in order to restore the liver to its normal shape. After removing the liver from the body it was placed for 10 min in the first beaker, which contained 150 ml Hank I buffer. The liver was then transferred to the second beaker and perfused for 15-30 min in 150 ml Hank II buffer containing 78 mg collagenase. The recirculation continued until the liver became soft and the cells in the liver sac had dissociated.

The liver was transferred into a Petri dish containing 80 ml Krebs Albumin buffer, and the cells were dispersed by using two forceps. Within two minutes, the cell suspension was filtered through sterile cotton gauze to remove clumped cells and any remaining connective tissues. The filtrate was left for 2-3 min to allow the cells to settle under gravity. The supernatant was then removed by aspiration, and the cells were washed by using 50 ml of Krebs Herpes buffer.

2.8.2 Isolated hepatocytes viability:

The viability of the isolated rat hepatocytes was directly determined after the perfusion using Trypan blue. 10 µl of cell suspension was added to 990 µl of Trypan blue and the results were calculated by loading the sample on both sides of a haemocytometer. The examination was done using a light microscope, and the cells were counted in 18 haemocytometer sections of 0.1 mm³ each.

Percentage of viability = No. of cells excluding Trypan blue / Total No. of cells X 100

The viable cell count = (No. of cells excluding Trypan blue / 18) X 10⁴ X 100

100 is the initial dilution and 10^4 is the conversion factor.

The standard level of cell suspension density was 2×10^6 cells/ml.

2.8.3 Incubation of rat hepatocytes:

Three round bottom flasks were used, the first was cells without drug as a cell control, the second was cells with drug, and the last was drug without cells as a drug control. About 12 ml of cell suspension was added into both flasks 1 and 2 whereas 12 ml of Krepes Hepes buffer (KH) was added to flask 3. Then 60 μ l of 20 mM drug was added to flasks 2 and 3, and the cells were incubated at 37 °C for 2 hours with 95% O₂ and 5% CO₂, and the flasks were rotated at 60 rpm (Figure 2.1). Samples were taken at regular time intervals (0, 15, 30, 60, 90 and 120 min) in order to assess viability, total cell protein (Lowry Assay), glutathione and drug metabolism. The first time point (0 minute) was taken immediately after adding drug.



Figure 2.1. Picture showing the water bath and rotor equipped for the incubation of the hepatocytes.

2.8.4 Effects of lead compounds on incubated hepatocytes viability.

The cell viability was measured by taking 100 μ l of Trypan blue solution (0.1% w/v in PBS, pH 7.4) in which was added to 100 μ l of cell suspension. The cells were loaded onto a haemocytometer, and 50 cells (live and dead) were counted from the centre of the grid. The percentage was calculated by multiplying the live cells x 2.

2.8.5 Total cell protein measurement (Lowry Assay):

The assay was performed according to Lowry *et al.* (1951). At predetermined times, 0.5 ml of cell suspension was taken from each flask and transferred to 15 ml Falcon tubes, then immediately centrifuged for 4 minutes at 50 x g. The supernatant was removed and the pellet was re-suspended in 0.5 ml 10% trichloro acetic acid (TCA). The samples were left on ice for at least 10 min and then centrifuged again at 500 x g. The supernatant was removed, transferred to microfuge tubes and frozen at -20 °C for GSH measurement by fluorimetry following derivatisation with *O*-phthaldehyde (OPT). The pellet was re-suspended in 0.5 ml of 0.5 M NaOH and digested for 18 hours at 37 °C for use in the Lowry assay.

The protein standard (200 μ g/ml bovine serum albumin in 0.5 M NaOH) was used for the standard curve and stored at -20 °C. On the day of the assay, the standard curve and two solutions were made up: solution A and solution B (appendix I).

The cell samples were diluted 20-fold with 0.5 M NaOH (50 μ l of sample + 950 μ l NaOH). Then 5 ml of solution A was added to all standards and samples, and mixed. The samples were left at room temperature for 10 minutes after which 0.5 ml of solution B was also added and mixed immediately and very thoroughly. The samples must be left for at least 30 minutes and at most 90 minutes before the absorbance is read at 725 nm.

2.8.6 Determination of Reduced Glutathione by Fluorimetry:

The solutions that were used in this assay are Sodium Phosphate buffer (pH 8.0), 10% TCA, and 1 mg/ml *O*-phthaldehyde (OPT) in methanol or ethanol freshly dissolved

(should be kept in the dark by wrapping the bottle in tin foil). The standard was made up as described in appendix I.

25 μ l of both standards and samples was taken into clean test-tubes, and 2.3 ml of phosphate buffer and 100 μ l of 1 mg/ml OPT in methanol were added and mixed well. The tubes were left at room temperature in the dark for at least 15 minutes and not for more than 40 minutes after which the fluorescence was read at 350 nm excitation and 420 nm emission using water to zero the fluorimeter.

2.9 Transport assays:

2.9.1 Transport assay in *T. brucei brucei*:

Transport assays for [3 H] pentamidine, [3 H] choline, and [3 H] curcumin by *T. brucei brucei* blood stream forms were performed exactly as described previously (De Koning, 2001b; Wallace *et al*, 2002). Briefly, trypanosomes were isolated from blood taken from infected rats as described in section 2.1.2. Cells were washed twice in Assay Buffer (pH 7.3) for 10 minutes at 2200 x g and 4 °C, and then re-suspended at $\sim 1 \times 10^8$ cells/ml and left at room temperature for 20 minutes before using. Cells (100 μ l) were then incubated, in 1.5 ml Eppendorf tubes containing a mixture of 300 μ l of dibutyl-phthalate oil (Merck)/mineral oil (Sigma) 7:1 (v/v), with the same volume of the radiolabel at two times the final concentration in presence or absence of potential effectors. Transport was terminated by adding 1 ml of ice-cold stop solution (vast excess of unlabeled permeant in Assay Buffer at 0 °C), and followed by rapid centrifugation at 13,000 rpm for 30 seconds through an oil layer. The samples were then frozen in liquid nitrogen and the tubes were cut in scintillation tubes containing 300 μ l of 2% of sodium dodecyl sulphate (SDS). The samples were left for 20 minutes to allow the pellet to be dissolved, and then 3 ml of scintillation fluid (OPTIPHASE HISafe III; Perkin-Elmer) was added to each tube, which were left overnight at room temperature (see Figure 2.2). All experiments were performed in triplicate, and the cell pellet radioactivity was determined using a 1450 MicroBeta Trilax scintillation counter.

2.9.2 Transport assay in *Leishmania mexicana* promastigotes:

Transport of radiolabelled permeants into *Leishmania mexicana* promastigotes was measured exactly as described in section 2.9.1 for *T. brucei brucei* bloodstream forms with one exception, which is that the cells were grown *in vitro* for two days in 200 ml of HOMEM medium supplemented with 10% FCS.

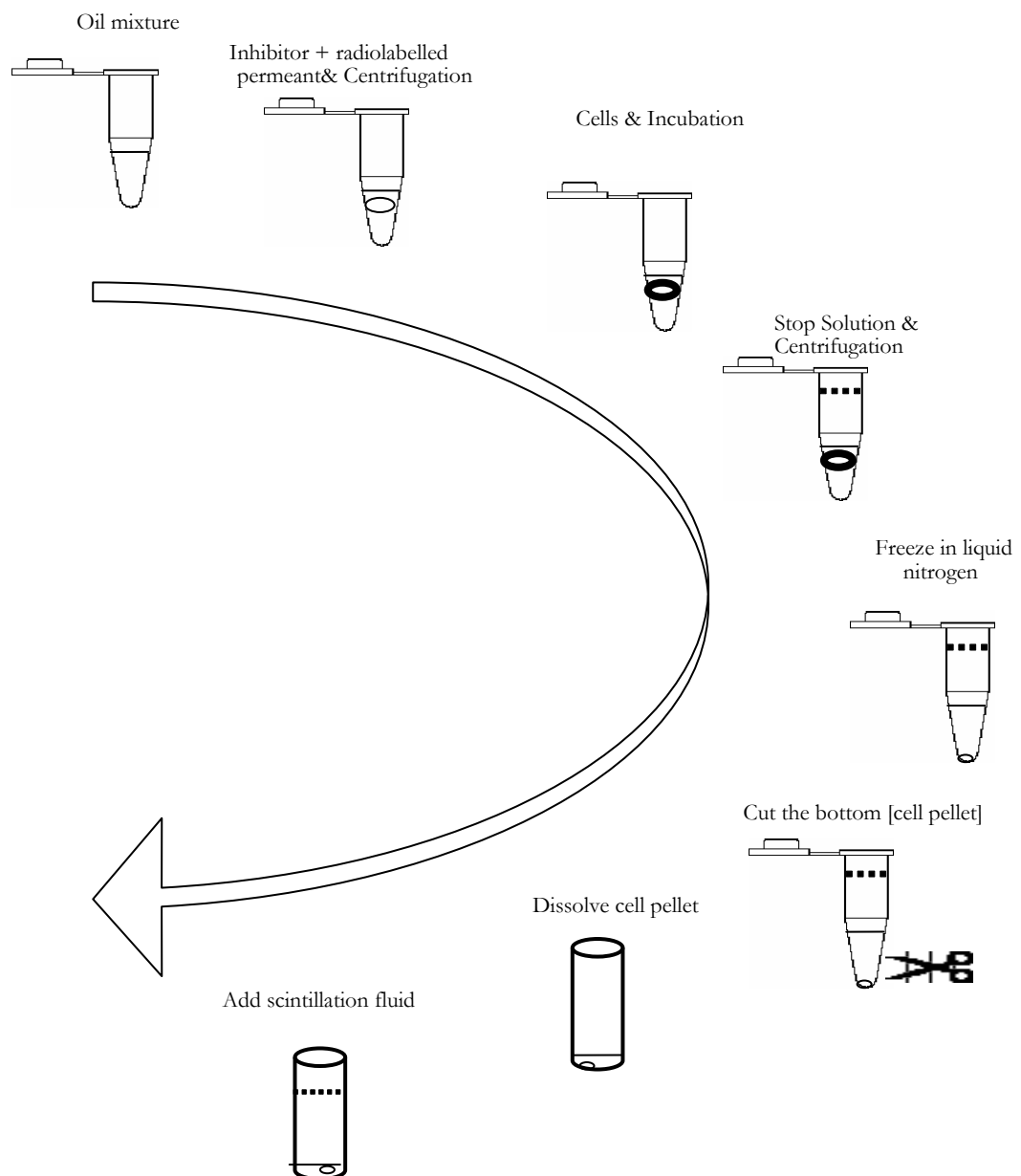


Figure 2.2 A Schematic of the rapid oil-stop protocol used in the uptake assays. (Adapted from Al-Salabi M. PhD Thesis, 2006, Glasgow University).

2.10 FACS analysis:

T. brucei brucei bloodstream forms were incubated in 25 ml flasks (10 ml final volume) in HMI-9 medium with different concentration of test compounds. The flasks were kept at 37 °C with 5% CO₂ atmosphere for up to 72 hours. At different time points, 1 ml was taken from each culture and added in Eppendorf tubes for assessment of DNA content, cellular permeability, and mitochondrial membrane potential.

2.10.1 DNA content:

This method was performed as described by (Mutomba *et al.*, 1997; Hammarton *et al.*, 2003b). About 1 ml of treated and non-treated cells ($\sim 2 \times 10^6$ cells/ml) was centrifuged at 1000 x g for 10 minutes at 4 °C. The pellet was re-suspended and fixed in 1 ml of 70% methanol and 30% PBS (pH 7.4) and left at 4 °C overnight. The cells were washed twice with 1 ml PBS by spinning at 2500 x g for 10 minutes at 4 °C, and re-suspended in 1 ml PBS containing Propidium iodide (Sigma) and RNase (Sigma) both are at 10 µg/ml, and incubated in the dark at 37 °C for 45 minutes. A sample cells incubated without drug was used as a positive control, and the DNA content of samples stained by PI was analyzed with a Becton Dickinson Fluorescence Activated Cell Sorter Calibur (FACSCalibur) using the FL2-Area detector and CellQuest software.

2.10.2 Cellular permeability:

Plasma membrane integrity was measured as previously described (Figarella *et al.*, 2005; Uzcátegui *et al.*, 2007) using propidium iodide staining. One millilitre of treated and untreated cells ($\sim 2 \times 10^6$ cells/ml) in HMI-9/FCS medium was taken and spun at 2500 x g for 10 minutes at 4 °C, and resuspended in the same volume of propidium iodide solution (5 µg/ml) in the same medium. The samples were left in the dark for 10 minutes at room temperature, and immediately transferred into FACS tubes and analyzed with FACSCalibur as mentioned in section 2.10.1. Digitonin 6 µM was used as a positive control for plasma membrane disruption.

2.10.3 Determination of the mitochondrial membrane potential:

The mitochondrial membrane potential of treated and untreated cells was evaluated using Tetramethylrhodamine ethyl ester (TMRE) (Figarella *et al.*, 2006; Denninger *et al.*, 2007). Briefly, following incubation of bloodstream trypanosomes with and without test

compounds for a determined time, 1 ml of cells ($\sim 10^6$ cells/ml) was transferred to Eppendorf tubes and centrifuged for 10 minutes at 2500 x g, and then washed once in 1 ml PBS. Thereafter, the pellet was resuspended in 1 ml PBS containing 25 nM of TMRE, and incubated at 37 °C for 30 minutes. Two positive controls were used: valinomycin 100 nM and troglitazone 10 μ M. The samples were then analyzed with flow cytometry using the FL2-Height detector and CellQuest software.

2.11 Measurement of reactive oxygen species (ROS):

This assay was performed using black bottom 96-well plates. 200 μ l of test compounds, at twice the final concentration, were added to the wells in the first column in the plate as shown in Figure 2.3 (4 drugs in each plate). Then 100 μ l of Phosphate buffer saline (PBS), pH 7.4, was added to all wells, leaving the last column empty. After which, 100 μ l of hydrogen peroxide (H_2O_2) at 100 μ M was added to all the wells in the last column. Drug doubling dilutions were performed in the PBS from 50 μ M to 0.01 μ M. 100 μ l of PBS, without cells, was added to the first four rows, and the same volume of culture at 2×10^6 cells/ml in PBS was added to each well of the other rows. Immediately, and in the dark, 2 μ l of 1 mM dichlorofluorescein (DCF) was added to all wells. The plates were then incubated in a FLUOstar OPTIMA fluorimeter at 37 °C for *T. brucei* blood stream forms, and the fluorescence was monitored over time at 485 nm excitation and 520 nm emissions.

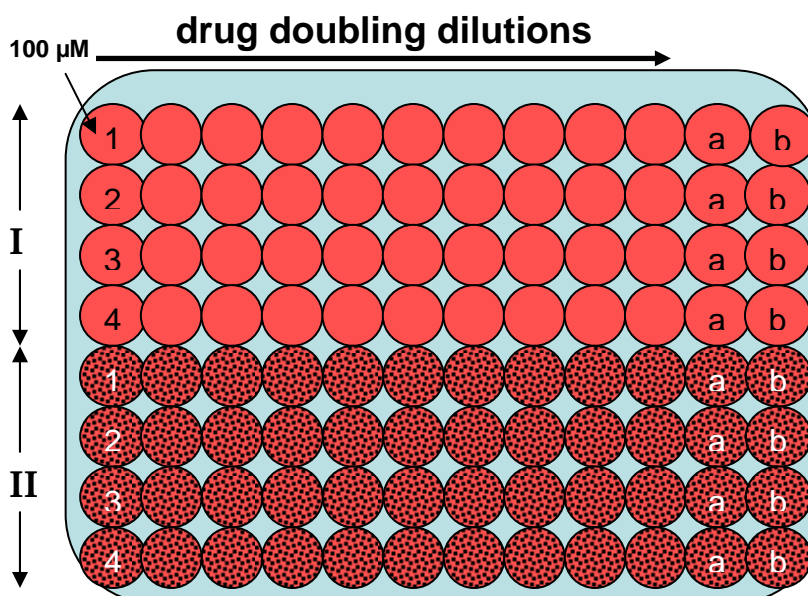


Figure 2.3. A diagram of a 96-well plate used in reactive oxygen species measurement. Four different drugs, 1; 2; 3; 4, were used in the plate. Serial drug doubling dilutions in phosphate buffer saline were performed in the presence (II) and absence (I) of cells at 1×10^6 cells/ml. Drug free (a) and $100 \mu\text{M}$ hydrogen peroxide were used as negative and positive controls, respectively.

2.12 Measurement of lipid content in *T. brucei*:

2.12.1 Lipid Extraction

Lipids were extracted according to the method of (Bligh & Dyer, 1959). Briefly, mid-logarithmic cells treated with either solvent or test compound for 8 h were collected by centrifugation ($800 \times g$, 10 min) and washed with PBS and resuspended in $100 \mu\text{l}$ PBS and transferred to a glass tube. $375 \mu\text{l}$ of 1:2 (v/v) CHCl_3 : MeOH added to each tube and the samples were vortexed and agitated vigorously for a further 10-15 min. The samples were made biphasic by the addition of $125 \mu\text{l}$ of CHCl_3 , and vortexed and then $125 \mu\text{l}$ of H_2O was added and vortexed again and spun at $1000 \times g$ at room temperature for 5 min. The lower phase of each sample was transferred to a new glass vial, and the solvent was dried under nitrogen and stored dry at 4°C until analysed by electrospray mass spectrometry (ES-MS and ES-MS-MS).

2.12.2 Nano-electrospray ionization tandem mass spectrometry

Samples were resuspended with chloroform/methanol (1:2 v/v) to the desired volume, depending on the desired resolution of peaks. An aliquot of total lipid extract was analyzed

with a Micromass Quattro Ultima triple quadrupole mass spectrometer equipped with a nanoelectrospray source. Samples were loaded into thin-wall nanoflow capillary tips (Waters) and analyzed by ES-MS in both positive and negative ion modes using a capillary voltage of 0.9 kV and cone voltages of 50V. Tandem mass spectra (MS-MS) were obtained using argon as the collision gas (~3.0 mTorr) with collision offset energies as follows: 35V, PC/SM in positive ion mode, parent-ion scanning of m/z 184; 45V, GPI_{no} in negative ion mode, parent-ion scanning of m/z 241; PE in negative ion mode, parent-ion scanning of m/z 196; 28V, PS in negative ion mode, neutral loss scanning of m/z 87; 45V; and 50V, all glycerophospholipids detected by precursor scanning for m/z 153 in negative ion mode. MS-MS daughter ion scanning was performed with a collision offset energy of 35V.

In positive ion mode, ions in the PC and SM spectra were annotated based on their [M+HNMe₃(+)]⁺, [M-140], and [GPA-H] daughter ion derivatives, respectively and compared with that of their theoretical values and previous analyses (Richmond & Smith, 2007). Annotation of all phospholipids is also based upon comparison with their theoretical values and other ES-MS and ES-MS-MS analyses conducted on whole cell extracts (Smith, T.K. unpublished data). Each spectrum encompasses at least 50 repetitive scans.

2.13 Data analysis:

The results were analyzed by using the GraphPad Prism software package, versions 3.0 and 4.0 by using non-linear regression, plotting the data typically to sigmoid curves. The IC₅₀ values were calculated by nonlinear regression. All transport experiments were performed in triplicate and kinetic parameters and IC₅₀ values are presented as means and standard error (S.E.) of at least 3 independent experiments. Wilcoxon's signed ranks test for two groups was used to assess the differences between the average EC₅₀ values of groups of compounds against different species or of different forms within one species such as *L. major* and *L. mexicana* promastigotes, *L. mexicana* promastigotes and amastigotes, and between *T. brucei*, *T. evansi* and *T. equiperdum* bloodstream forms. The differences were considered statistically significant when the probabilities of equality, *P* values, were ≤0.05. All the data obtained by flow cytometry were analyzed by using CELLQuest software to determine the fluorescence of each sample. 10,000 cells were analyzed per run, and the percentages of cells in each cell cycle phase were determined with excluding cell autofluorescence and cell debris.

Chapter three

3 Assessment of choline analogues as new anti-kinetoplastid lead compounds

3.1 Introduction:

Choline is a natural amine belonging to the B complex group of vitamins. It is naturally abundant in the lipids that make up the cell membrane and is also found in the neurotransmitter acetylcholine.

In most protozoa, choline phospholipids and sphingomyelin are essential. This makes choline salvage and metabolism as potential drug targets. The choline group of compounds consists of a large number of choline analogues. More than 420 analogues have been synthesized (Calas *et al*, 1997; Calas *et al*, 2000; Vial, 1996). Most previous studies of these compounds concentrated on activity against malaria parasites, especially *Plasmodium falciparum* (Ancelin *et al*, 2003; Ancelin *et al*, 1985; Roggero *et al*, 2004). The thiazolium T1 and the bisquaternary (G25) and the bithiazolium salts T3 and T4 were reported to exhibit high antimalarial activity against *P. falciparum in vitro* with EC₅₀ values of 70, 0.65, 2.3-9 and 0.65-2.9 nM, respectively (Hamze *et al*, 2005; Vial *et al*, 2004). G25 was also shown to inhibit the growth of *Plasmodium vivax* (Wengelnik *et al*, 2002) and G25, T3 and T4 display potent *in vivo* activities against *Plasmodium vinckei* in mice (Hamze *et al*, 2005; Vial *et al*, 2004). Choline compounds have also been reported to possess anti-babesial properties (Richier *et al*, 2006). In this regard, T16, a Bithiazolium analogue, was shown to potently inhibit the growth of *Babesia canis* and *Babesia divergens*, with EC₅₀ values of 7 and 28 nM, respectively (Richier *et al*, 2006).

The discovery that choline analogues inhibit the synthesis of new membranes and block the growth of the parasite has stimulated efforts to test this class of compounds for potential as antitrypanosomal and antileishmanial chemotherapy. Seven analogues were investigated in this study for effects on kinetoplastid parasites: G25 (Bisquaternary), T3 and T4 (Bithiazolium), T1 (Monothiazolium), M38, MS1 and M53 (bisalkylamidine) (Figure 3.1). We initially assessed these compounds for overall activity against *Leishmania* and *Trypanosoma* species, but also against Human Embryonic Kidney (HEK) cells in culture for a preliminary assessment of toxicity to human cells.

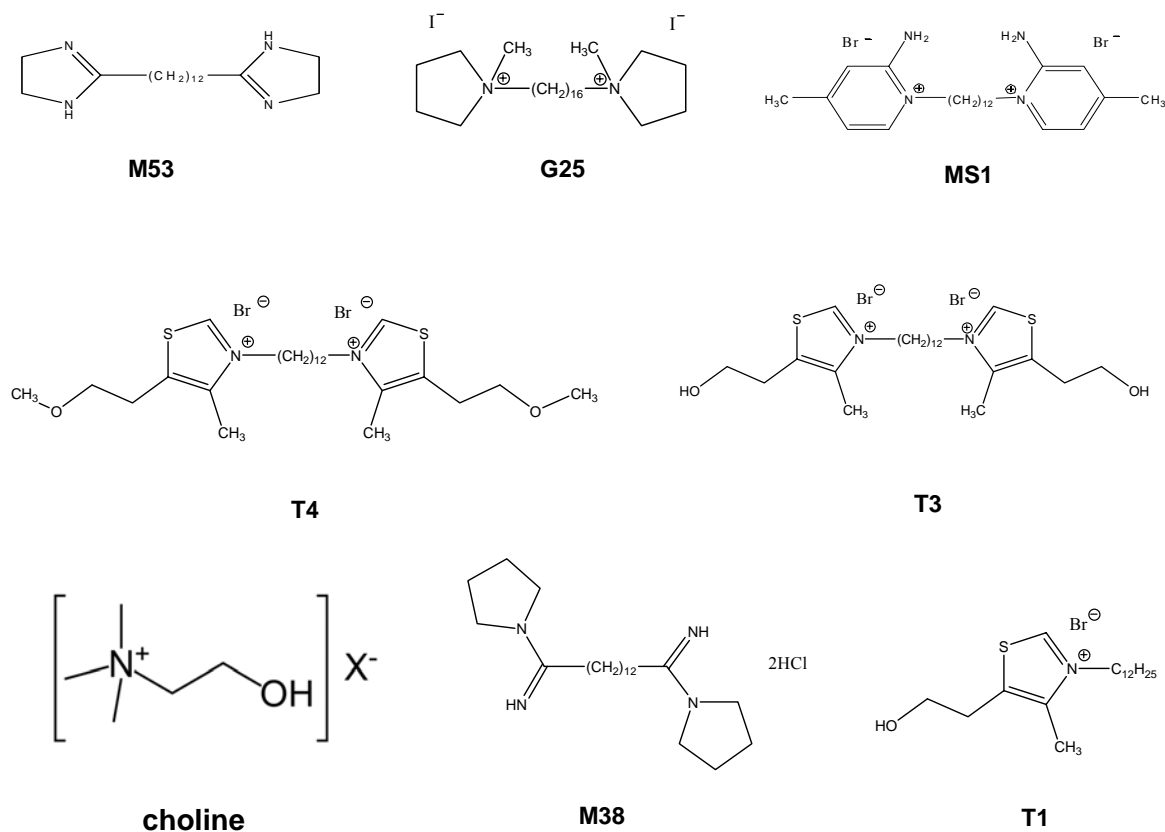


Figure 3.1. Chemical structures of choline and various choline analogues.

Colorimetric and fluorimetric methods using different stains like Alamar Blue, propidium iodide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) are widely used in-vitro to assess the anti-protozoan activities. Alamar Blue (Resazurin) is used as an indicator for oxidation-reduction reactions. It is extensively used in colorimetric (Mikus & Steverding, 2000) and fluorimetric (Raz *et al*, 1997) methods to test drug activity against protozoan parasites. This dye has no toxic effects on living cells (Fumarola *et al*, 2004) and it is used to evaluate the sensitivity of cells to drugs, based on the ability of the intracellular enzymes of these cells to reduce the non-fluorescent Blue Resazurin (Figure 3.2A) to the fluorescent pink Resorufin (Figure 3.2B) (O'Brien *et al*, 2000). This colorimetric assay, being simple and rapid, was used in this study to measure the sensitivity of different compounds against different protozoan species namely *T. brucei* (bloodstream forms), *L. major* (promastigotes) and *L. mexicana* (promastigotes and amastigotes).

An alternative fluorescent dye, propidium iodide (PI), was used in this study to evaluate drug action against the target cells. The main idea of this assay is based on the ability of

this dye to bind to nucleic acids. The dye is not able to cross the intact membrane of live cells, and its fluorescence thus reliably indicates the moment at which the integrity of the plasma membrane is breached (Gould *et al*; 2008).

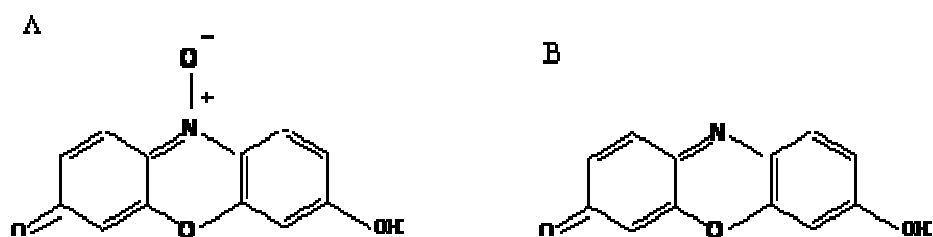


Figure 3.2 Structure of Alamar Blue dye. Non-reduced fluorescent form of Alamar Blue, Resazurin (A), and reduced Alamar Blue form, Resorufin (B).

In order to understand the activity of any anti-parasitic compound, transport studies should be performed to investigate by which mechanism this drug is taken up. There are at least four relevant uptake modes that should be considered: endocytosis, passive diffusion, receptor-mediated uptake and transporter-mediated uptake, with channels usually allowing only the flux of ions or very small molecules (De Koning, 2001a). Pentamidine, an aromatic diamidine used to treat Human African Trypanosomiasis (HAT) and antimony-resistant leishmaniasis, is accumulated by African trypanosomes to millimolar concentrations and taken up by three different transporters: the P2 adenosine/adenine transporter (encoded by the gene *TbATI*), a high affinity pentamidine transporter (HAPT1), and a low affinity pentamidine transporter (LAPT1). About 50-70% of pentamidine is taken up by the P2 transporter, depending on the concentration of the drug (Bray *et al*, 2003). In trypanosomes, drug resistance is very often associated with the loss of a transporter function, thus the presence of multiple transporters for pentamidine probably contributed to the observed lack of resistance for this drug, although resistance is common for some other diamidines (De Koning, 2001b).

For the choline-type test compounds, it was investigated whether they enter the parasite through a designated choline transporter, or through the known drug transporters HAPT1 and LAPT1.

3.2 In-vitro efficacy of choline-type compounds using Alamar Blue

To examine the effect of the trypanocidal and leishmanicidal choline analogues using Alamar Blue in-vitro, all experiments were performed at least four times for each test compound, and the mean values and SE were calculated and shown in the tables below. The no-drug control was considered to represent a 100% value for fluorescence, typically measuring 100-250 arbitrary units out of a scale of 1000. Background fluorescence was observed to be typically 25–30 units. All early experiments were performed on a Perkin-Elmer LS 55 fluorimeter equipped with a plate reader. Later experiments were performed with a BMG FLUOstar OPTIMA plate reader, and the arbitrary scale extends to 65,000. In this part, we investigated the suitability of Alamar Blue as a drug screening assay in-vitro with different species of protozoan parasites.

3.2.1 *Trypanosoma brucei brucei* (BSF)

Doubling serial dilutions of choline analogues were tested in order to determine the effective concentrations against three strains of *Trypanosoma brucei brucei*: Tb 427 wild type (TbAT1WT or *T. brucei* adenosine transporter 1), TbAT1 knock out (TbAT1KO), and the pentamidine-adapted clonal line (TbAT1-KO-B48). TbAT1-KO displays a high level of resistance to diminazene aceturate (Matovu *et al.*, 2003) and B48 additionally displays a very high level of resistance to both pentamidine and melaminophenyl arsenicals (Bridges *et al.*, 2007).

The trypanosomes were incubated in serial dilutions of drugs for 48 h prior to the addition of 20 µl of resazurin dye. The trypanosomes were then incubated for a further 24 h before the plates were read. Drug free incubations and diminazene were utilized as negative and positive controls, respectively. Alamar Blue results with these three strains showed a strong trypanocidal activity on the three strains. Representative results of the Alamar Blue assays are displayed in Figure 3.3 and comparisons of effects of the choline analogues on the different strains show that there is no cross-resistance with the existing trypanocides. For example, strain B48, over 100-fold resistant to pentamidine and strongly cross-resistant to melarsen oxide *in vitro* (Bridges *et al.*, 2007), displayed almost the same sensitivity to the choline analogues as the Tb427 wild-type strain. With the sole exception of the EC₅₀ value for T4 against the B48 strain (Table 3.1), no significantly different effects of choline agents were observed on control and resistant strains.

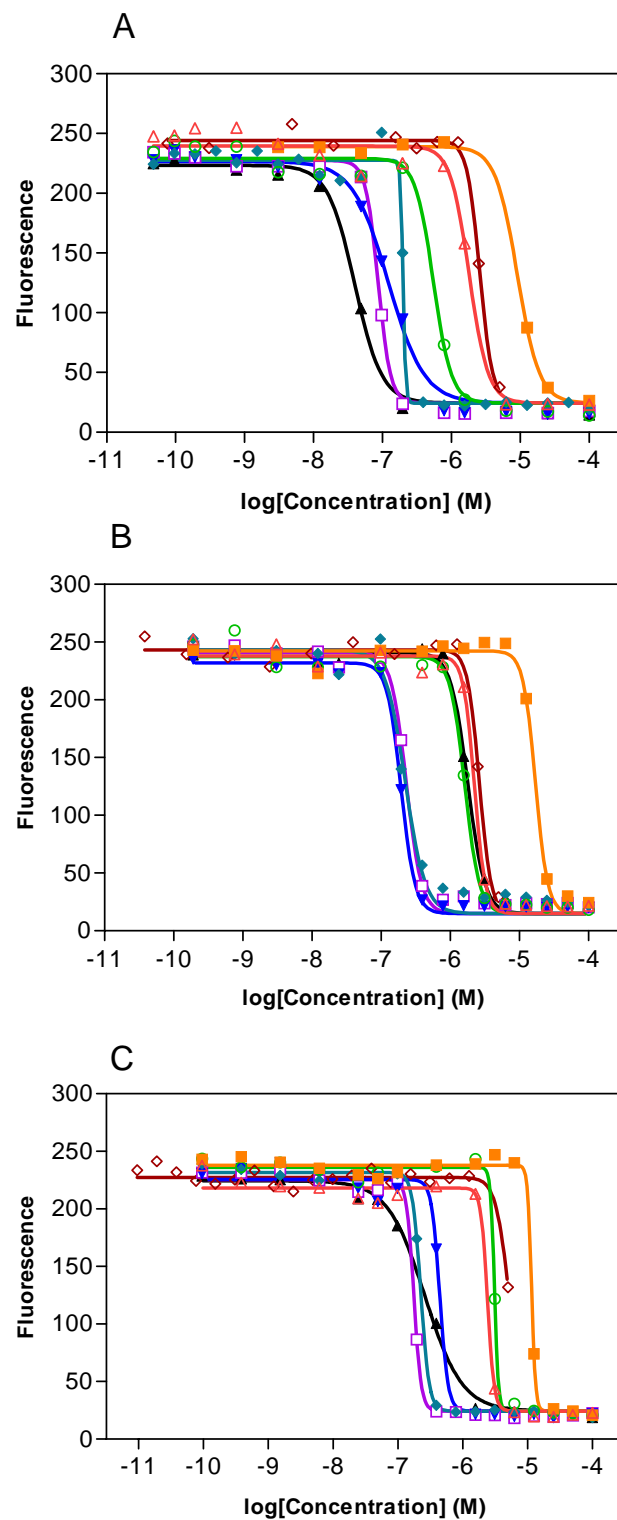


Figure 3.3. Sensitivity of bloodstream forms of three *T. brucei* clones, Tb427 (A), TbAT1-KO (B), and KO-B48 (C), to seven choline compounds.

T1 (Δ), T3 (\blacksquare), T4 (\circ), M38 (\blacklozenge), G25 (\blacktriangledown), MS1 (\square), and M53 (\blacklozenge). Diminazene (\blacktriangle) was used as a positive control. The data shown is a representative experiment using Alamar Blue method as described in section 2.3.1.

Some of the choline-type compounds display a similar EC_{50} values as the control (diminazene). Compound MS1 performed the best against the three strains, with EC_{50} values of 0.13 ± 0.02 (n=4), 0.25 ± 0.04 (n=4), and 0.18 ± 0.01 (n=6) μ M against the WT, KO, KO-B48 strains, respectively.

Table 3.1 Comparisons of EC_{50} values of choline analogues against *T. brucei* clones Tb-427, TbAT1KO, and KO-B48 evaluated by Alamar Blue. Values are the average \pm SE of 4-6 independent experiments.

compound	<i>T. brucei</i> (WT)	<i>T. brucei</i> (KO)	<i>T. brucei</i> (KO-B48)
	EC_{50} (μ M)	EC_{50} (μ M)	EC_{50} (μ M)
T1	1.8 ± 0.1	2.2 ± 0.5	2.3 ± 0.2
T3	7.9 ± 2	11 ± 2	10 ± 0.9
T4	0.61 ± 0.2	2.0 ± 0.9	$2.5 \pm 0.2^{**}$
M38	0.17 ± 0.06	0.28 ± 0.1	0.23 ± 0.01
G25	0.20 ± 0.04	0.37 ± 0.1	0.34 ± 0.03
MS1	0.13 ± 0.02	0.25 ± 0.04	0.18 ± 0.01
M53	2.6 ± 0.7	4.0 ± 0.2	4.7 ± 0.6
diminazene	0.10 ± 0.01	$2.4 \pm 0.3^{***}$	0.29 ± 0.1
Pentamidine ¹	0.0021 ± 0.0002	0.0079 ± 0.0011	0.27 ± 0.02

¹ values from Bridges et al., 2007.

**, $P < 0.01$ and **, $P < 0.001$ compared to the WT strain, using an unpaired Student's T test.

3.2.2 *Leishmania major* and *L. mexicana* promastigotes using Alamar Blue dye:

Promastigotes of *L. major* and *L. mexicana* were incubated with serial dilutions of drugs for 72 h, after which 20 μ l of Alamar Blue dye was added to each well. The cells were then incubated for a further 48 h before the fluorescence was measured. Drug free incubations and pentamidine were used as negative and positive controls, respectively. The general results in promastigotes of *L. major* and *L. mexicana* with this group of drugs are similar to those in *T. brucei* strains, and the EC_{50} values of the seven investigated drugs have been summarized in Table 3.2. All compounds, except T3 and T4, displayed an activity better than pentamidine (Figure 3.4). However, no EC_{50} values could be determined for M53, due to its limited solubility in aqueous buffers (5 μ M).

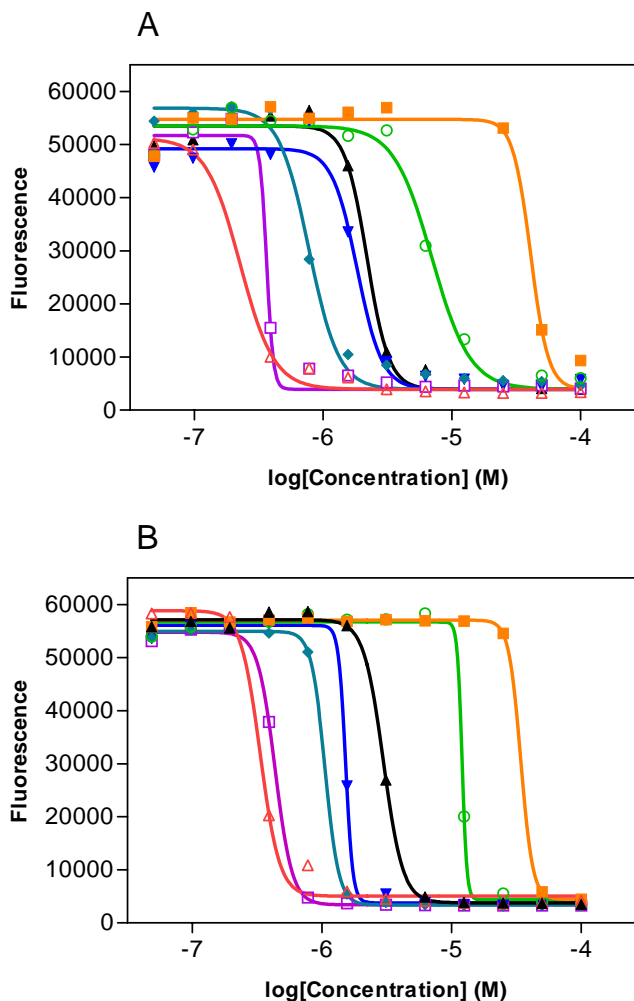


Figure 3.4. *In vitro* efficacy of different serial concentrations of choline compounds. T1 (Δ), T3 (■), T4 (○), M38 (◆), G25 (▼), and MS1 (□), against *L. major* (A) and *L. mexicana* (B) promastigotes using the Alamar Blue method. Pentamidine (▲) was used as a positive control.

The data in Table 3.2 showed that T1 was the most effective compound against promastigotes of both species, *L. major* and *L. mexicana*, with EC₅₀ values of 0.14 ± 0.03 (n=5) and 0.28 ± 0.08 (n=5), μM respectively, followed by MS1 and M38. The sensitivity to these compounds was slightly lower in *L. mexicana* than in *L. major* promastigotes (Table 3.2). T1 also had a potent antileishmanial activity against amastigotes of *L. mexicana*, with an EC₅₀ value of 1.7 ± 0.4 μM (n=5). For comparison between the two forms of *L. mexicana*, promastigotes and amastigotes, the results shown in Table 3.2 indicated that the activity of all the tested choline-type compounds were lower in the latter stage than in the former stage. Pentamidine, a drug used currently against leishmaniasis,

had an EC₅₀ of 14 ± 3 (n=5) against *L. mexicana* amastigotes and presented a lower potency than all choline agents except T3.

Table 3-2 Effective concentration (EC₅₀) of choline compounds on promastigotes and amastigotes of the two species of *Leishmania* evaluated by Alamar Blue assay. NS, not significant; ND, not determined. NE, no effect at indicated concentration. GI, growth inhibitory at a concentration of approx 3 μM. Values are the average ± SE of 3-5 independent experiments.

compound	<i>L. major</i> (promastigote)	<i>L. mexicana</i> (promastigote)	P-value*	<i>L. mexicana</i> (amastigote)	P-value**
	EC ₅₀ (μM)	EC ₅₀ (μM)		EC ₅₀ (μM)	
T1	0.14 ± 0.03	0.28 ± 0.08	NS	1.7 ± 0.4	P<0.02
T3	24 ± 5	33 ± 3	NS	38 ± 10	NS
T4	4.3 ± 0.9	6.9 ± 0.5	NS	13 ± 4	NS
M38	0.68 ± 0.07	0.95 ± 0.1	NS	2.9 ± 0.9	P<0.05
G25	1.1 ± 0.2	2.1 ± 0.3	P<0.02	4.8 ± 1	NS
MS1	0.29 ± 0.03	0.42 ± 0.03	NS	3.0 ± 0.6	P<0.01
M53	GI	NE, 5 μM	ND	3.4 ± 1.7	ND
pentamidine	2.9 ± 0.4	2.9 ± 0.5	NS	14 ± 3	NS

*= significant differences between *L. major* and *L. mexicana* promastigotes (Student T-test).

** = significant differences between *L. mexicana* promastigotes and amastigotes (Student T-test).

3.3 Cytotoxic activities of choline analogues using Human Embryonic Kidney (HEK) cells

The toxic effect of choline compounds, at concentrations up to 400 μM of the choline analogues on HEK cells was evaluated after an incubation time for 40 h in-vitro using the Alamar Blue (AB) method described in section 2.1.5. Our results indicate that there is only a low toxicity associated with some of the choline compounds. These results also indicate that these compounds have different levels of toxicity. However, the Selectivity Index (S.I., EC₅₀ (human cells)/EC₅₀ (parasite)) is very high for some of them (Table 3.3), mostly depending on their antiparasitic EC₅₀ values rather than their activity against HEK cells. M38 and G25, in particular, displayed an excellent S.I. against both trypanosomes and leishmania promastigotes *in vitro*. T1 displayed a promising Selectivity index only against promastigotes. For clinical application, it is very important to consider a Selectivity index

(May *et al.*, 2006). Obviously, a higher index increases the chance of a non-toxic but curative dosage of a test drugs.

Table 3-3. EC₅₀ values and Selectivity Index of Human Embryonic Kidney cells treated by choline compounds in *Trypanosoma* and *Leishmania*.

ND, not determined. For M53, no toxicity could be observed at the limit of solubility, thus no Selectivity Index could be calculated.

drug	HEK EC ₅₀ (μM)	<i>T. brucei</i> Tb 427	<i>T. brucei</i> TbAT1KO	<i>T. brucei</i> KO-B48	<i>L. major</i> promastigote	<i>L. mexicana</i> promastigote	<i>L. mexicana</i> amastigote
T1	41.9	23	19	18	303	152	25
T3	597	63	44	49	70	15	57
T4	508	821	487	201	116	33	39
M38	247	1409	869	1064	364	252	86
G25	364	1480	801	885	278	134	63
MS1	65	490	255	365	226	161	22
M53	ND	ND	ND	ND	ND	ND	ND

3.4 Comparison of Alamar Blue and propidium iodide-based assays for determination of EC₅₀ values:

We have recently developed an alternative to the standard Alamar Blue assay, using the fluorophore propidium iodide (PI). This protocol has several advantages over the Alamar Blue assay, including that the outcome of the test is not dependent on the metabolic activity of the parasites. Conceivably, the test compounds themselves could influence the reduction of the AB dye to its fluorescent state (resorufin), or accelerate it to colourless, non-fluorescent hydroresorufin (Gould *et al.*, 2008). With the new protocol available late in this project we decided to verify the antiparasitic activity of the choline analogues.

For this assay, used to determine EC₅₀ values for test compounds, propidium iodide dye is added at the end of the incubation with the test compound; it is not, as Alamar Blue, incubated with the parasites while in culture. Digitonin was added together with the PI, in order to permeabilise the cells to PI. The level of fluorescence was, under those conditions, directly proportional to the number of cells in the well (Gould *et al.*, 2008).

The Alamar Blue assay and propidium iodide assay were performed using the same conditions and 96-well plates, as described in chapter 2, section 2.4. In the propidium iodide assay, cells of *T. brucei* bloodstream forms or promastigotes of *L. major* and *L. mexicana* were incubated for 72 h in the appropriate medium. Then 20 μ l of propidium iodide with digitonin (final concentration 9 μ M and 20 μ M, respectively) was added, followed by 1 h incubation in the same atmosphere.

The data show that the EC₅₀ values obtained with the propidium iodide plus digitonin assay were very close to those acquired using the Alamar Blue assay for bloodstream forms of *T. brucei* WT (Figure 3.5A), *L. major* promastigotes (Figure 3.5B) and *L. mexicana* promastigotes (Figure 3.5 C).

In order to investigate whether the different incubation periods of *L. major* promastigotes influenced results, the more potent choline compounds were tested by setting up four identical plates (as described previously) at the same time, and after predetermined times (2, 3, 4, and 5 days) propidium iodide and digitonin were added, and fluorescence read after 1 h. The data show that there are no significant differences in EC₅₀ values using the propidium iodide assay after 2, 3, 4, or 5 days of incubation with choline compounds (Figure 3.6). However, some differences were noted in pentamidine between the first and last two days probably as a result of the very slow action of pentamidine on these parasites. The same values were obtained from each plate after freezing at -20 °C for 24 h after the addition of PI/digitonin, showing that an additional freeze thaw cycle did not cause further cell lysis.

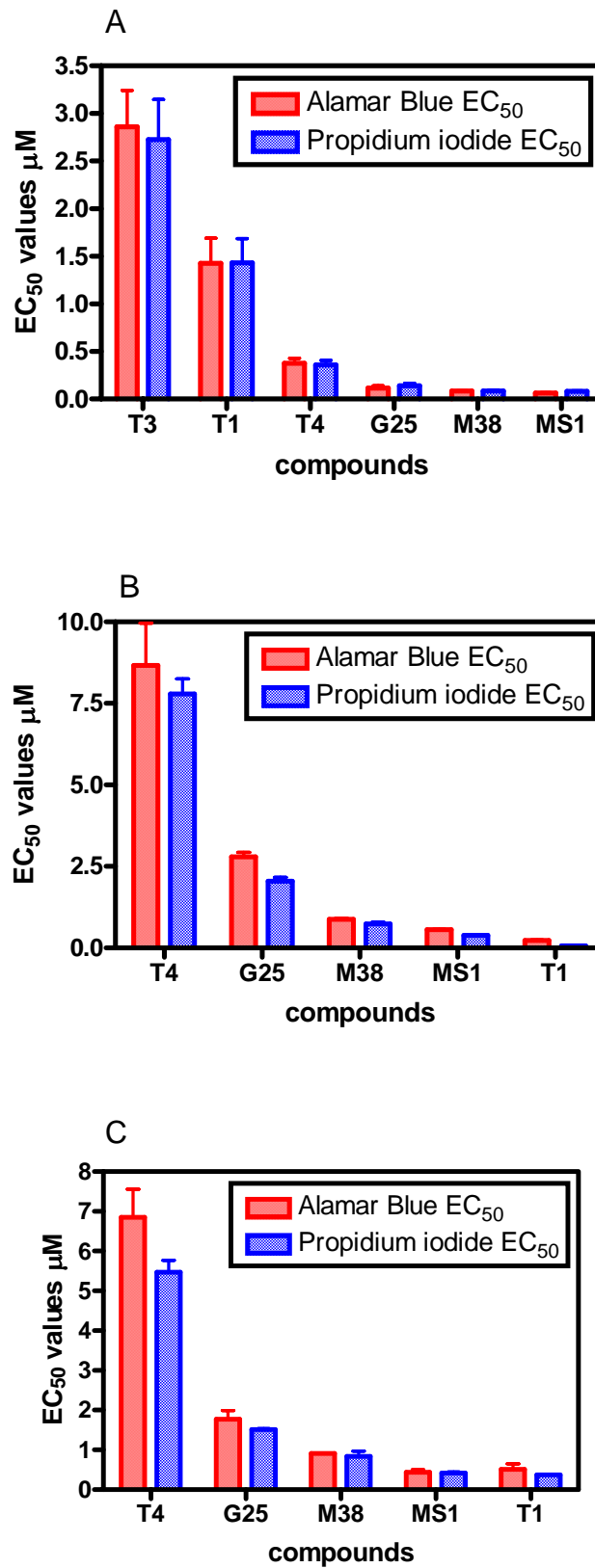


Figure 3.5. Comparison of EC₅₀ values of choline compounds measured by propidium iodide and Alamar Blue methods in three different types of parasites, bloodstream forms of *T. brucei* s427 (A); promastigotes of *L. major* (B) and *L. mexicana* (C). Values are the average ± SE of 3 independent Alamar Blue and PI experiments.

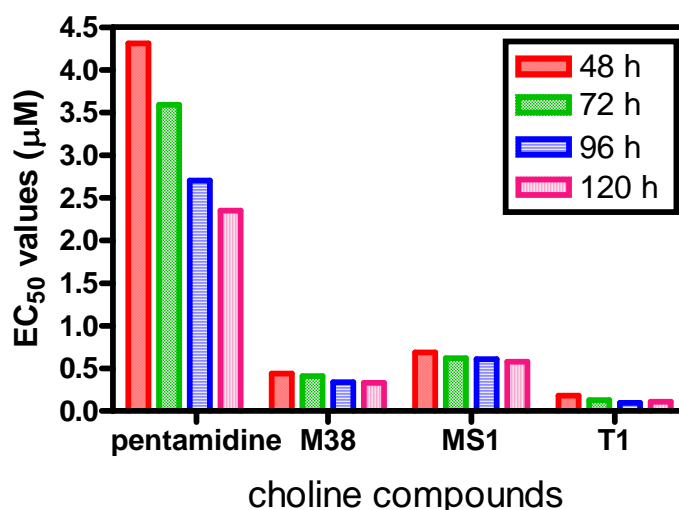


Figure 3.6. Effect of the incubation time of *L. major* promastigotes treated by some choline drugs on the development of propidium iodide fluorescence. Four plates were set up at the same time and incubated for 2 (red) 3 (green), 4 (blue), and 5 (pink) days. Pentamidine was used as a positive control.

The results demonstrate that 48 h is a sufficient incubation time for drug screening assay using propidium iodide with promastigotes of *L. major*. Therefore, this incubation period might be valid for all species of *Leishmania*, although this needs further experimental confirmation.

To investigate whether the 20 μM of digitonin used killed all the cells, doubling serial dilutions of digitonin, starting at 25 μM , were incubated in the presence of 9 μM of propidium iodide with promastigotes of *L. major* at a cell density 5×10^7 cells/ml. The cells were monitored for 2 h using a FLUOstar OPTIMA plate reader. The measurements shown in Figure 3.7 indicate that 6.2 μM of digitonin is able to kill all the promastigotes within the first 15 min of incubation.

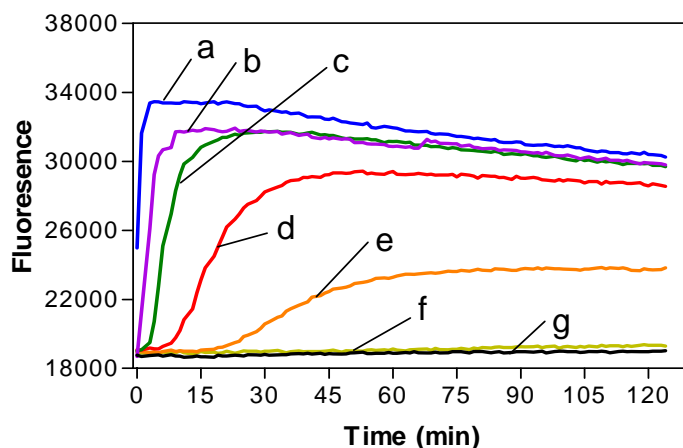


Figure 3.7. Development of fluorescence intensity in *L. major* promastigotes over 2 h due to binding of propidium iodide with DNA of permeable cells using different concentrations of digitonin.

(a) 25 μM ; (b) 12.5 μM ; (c) 6.25 μM ; (d) 3.13 μM ; (e) 1.56 μM ; (f) 0.78 μM ; and (g) no-drug control.

3.5 Assessment of choline uptake in *T. b. brucei* and *L. mexicana*.

In order to fully understand the mechanism of action of the choline analogues, we investigated their effect on choline uptake by the parasite using the classical uptake technique. It was ascertained whether the choline compounds tested in this study enter the parasite through a designated choline transporter or not.

Uptake of [^3H]-choline into *Leishmania mexicana* promastigotes and *Trypanosoma brucei* bloodstream forms were performed using a rapid oil-stop protocol as described in section 2.9. All experiments were performed in triplicate and radioactivity in the cell pellets was determined using liquid scintillation counting. Preliminary transport studies were performed to investigate choline uptake in *T. brucei* bloodstream forms and *L. mexicana* promastigotes.

Assessment of choline transport by *T. brucei* bloodstream forms showed that no uptake of 0.25 μM [^3H]-choline was detectable in the presence or absence of 1 mM of unlabelled choline over 60 s (Figure 3.8A). These results suggested that *T. brucei* bloodstream forms do not express choline transporters. In contrast, the transport of 0.25 μM [^3H]-choline by *L.*

mexicana promastigotes was linear for at least 20 s, and plateaued at 0.15 pmol/10⁷ cells after 20 s of incubation (Figure 3.8B).

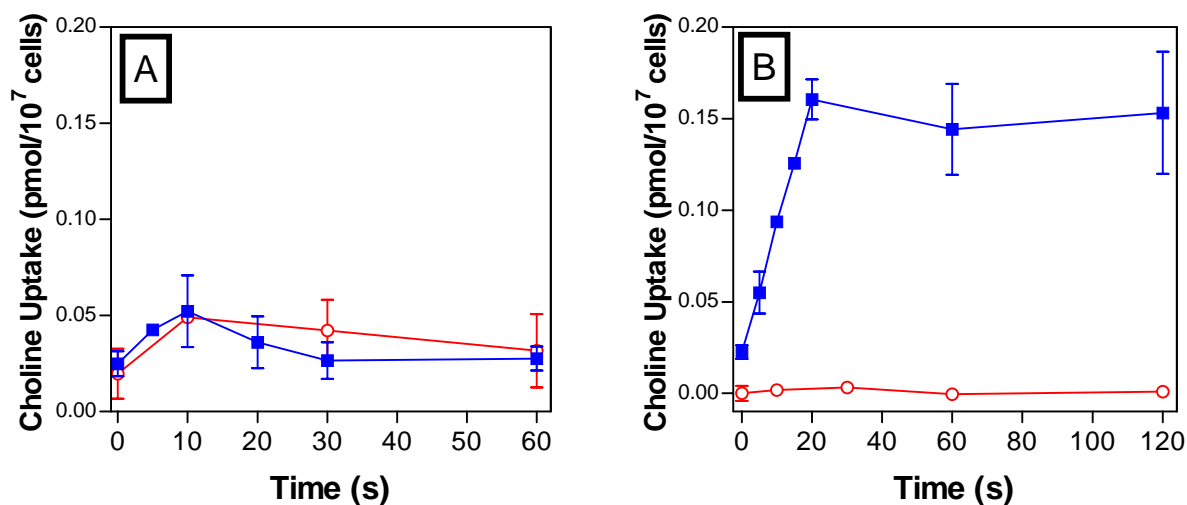


Figure 3.8. Time course of 0.25 μM [³H]-choline uptake by strains of trypanosomes and Leishmania.

(A) *T. brucei* 427 bloodstream forms and (B) *L. mexicana* promastigotes over 120 seconds in the presence (○) or absence (■) 1 mM unlabelled choline. Assay was performed in triplicate and representative of 4 similar experiments. Error bars are S.E. In Figure A, linear regression through either data set produced a line (not shown) with a slope that was not significantly different from zero (F-test; GraphPad Prism).

We investigated the effect of choline analogues on [³H]-choline uptake in *L. mexicana* promastigotes. Transport of [³H]-choline in *L. mexicana* promastigotes, assessed after 10 seconds of radiolabel incubation, was inhibited by T1 and T3 (Figure 3.9). These two compounds were effective inhibitors of [³H]-choline transport with average K_i values of $4.0 \pm 0.3 \mu\text{M}$ and $0.41 \pm 0.1 \mu\text{M}$ ($n=3$), respectively. Indeed, T3 displayed a very similar affinity as choline itself, which displayed an average K_m of $0.49 \pm 0.2 \mu\text{M}$ and V_{max} of $0.11 \pm 0.04 \text{ pmol} (10^7 \text{ cells})^{-1} \text{ s}^{-1}$ ($n=4$). The inhibition curves with T1 and T3 were consistent with competitive inhibition, displaying Hill slopes near the theoretical value of -1. The effects of the other choline analogues on [³H]-choline uptake were not determined.

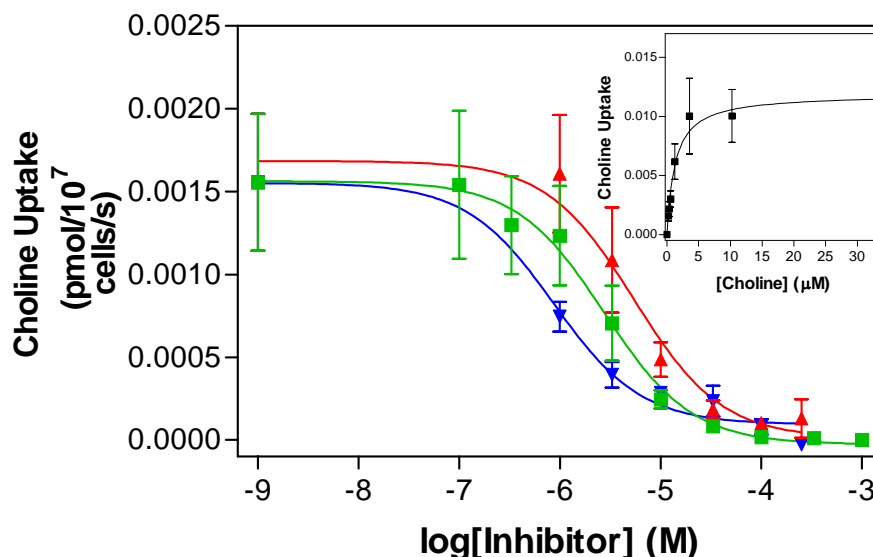


Figure 3.9. Inhibition of 0.25 μM [^3H]-choline uptake in *L. mexicana* promastigotes by various concentrations of unlabelled choline (\blacksquare), T1 (\blacktriangle) and T3 (\blacktriangledown), with IC_{50} values of 2.6 μM , 5.4 μM and 0.87 μM , respectively. The inset shows the conversion of choline inhibition data to Michaelis-Menten plot of total choline uptake, with a K_m value of 1.3 μM and a V_{max} of 0.012 $\text{pmol} (10^7 \text{ cells})^{-1} \text{s}^{-1}$.

3.6 Choline analogues as inhibitors of pentamidine transport in *T. b. brucei*

The best characterised drug transporters in *T. brucei* are the HAPT and LAPT transporters that are implicated in the uptake of diamidine drugs such as pentamidine (de Koning, 2008). With the exception of the monocationic T1, the choline analogues are structurally similar to pentamidine in that they are dications in which the positively charged end groups are joined through a flexible aliphatic linker chain. In order to determine whether choline compounds can interfere with the activity of pentamidine transporters in *T. brucei* bloodstream forms and might possibly be substrates for these transporters, pentamidine uptake assays were performed using [^3H]-pentamidine at 40 nM for HAPT1 and 1 μM for LAPT1, as their respective K_m values are 36 ± 6 nM and 56 ± 8 μM (De Koning, 2001b). Under these conditions the two transporters can be studied in virtual isolation. These assays were performed in three independent experiments, each performed in triplicate. The results indicated that G25 and M38 did not inhibit the [^3H]-pentamidine uptake at the concentrations at which they are pharmacologically active, and are clearly poor substrates of *T. brucei* HAPT1 (Figure 3.10), with K_i values 44 ± 10 μM ($n=4$) and 45 ± 4 μM ($n=3$) respectively. Similar results were obtained for these two compounds of LAPT1 (Figure 3.11), with K_i values 560 ± 200 μM ($n=3$) for G25 and 200 ± 70 μM ($n=3$) for M38. T4 was also tested and the IC_{50} values were 210 ± 50 μM ($n=3$) and >1000 μM ($n=1$) for

HAPT1 and LAPT1, respectively. In each [^3H]-pentamidine transport experiment, unlabeled pentamidine was used as a positive control and inhibited HAPT and LAPT with average K_m values of $0.035 \pm 0.005 \mu\text{M}$ ($n=4$) and $61 \pm 8 \mu\text{M}$ ($n=5$), respectively, values nearly identical to those published previously (De Koning, 2001b).

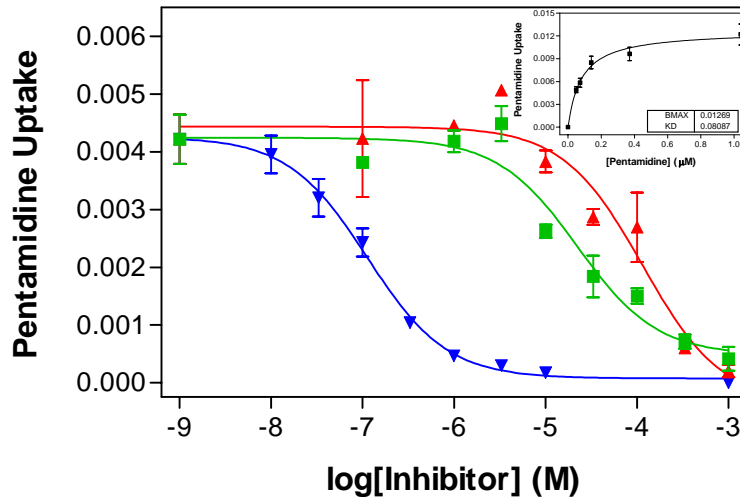


Figure 3.10. Inhibition of $0.04 \mu\text{M}$ [^3H]-pentamidine uptake (high affinity pentamidine transporter) in *T. brucei* 427 bloodstream forms by various concentrations of G25 (■), M38 (▲) and unlabeled pentamidine (▼), with IC_{50} values of $22.2 \mu\text{M}$, $115 \mu\text{M}$ and $0.11 \mu\text{M}$, respectively. The inset shows the conversion of the pentamidine inhibition plot to a Michaelis-Menten curve.

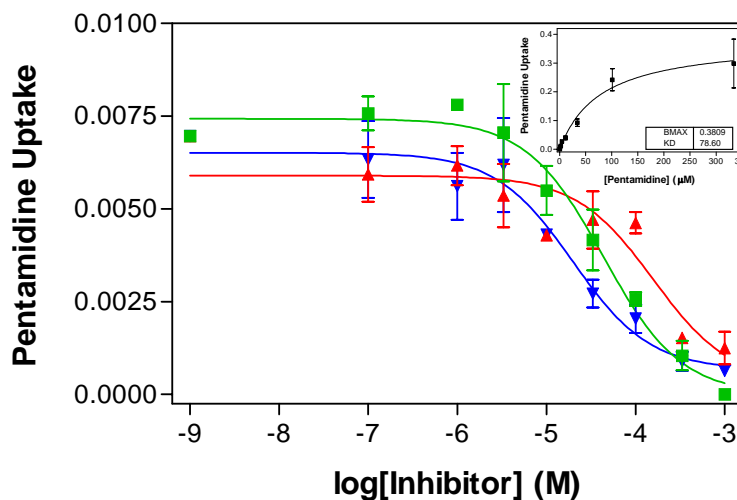


Figure 3.11. Inhibition of $1 \mu\text{M}$ [^3H]-pentamidine uptake (low affinity pentamidine transporter) in *T. brucei* 427 bloodstream forms by various concentrations of G25 (■), M38 (▲) and unlabeled pentamidine (▼), with IC_{50} values of $43.5 \mu\text{M}$, $158 \mu\text{M}$ and $19.6 \mu\text{M}$, respectively. The inset displays the conversion of the pentamidine inhibition data to a Michaelis-Menten plot.

The IC₅₀ values of choline compounds on HAPT1 were approximately 1000 lower affinity than that for pentamidine, and on LAPT displayed also lower affinity than pentamidine. It is unlikely that these pentamidine transporters mediate the uptake of these compounds, as the flux would be very low at the submicromolar concentration of which the compounds act on the trypanosomes.

3.7 Discussion

It was demonstrated in this study that choline analogues have anti-parasitic activities against *Trypanosoma* and *Leishmania species*. In particular, the activities of M38, G25 and MS1 against bloodstream forms of three different strains of *Trypanosoma brucei brucei* (Tb427 WT, TbAT1-KO and TbAT1 KO-B48) was very promising and close to the level of the positive control, diminazene aceturate. TbAT1KO has a high level of resistance to diminazene, but not to pentamidine and arsenical drugs like melarsen oxide, melarsoprol, and melarsamine hydrochloride, whereas TbAT1KO-B48 has a very high resistance level to pentamidine. Considering the severe problems with drug resistance in the field (Delespaux & De Koning, 2007), the apparent lack of cross-resistance between the choline analogues and the diamidine and arsenical classes of trypanoscides is an absolute condition for the further development of any agent against African trypanosomiasis. It must also be considered that this study only assessed a small sample of the existing choline analogue library. As such, the activities identified are highly encouraging. The same is true for the antileishmanial activities reported here, with the activity of T1 ten-fold better than pentamidine.

Many methods like microscopic counting (Rolon *et al*, 2006), colorimetric MTT assay (Mosmann, 1983; Ferrari *et al*, 1990), acid phosphate activity (Bodley *et al*, 1995), Alamar Blue assay (Raz *et al*, 1997; Mikus & Steverding, 2000; Rolon *et al*, 2006) and propidium iodide assay (Gould *et al*, 2008) have been described to determine the drug susceptibility of different pathogenic cells. Alamar Blue, an endpoint measurement for cytotoxicity, is a fast, sensitive and simple method for drug screening, and the cell viability can be measured by performing cell counts during the incubation time. This method was found to be an acceptable tool for measuring the drug sensitivity of the human pathogenic African trypanosomes, *T. b. gambiense* and *T. b. rhodesiense in vitro* (Raz *et al*, 1997) and against *L. major* promastigotes (Mikus & Steverding, 2000), and *L. mexicana* amastigotes (Al Salabi & De Koning, 2005).

Yet, problems with the Alamar Blue assay exist. It needs to be optimized for all type, as they can metabolise the dye at different rates, but over incubation must be avoided as cells can metabolise the fluorescent resorufin to non-fluorescent and colorless hydroresorufin (O'Brien *et al*, 2000). Compared with *T. brucei* bloodstream forms and human embryonic kidney cells, promastigotes of *L. major* and *L. mexicana* and amastigotes of *L. mexicana* reduced Alamar Blue dye more slowly (Gould *et al*, 2008). The same result have been obtained by Mikus & Steverding (2000) and Rolon *et al.*, (2006) when they found that the reduction of Alamar Blue is more slowly in *L. major* promastigotes and *T. cruzi* epimastigotes, respectively. In this study, the optimal cell density and incubation period for *T. brucei* were found to be 10^5 cells/ml and 48 h plus 24 h with Alamar Blue, respectively. In all *Leishmania* species these parameters were 10^6 cells/ml and 72 h plus 48 h with Alamar Blue. Raz *et al*, (1997) found that the optimal period for Alamar Blue incubation for *T. b. rhodesiense* and *T. b. gambiense* was 66 h Plus 6 h or 24 h, respectively, after adding the dye. The differences in the rate of Alamar Blue metabolism between the different types of cells may in past attributed to differences in incubation, the temperature and the doubling time.

Choline compounds designated T1, M38, G25 and MS1 inhibited the growth of *Leishmania species* with EC_{50} values less than 2.0 μ M. Interestingly the EC_{50} value of less than 2.0 μ M obtained for some of the compounds studied is lower than that of pentamidine, a drug used in the routine treatment of the disease. This finding is reassuring considering the current need for novel anti-parasitic drugs to replace the routinely used drugs for which the parasites have developed resistance. Choline compounds, with the exception of compound T3 and M53, also showed significant anti-parasitic activity against trypanosomes.

The level of potency of these choline compounds appears to be determined, among other things, by the presence or absence of a second cationic ring. Against the *T. brucei* bloodstream forms, the bisthiazolium T4 (0.61 μ M) was observed to be 3 times more active than the monothiazolium T1 (1.8 μ M). A similar trend in activity against *Plasmodium* species has previously been reported (Hamze *et al*, 2005). The presence of an aliphatic side chain $[(CH_2)_{12}]$ in compound T1 could probably account for the moderate anti-parasitic activity of this compound whilst the dicationic nature of T4 might contribute to the significant increase in the anti-parasitic activity of this compound.

Up to a concentration of 400 μM , the choline analogues were found to be non-toxic to HEK cells. It must be emphasized that it is very important to consider the selectivity index [$\text{LD}_{50}/\text{EC}_{50}$] of a drug before clinical use (May *et al*, 2006). Higher index means the drug is less toxic to host cells. Some of the compounds tested in the current study were found to have a lower therapeutic index. This observation is quite important since it implies that some of the choline compounds may lend themselves as novel anti-leishmania and/or anti-trypanosomic drugs. It is now imperative that a much larger group of choline compounds is evaluated for activities against these parasites.

The propidium iodide fluorescence procedure was used in this study because it is more rapid than Alamar Blue and is not influenced by the level of cellular metabolism. The staining by PI takes 1 h prior to fluorescence reading compared with the Alamar Blue which is 24 or 48 h depending on the cell type. Each of the dyes has its own mode of indicating the number of cells. The main idea of the PI assay is that the use of digitonin, to permeabilise all the cells in each well, allows the PI dye to cross the cell membrane of cells to bind the nucleic acids, upon which it fluoresces. This method is sensitive and the level of fluorescence correlates well with cell numbers (Gould *et al*, 2008). Importantly, the EC_{50} values obtained here with the PI method were very similar to those obtained using Alamar Blue.

Data obtained in this study strongly suggest that *T. brucei* bloodstream forms do not express specific choline transporters acting in the micromolar concentration range. This observation is consistent with the results obtained by (Rifkin *et al*, 1995). How the choline compounds traverse the cell wall of the parasites is therefore a question that remains to be answered. Further investigations are needed in this direction. Generally, there are at least four different uptake modes in protozoan parasites: endocytosis, passive diffusion, receptor-mediated uptake and transporter-mediated uptake (De Koning, 2001a).

Labeled choline was taken up by *L. mexicana* promastigotes and it was inhibited by unlabeled choline and some choline analogues like T1 and T3 with apparent K_m values of 0.49, 4.0 and 0.41 μM , respectively. This result indicates that promastigotes of *Leishmania* spp express choline transporter(s). Such a carrier was previously reported by (Zufferey & Mamoun, 2002), with a K_m value of 2.5 μM .

Pentamidine one of the controls in this study is known to accumulate in trypanosomes to millimolar concentrations and studies indicate that it is taken up by three different transporters: the P2 adenosine/adenine transporter; a high affinity pentamidine transporter

(HAPT1); and a low affinity pentamidine transporter (LAPT1 (De Koning, 2001b). Since uptake of pentamidine into the parasites seemed not to be inhibited by any of the choline analogue at pharmacologically relevant concentrations it is not likely that the choline compounds are taken into the cell through either the low or high affinity transporters. Uptake through the P2 transporter was not specifically addressed but must be considered highly unlikely given the structures of choline compounds, which do not contain the well-established P2 recognition motif (Luscher *et al*, 2007; De Koning *et al*, 2005).

Chapter Four

4 A study of the mechanism of action of the choline analogues in kinetoplastida

4.1 Introduction

In this chapter we investigate how the choline compounds interact with *Trypanosoma brucei* cells. The first parameter is to study the effect on growth, giving information about the rate at which the compounds affect the cell population, and whether the effect is directly on the viability of the parasites (parasitocidal effect), or rather on their ability to divide (parasitostatic effect).

There are many ways to study growth curves in parasitic protozoa. In this study, assays like direct microscopic cell counts, spectrophotometric lysis assays and viability assays with propidium iodide were used to assess the cells' viability and growth. Flow cytometry was used to analyze cell cycle progression, identifying trypanosomes in the G1, S, and G2/M stages. The kinetoplast and nucleus undergo division separately, with the division of the former proceeding first (see Figure 4.1). In the G1 phase of the *T. b. brucei* life cycle, all the cells have one kinetoplast and one nucleus, the pro-basal body becomes mature, a new one is formed, and the daughter flagellum is starting to grow out. The kinetoplast S phase is shorter than the nuclear S phase and initially results in cells with two kinetoplasts and one nucleus, rapidly followed by nuclear division to cells with two kinetoplasts and two nuclei (G2 phase). These cells then enter M phase with the formation of a furrow and longitudinal division into parent and daughter cells (Hammarton *et al*, 2003a).

Cell membrane permeability was assayed using the fluorescence dye propidium iodide, which does not cross intact membranes. At the moment of cell permeabilization, this fluorescent marker can stain the nuclei of necrotic cells due to plasma membrane disruption. Propidium iodide fluorescence was monitored both in real time in a fluorimeter (up to 8 hours) and using flow cytometry after exposure of cultures to low concentrations of the test compounds for a prolonged time. We further used flow cytometry to assess the mitochondrial membrane potential Ψ_m , using the fluorophore Tetramethylrhodamine ethyl ester (TMRE). By studying cellular permeability, Ψ_m and DNA content in parallel we aimed to establish whether changes in cell cycle, DNA integrity or mitochondrial function occur before loss of cell integrity. We also examined whether the cell death process by choline compounds is mediated by the production of reactive oxygen species in *T. brucei*. Finally, we investigated whether incubation with these choline analogues alters the plasma membrane lipid composition of *T. b. brucei* through inhibition of choline lipid synthesis.

The mode of action of similar choline analogues has been investigated previously in *Plasmodium falciparum* (Ancelin *et al*, 1985; Ancelin & Vial, 1986; Richier *et al*, 2006; Roggero *et al*, 2004). For example, the quaternary ammonium choline compounds were found to inhibit very early and specifically the synthesis of phosphatidylcholine and concomitantly block the *in vitro* growth of *Plasmodium falciparum* (Ancelin & Vial, 1986). Ancelin *et al*. (1985) first demonstrated that the choline analogues hemicholinium 3 and decyltrimethylammonium inhibit phosphatidylcholine (PC) biosynthesis in *P. falciparum* and Ancelin and Vial (1986) subsequently showed that these quaternary ammonium compounds acted on the choline transporter of the infected erythrocytes, characterised by Ancelin *et al*. (1991), thereby affecting the de novo PC biosynthesis pathways. Yet, it has also been reported that some antimalarial choline analogues enter infected erythrocytes through the so-called New Permeation Pathways (NPP) and enter the parasites through a choline transporter characterised by Biagini *et al* (2004). Indeed, these authors showed that the choline analogue T16 and the diamidine pentamidine enter the infected cell through the NPP, followed by uptake into the parasite by the *P. falciparum* choline transporter (Biagini *et al*, 2004). As their primary impact, these compounds did not affect choline phosphorylation, the first step of the Kennedy pathway (see Chapter 3). Indeed, the quaternary ammonium compound G25 was shown to inhibit the process of de novo PC biosynthesis and at higher concentrations also the decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) by phosphatidylserine decarboxylase (Roggero *et al*., 2004). At a concentration of 1 μ M G25 caused a significant inhibition (56%) of phosphatidylcholine biosynthesis, and almost complete inhibition at 10 μ M (Roggero *et al*, 2004). This seems to suggest that choline analogues have more than one impact on lipid metabolism, and might exert their antimalarial effects in different ways.

This chapter will focus on biological activities of seven choline analogues on *T. b. brucei* with the aim of establishing how they effect this parasite and might exert their toxic effect.

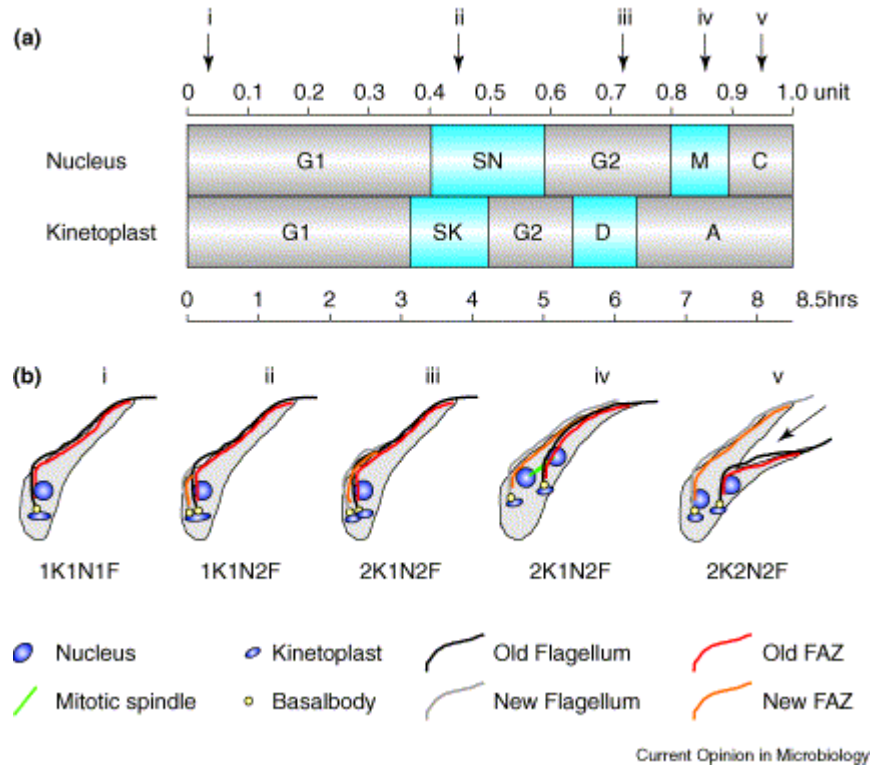


Figure 4.1. Cell division cycle of *T. b. brucei*.

Reproduced from McKean (2003). (a) The trypanosome cell cycle is separated into nuclear and kinetoplast components. Cell cycle duration for exponentially growing procyclic trypanosomes is 8.5 h . Kinetoplast replication (S) initiates before nuclear S phase, and is considerably shorter and consequently kinetoplast segregation (D) occurs before the onset of nuclear mitosis (M). The phase annotated on the kinetoplast cycle as ‘A’ refers to the ‘apportioning’ phase during which basal bodies continue to move apart. (b) Schematic representations of trypanosome cells taken from various time points through the cell cycle. The black arrow indicates the direction and position of the cleavage furrow.

4.2 Choline analogues reversibly inhibit growth of kinetoplastid parasites but do not lead to rapid cell lysis

In order to study the effect of choline compounds on the growth of *T. brucei* bloodstream forms (BSF), it was first necessary to construct a normal growth curve by collecting and counting cells at regular times of days. For BSF growth curves, a cell culture was set up at log phase 1×10^5 cells/ml in HMI-9 supplemented with 10% foetal calf serum. Using a haemocytometer, cell numbers were determined every 24 h until the cells stopped growing (stationary phase). Figure 4.2 shows a reference growth curve of *T. b. brucei* seeded at 10^4 cells/ml. Cells grew logarithmically for three days, after which the culture entered stationary phase and cells started dying by day 5.

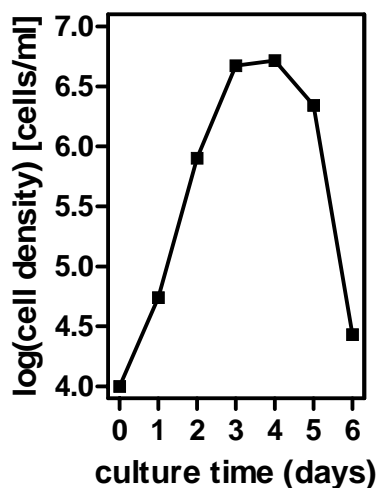


Figure 4.2. Graph showing the normal growth curve of *T. brucei* 427 bloodstream forms. Cells were grown in HMI-9 medium at 37 °C & 5% CO₂. Cells were counted once a day for 6 days using a hemocytometer.

Next, to investigate the effect of choline analogues on the viability and proliferation of a population of *T. brucei* BSF, cell counts were performed on cultures incubated with the various choline analogues. This method is easy and inexpensive, and allows an initial assessment of the effects of the compounds on the growth and survival of trypanosomes.

Cells were seeded up to 24 h at 1×10^5 cells/ml in the presence and absence of different concentrations of 6 choline analogues (T1, T3, T4, M38, G25 and MS1) which were selected from the Alamar Blue results (described in chapter 3) as the most promising choline compounds against *T. brucei* BSF. When these cells were incubated with the

indicated compounds at $3 \times EC_{50}$, G25 and T4 were the least effective in reducing the growth rate, which continued normally for up to 24 and 32 h, respectively. In contrast, T1, T3, M38 and MS1 appeared to inhibit growth almost immediately, even though the compounds did not start to reduce the cell population until approximately 30 h (data not shown). From this experiment it cannot distinguish between the two possibilities that T1, T3, M38 and MS1 rapidly induced cell cycle arrest, leading to an effective freeze of the cell density, or killed a proportion of the cell population at a rate similar to the growth rate.

At $10 \times EC_{50}$, T1 ($EC_{50} = 1.8 \mu\text{M}$) killed the cells in less than 4 h, whereas MS1 ($EC_{50} = 0.13 \mu\text{M}$), induced cell death after 24 h and T3 ($EC_{50} = 7.9 \mu\text{M}$) in 28 h (Figure 4.3A). Thus, there was no correlation between EC_{50} value and the rate of decline of the cell population. In a next experiment, the cells were incubated with $20 \mu\text{M}$ of each of the compounds for 24 h, and the results in Figure 4.3B demonstrate that T1 and MS1 are fast acting drugs at this concentration, and they killed the population of BSF *T. b. brucei* in about 8 h. The onset of apparent cell death in the trypanosome population was between 2 and 4 hours. In contrast, the other choline compounds caused a slower reduction in cell numbers, although most appeared to have an effect within one cell cycle, usually estimated at 8 hours under the conditions used. This is important to note as the cell population is not synchronised and the compounds might act on a specific point of the cell cycle.

Overall, we can say that most of the choline compounds appear to gradually reduce the trypanosome population, even at concentrations of $10 \times EC_{50}$, with perhaps the exception of T1, which displayed a more drastic trypanocidal effect. This may well be related to the fact that T1 is the only monocation, the other analogues all being symmetrical dimers of choline lipid analogues (see Figure 3.1). It is thus to be expected that the uptake dynamics of T1, for instance, might differ from the other compounds.

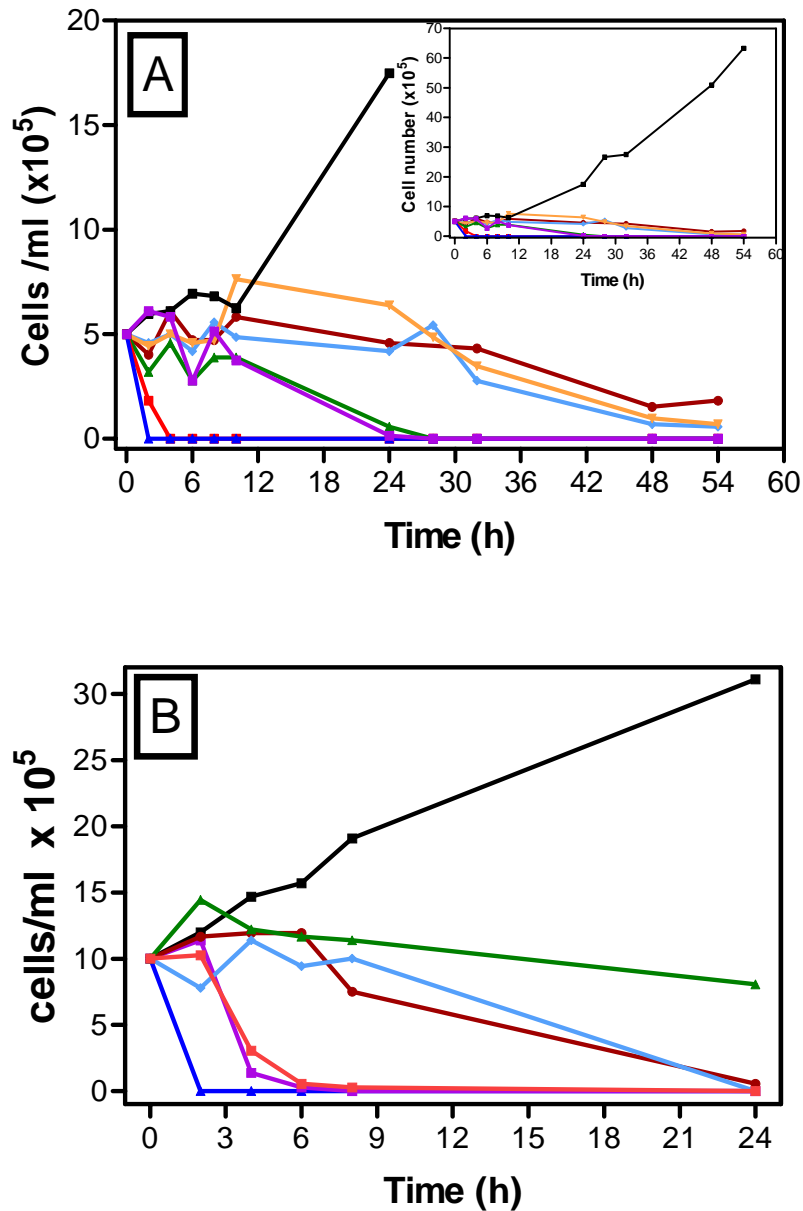


Figure 4.3. *In vitro* effect of choline analogues on proliferation of bloodstream forms of *T. brucei* 427.

The figure shows incubation *in vitro* with choline compounds at 10x their EC₅₀ value (A) and 20 μM (B). Drug free (■), 0.5 μM PAO (▲), T1 (■), T3 (▲), T4 (▼), M38 (◆), G25 (●) and MS1 (■). The inset shows the same figure in lower magnification.

An alternative method of assessing the effects of compounds on a trypanosome population is the spectrophotometric assay used by Fairlamb and others (Fairlamb *et al*, 1992; Carter & Fairlamb, 1993; Bridges *et al*, 2007) to monitor cell lysis in response to melarsen oxide. The advantage of this assay over the cell count is that it allows the real time monitoring of up to eight trypanosome cultures using our HP-8453 spectrophotometer (Hewlett-Packard). Fairlamb and his collages defined the cell lysis as “all the trypanosomes were observed to have become swollen and immotile with loss of their normal refractile appearance” (Fairlamb *et al*, 1992). The assay really monitors light scatter by the highly motile trypanosomes in a cuvette, and a reduction in absorbtion may thus equally signify cell death or loss of motility as a result of, e.g., ATP depletion. The assay is performed at 37 °C in a full culture medium, but because it is not performed under CO₂ atmoshere, it is only useful for up to 8 hours.

The results showed that, at 10 µM, there is no significant activity of the choline analogues studied within the 8 hours of incubation time the experiment allows. Only MS1 started its effect after 6 hours of incubation (Figure 4.4A). The same experiment was repeated with the increased drug concentration to 20 µM. The results showed that MS1 at this concentration started to affect cells after 2 hours of incubations, followed by T1 after 6 hours (Figure 4.4B). The results obtained by the spectrophotometric assay confirmed those were resulted from the cell count and additionally showed that the choline compounds had no immediate effects on cell motility.

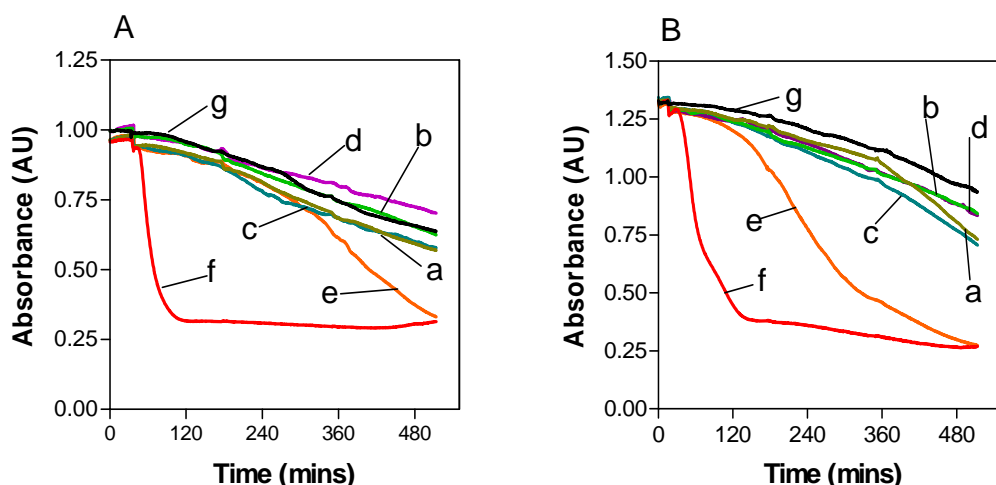


Figure 4.4. Inhibitory curves of choline analogues on mobility of *T. brucei* BSF measured by light scattering. The figure shows the effect of 10 µM (A) and 20 µM (B) of (a) T1; (b) T3; (c) T4; (d) G25; (e) MS1 on the parasites. (f) 0.5 µM Phenylarsine oxide and (g) drug free were used as positive and negative controls, respectively.

The spectrophotometric assay was also performed to test whether the co-administration of 1 μM pentamidine with 20 μM of some choline analogues has any effect on the cell lysis or not. This effect could be either synergistic, accelerating cell lysis, or antagonistic as 1 μM pentamidine fully inhibits the HAPT and P2 drug transporters (Matovu *et al.*, 2003). For these investigations, the T1 and MS1 compounds were used, and their activities were tested in the presence and absence of 1 μM pentamidine. There was no clear difference in the effect of T1 and MS1 in the presence of this concentration of pentamidine (Figure 4.5). Pentamidine at 1 μM itself had no effect (data not shown). The experiments were repeated with increased concentrations of pentamidine. The results show that the drug activity of MS1 increased slightly in the presence of 1, 10, 100 μM of pentamidine (Figure 4.6). From this result it can be concluded that uptake of the choline analogues is not dependent on the P2 or the HAPT drug transporters. While some synergism was observed between MS1 and pentamidine this was at pentamidine concentrations that would be unattainable in patients. Surprisingly, 1 mM pentamidine completely prevented the effects of 20 μM MS1 (Figure 4.6). This may be attributable to the pentamidine blocking the uptake mechanism for the drug rather than antagonism at the site of action, although this cannot be excluded.

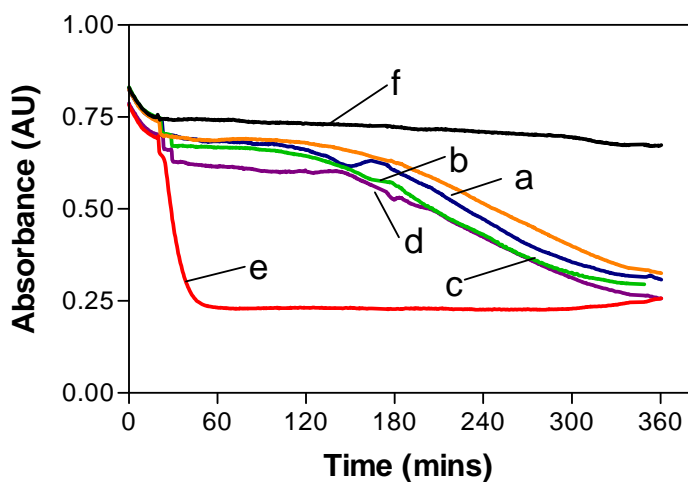


Figure 4.5. Effect of choline analogues on *T. brucei* light scatter in the presence or absence 1 μM of pentamidine. 50 μl of 400 μM of drugs were added to 950 μl of cell culture after 15 min of recording. Conditions from $t = 0$ min: a, 20 μM T1; b, 20 μM T1 plus 1 μM pentamidine; c, 20 μM MS1; d, 20 μM MS1 plus 1 μM pentamidine; e, 0.5 μM Phenylarsine oxide; f, no drug control. The figure shown is representative of two similar repeats.

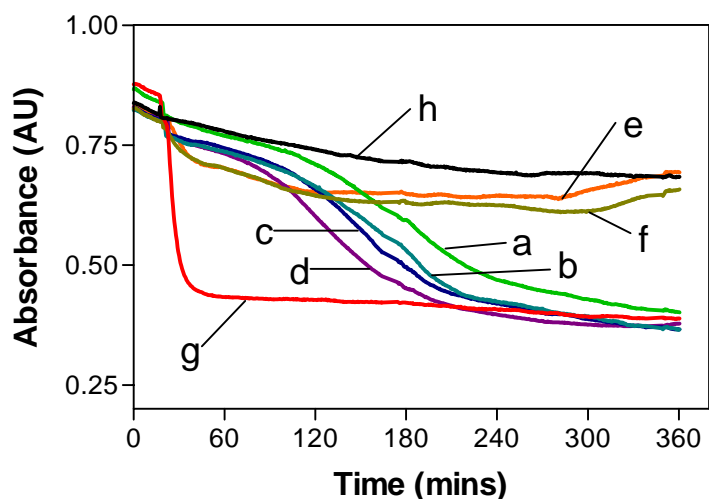


Figure 4.6. Effect of serial dilutions of pentamidine on the activity of 20 μM of MS1 for light scatter by *T. brucei*, measured at 750 nm by light absorbance. Traces: a, 20 μM MS1; b, 20 μM MS1 plus 1 μM pentamidine; c, 20 μM MS1 plus 10 μM pentamidine; d, 20 μM MS1 plus 100 μM pentamidine; e, 20 μM MS1 plus 1 mM pentamidine; f, 1 mM pentamidine; g, 0.5 μM Phenylarsine oxide; h, drug free control. The shown experiments are representative of two similar repeats.

Next, light scatter assays were used to explore the minimal duration of contact of the choline drugs with the parasites in order to exert a non-reversible trypanocidal effect. T1 and MS1 were tested for reversibility of their activity. The cells were incubated with these drugs and the absorbance was monitored for up to 120 min, the cells were then washed twice in fresh HMI-9 medium and incubated for a further 5 hours with or without drugs, as described in section 2.6.2. The results in Figures 4.7A and 4.7B indicated that an incubation of T1 and MS1 did not affect cell viability when the cells were subsequently resuspended in fresh media without drug. A tentative conclusion can be drawn that trypanosomes must be continuously exposed to these test compounds until cell death occurs.

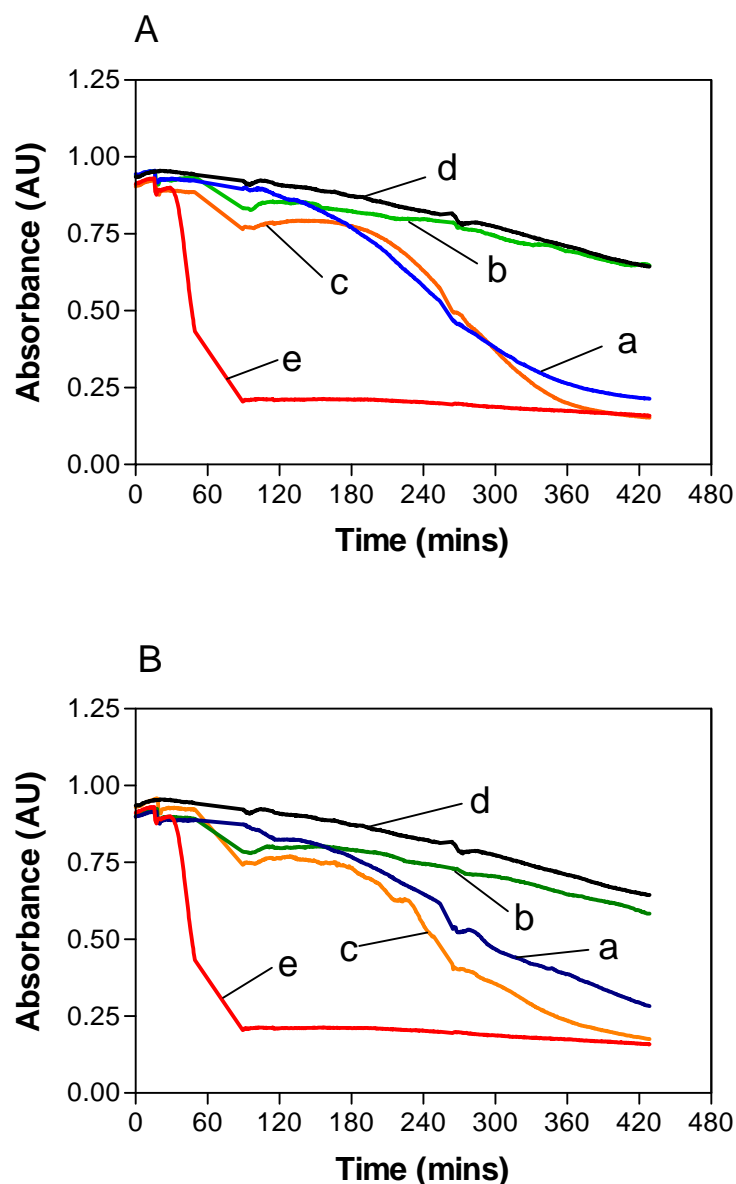


Figure 4.7. Reversibility curves of *T. brucei* s427 bloodstream forms with 20 μM of (A) T1 and (B) MS1.

Drugs were added to cells $\sim 5 \times 10^7$ cells/ml in HMI-9 medium after 15 min of recording. Monitored for 2 h at 750 nm and after then washed twice and re-suspend in fresh medium and monitored again for 5 h. Traces: a, No wash; b, cells washed after 2 h and resuspended in fresh medium; c, washed after 2 h and resuspended in 20 μM of test compound; d, no drug control; and e, 0.5 μM phenylarsine oxide.

While the spectrophotometric assay used above allows real-time monitoring of cell populations, the results are relatively hard to interpret as a response could equally signify reduced motility or cell lysis. We thus recently developed a new assay (Gould et al., 2008) based on propidium iodide (PI), which allows monitoring of a breach of plasma membrane integrity as a proxy for cell death. This assay, based on fluorescence being developed on cellular penetration of PI, is also much more sensitive to small changes and can be

performed in a 96 –well format, allowing the monitoring of many cultures in parallel, under a CO₂ atmosphere. Parasites were exposed to various drug concentrations and the fluorescence was monitored for up to 8 hours in *T. brucei* and 20 hours in *Leishmania* under appropriate culture conditions.

For the most active compounds, T1, M38, G25, and MS1, time-dose responses were investigated. Concentrations of 100, 50, 25, and 12.5 μM T1 killed *T. brucei* BSF in 15, 30, 90, and 180 min respectively (Figure 4.8A), whereas these times were 30, 90, 150, and 270 min respectively for MS1 (Figure 4.8B). M38 and G25 showed trypanocidal activity only at 100 and 50 μM, after 3 and 4 h, respectively (data not shown). Thus it is clear that these drugs only display fast trypanocidal activity at high concentrations, in excess of 100-fold their EC₅₀ values. The lowest concentration tested (6.25 μM) did not affect trypanosome viability for any of the 4 compounds tested.

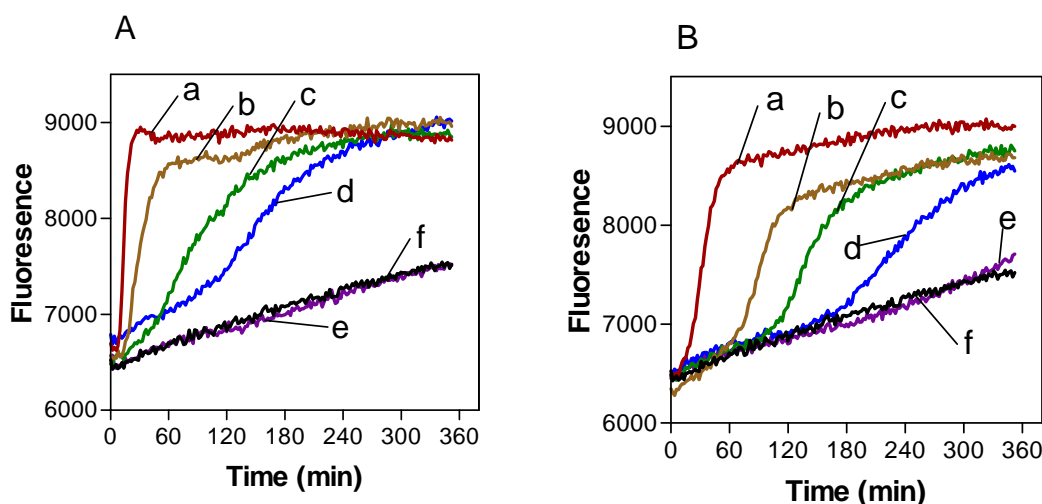


Figure 4.8. Dose response curves of two choline compounds.

Figure shows the effect of T1 (A) and MS1 (B) when incubated with *T. brucei* bloodstream forms. Cells were cultured in HMI-9 medium supplemented with 10% FCS and treated with indicated drugs at different concentrations, 100 μM (a), 50 μM (b), 25 μM (c), 12.5 μM (d), 6.25 μM (e), and drug free (f). Propidium iodide at 9 μM was added and the fluorescence was monitored over time at 544 nm excitation and 620 nm emissions.

The same protocol was also utilized to measure the dose response in promastigotes of *L. major* and *L. mexicana*. The EC₅₀ values listed in Table 3.2 showed that, using the standard Alamar Blue protocol, *L. major* promastigotes were very slightly more sensitive than *L. mexicana* promastigotes. The results depicted in Figure 4.9, however, show that the *L.*

mexicana promastigotes were killed more quickly by T1 and MS1. As with *T. b. brucei*, these compounds rapidly killed the parasites only at relatively high concentrations, considering the EC₅₀ values were between 0.14 and 0.42 μ M (Chapter 3).

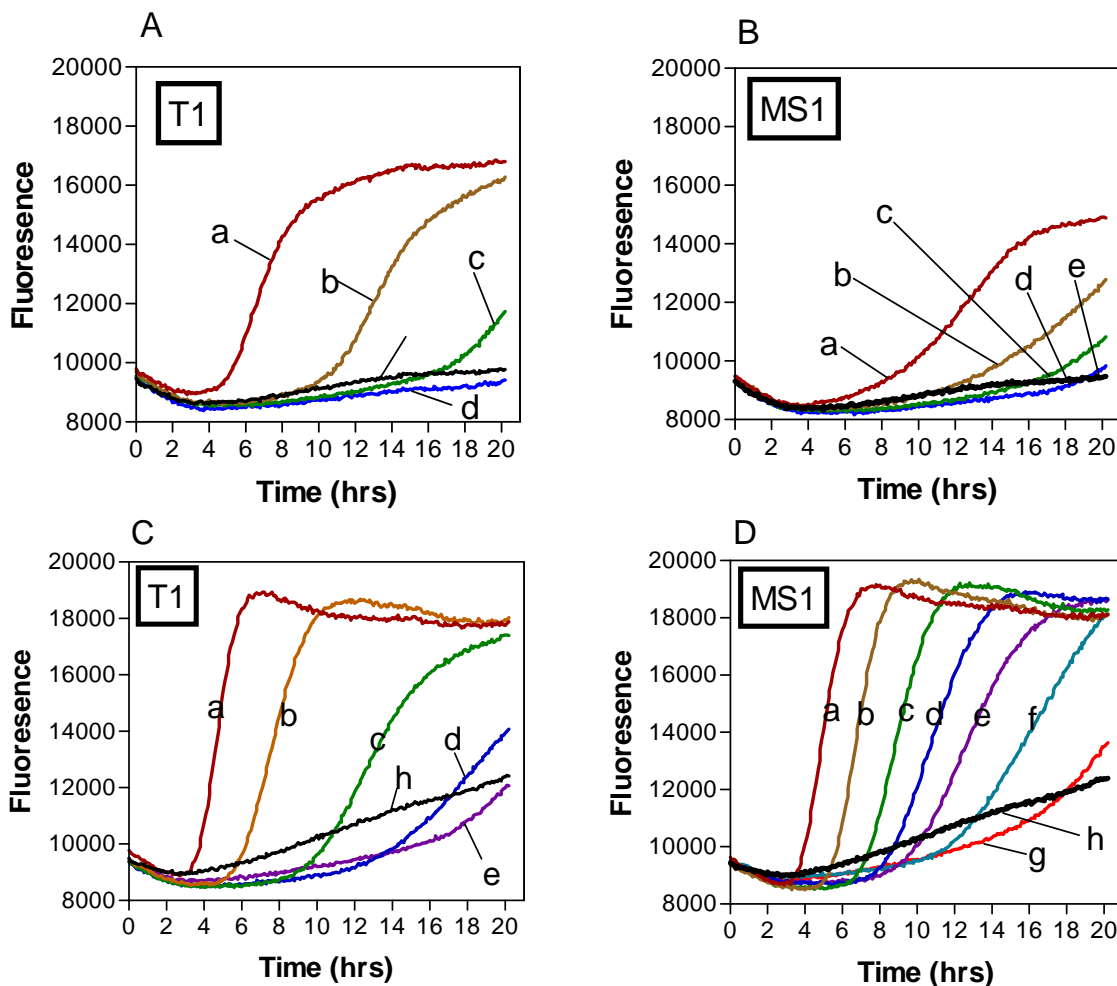


Figure 4.9. Comparison of dose response over time between promastigotes of *L. major* (A,B), and *L. mexicana* (C,D) for two choline analogues: T1 and MS1. Cells were seeded at 5×10^6 cells/ml in HOMEM+10% FCS. The drugs were added at: 100 μ M (a), 50 μ M (b), 25 μ M (c), 12.5 μ M (d), 6.25 μ M (e), 3.125 μ M (f), 1.56 μ M (g), and drug free (h). The final concentration of propidium iodide was 9 μ M.

The compounds M38 and G25 displayed almost no activity against *L. major* promastigotes over the course of the experiment, even at 100 μ M. The same experiment with *L. mexicana* showed approximately 50% lysis after 18 h with 25 μ M M38 or with 12.5 μ M G25 (data not shown).

Complete lysis of parasite cell populations of the choline analogues in the above dose response assays, over the 20 hours of the experiment, occurred generally at over 100-fold the EC₅₀ value. Therefore, these results suggest that incubation with such a high concentration of the choline analogues rapidly kills the trypanosomatid parasites through a different mode of action than incubation at low or sub-micromolar concentrations.

4.3 Effect of choline lead compounds on the cell cycle

We examined the *in vitro* antitrypanosomal effects of choline analogues on the cell cycle, plasma membrane integrity and DNA content of bloodstream forms of *T. b. brucei*.

The permeability of the plasma membrane was assessed in treated and untreated cell cultures at $\sim 2 \times 10^6$ cells/ml. The cells were spun at 2500 x g for 10 minutes at 4 °C, and re-suspended in propidium iodide solution (5 µg/ml). After 10 minutes in the dark and at room temperature, the samples were analysed with FACSCalibur using FL2-Area detector and CellQuest software. Three different concentrations were used for each drug: 1, 2, and 5 µM, and the fluorescence was measured at 4 time points 4, 24, 48, and 72 hours. The positive control, 6 µM digitonin, was used to permeabilize the cell plasma membrane. This assay uses the same principle of propidium iodide fluorescence as described above but allows far longer incubation times as the PI is only added at the termination of the incubation, instead of the cells being continuously exposed to the dye, which can itself affect cell division (Gould *et al.*, 2008). In addition, the flow cytometric analysis also gives a snapshot of the DNA content of the permeable cells, which would show extensive DNA degradation.

Lines I and II of Figure 4.10 show the controls for this assay. Panel I-A shows the drug-free control after 72 hours of incubation, with virtually no fluorescence detectable in the 10,000 cells counted, as the plasma membrane is intact, excluding the PI. Panel II-A shows a sample of the same cell population but treated with digitonin to permeabilise the plasma membrane, generating an intense fluorescence that peaks at 200 units, corresponding to cells with a 'normal' diploid set of chromosomes and a smaller peak at twice the intensity of cells undergoing cell division, with a 2K2N set of chromosomes. The combination of panels I-A and II-A thus shows that at 72 hours the plasma membranes of the control culture were intact and that the cells had a normal distribution of 1K1N and 2K2N cells. This result was repeated for cells exposed to 1% DMSO as the solvent of the choline test

compounds (Panels I-B and II-B), whereas Panels I-C and II-C show that the treatment with 6 μM digitonin does indeed permeabilise control ('healthy') cells.

At a concentration of 5 μM , none of the compounds used in this study (see Chapter 3) had a noticeable effect on plasma membrane permeability after incubation for 4 hours (data not shown), in agreement with the earlier conclusion (section 4.2) that these compounds act slowly on trypanosome viability.

Treatment with 1 μM compound G25 was illustrated in Figure 4.10 (rows III and IV) as an example for the analysis of the flow cytometry data. The cells analysed in row IV were taken from the same cultures as those in row III but treated with 6 μM digitonin to display what the DNA content would look like if the cells were fully permeable. At 24 h (panel III-D), few cells were permeable to propidium iodide and the population consisted mostly of cells in a normal distribution of 1K1N and 2K2N cells (compare panels II-A and IV-D). Panels E show that at 48 h a majority of cells are permeable (small but clear difference between III-E and IV-E) and there is evidence of very extensive degradation of nucleic acids as the majority of cells display a very low level of fluorescence (broad peak at ~ 50 units, corresponding to $\leq 25\%$ of normal nucleic acid content in the cell). At 72 h (panels F), all cells were permeable to PI and extensive DNA damage was virtually universal.

The results for all seven choline analogues have been summarised in Figure 4.11, plotting non-permeable ('viable') cells against incubation time for various concentrations (1 μM , 2 μM and 5 μM) of the test compound. The percentage viable cells was defined as the percentage of 1K1N and 2K2N cells in the digitonin-treated population, minus the percentage of 1K1N + 2K2N cells in the non-digitonised population. In the example of Figure 4.10, 24 h of treatment with 1 μM G25 resulted in $79.8 - 11.5 = 68.3\%$ viable cells, which was reduced to $29.8 - 19.8 = 10\%$ after 48 h and 0% after 72 h, compared to 72.3% for the 1% DMSO control (Panels I-B and II-B).

Compound T3 (Figure 4.11B) showed very little effect on cell permeability at concentrations up to 5 μM – perhaps unsurprising since its EC_{50} value was determined at $\sim 8 \mu\text{M}$ (Chapter 3). The effect of T1 (Figure 4.11A) was also marginal up to 72 h at 1 μM (10.9% of cells permeable) and 2 μM (15.9%), compared to 9.85% for the drug free control. At 5 μM , however, there was a clear and increasing effect on cell viability after 24, 48, and 72 h. Compounds T4 (Figure 4.11C), M38 (Figure 4.11D) and G25 (Figure 4.11E) also had a dose-dependent effect on cell viability. At the lowest concentration of 1

μM , MS1 permeabilised almost all cells within 24 h , reflecting its low EC_{50} value of 0.13 μM . M53 had only a small effect on cell viability after 72 h at 5 μM , i.e. $2\times\text{EC}_{50}$ (Figure 4.11G).

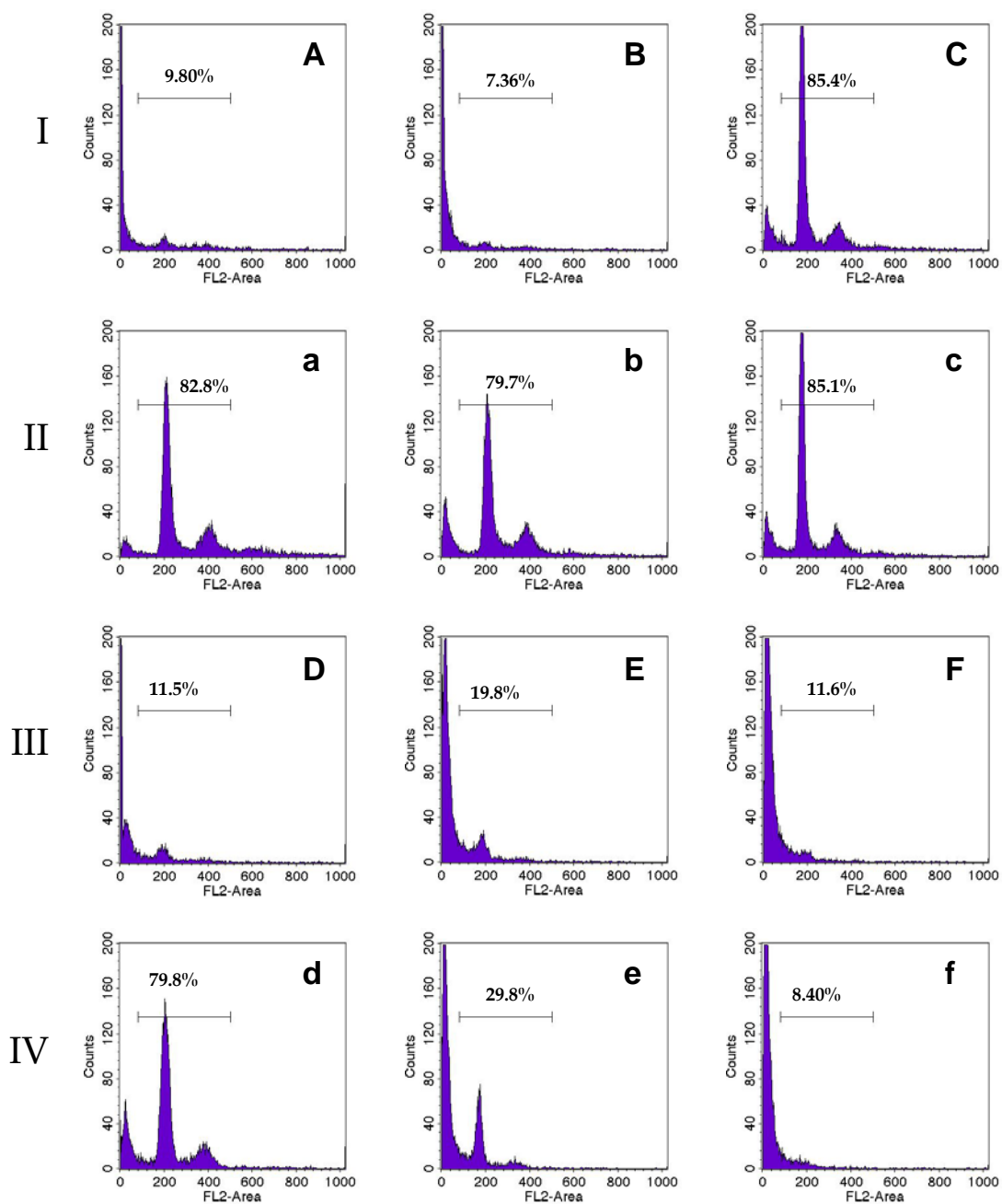


Figure 4.10. Flow cytometry analysis of cellular permeability of *T. brucei* bloodstream form stained with 5 $\mu\text{g}/\text{ml}$ propidium iodide.

(I): controls after 72 h , A, drug free control; B, 1% DMSO (v/v); and C, 6 μM digitonin. **(III):** treated cells with 1 μM G25 at: D, 24 h ; E, 48 h ; and F, 72 h . **(II)** and **(IV):** the same samples in I and III respectively after adding 6 μM digitonin and analyzed after 10 min. 10,000 events were analysed. The data shown are representative of three independent experiments of three different concentrations of seven choline compounds (see appendix III for the equivalent data on the other choline compounds).

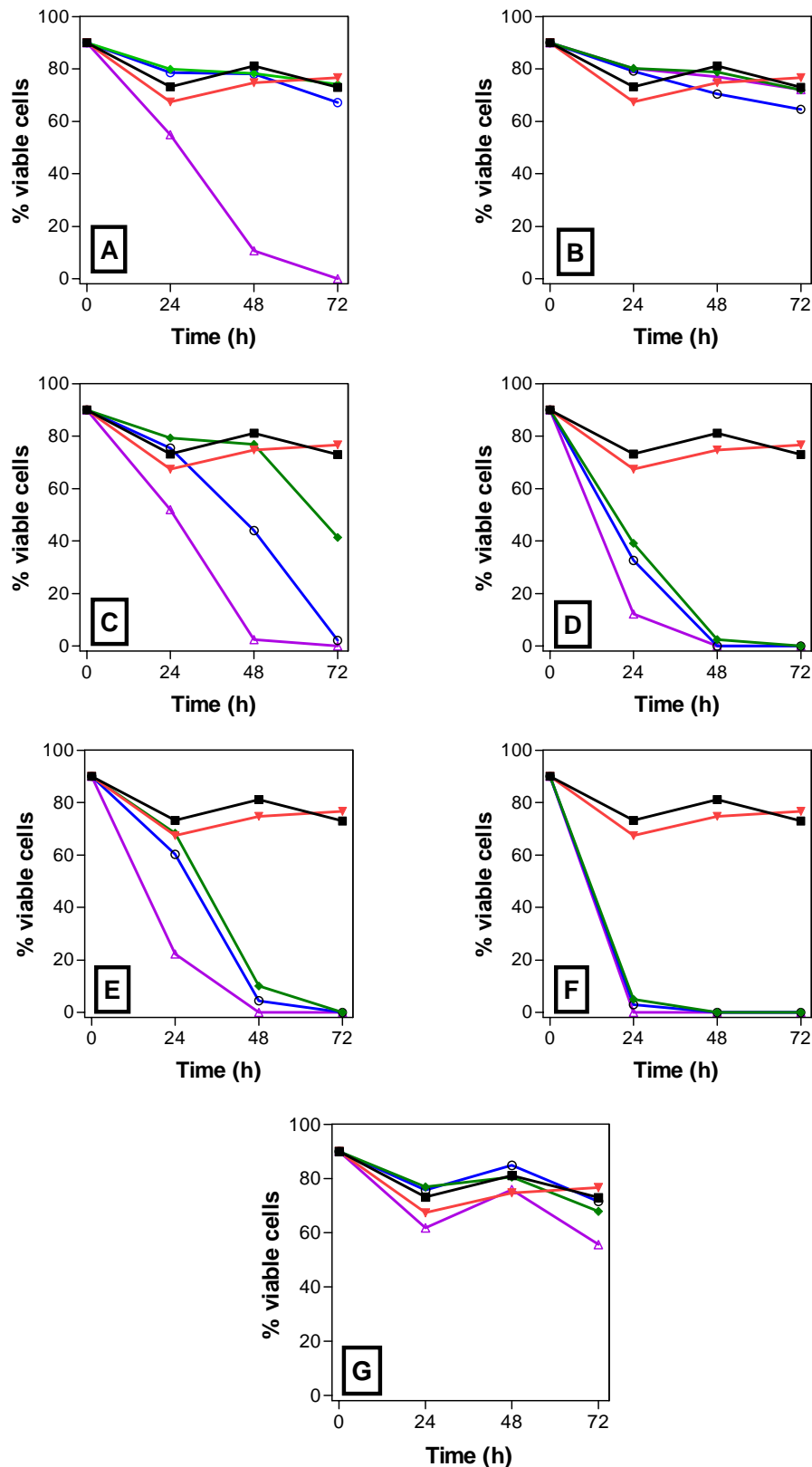


Figure 4.11. Flow cytometry analysis of *T. brucei* treated with choline analogues.

Drug free (■), T1 (A), T3 (B), T4 (C), M38 (D), G25 (E), MS1 (F), and M53 (G). Bloodstream form of *T. brucei* s427 were incubated in presence of 1% DMSO (▼), 1 μM (◆), 2 μM (○), and 5 μM (△). Cells were taken at 24, 48, and 72 h and stained with 5 μg/ml propidium iodide and analyzed by flow cytometry as described in section 2.10.2.

The second step for investigating whether choline analogues have any effect on the cell cycle was to specifically measure the DNA content, with emphasis on the ratio between 1K1N and 2K2N cells as well as the appearance of cells with higher chromosome copy numbers. For this method, untreated and treated cells with 5 μM , 7.5 μM , and 10 μM of choline test compound were lysed at 8 and 24 h in 70% methanol, 30% PBS and kept in the fridge till analysis. Cells were then stained with 10 $\mu\text{g/ml}$ Propidium iodide solution containing RNase at 10 $\mu\text{g/ml}$, and incubated in dark at 37 $^{\circ}\text{C}$ for 45 minutes before analyzing the DNA content by flow cytometry. The results, summarized in Table 4.1, indicated there was no clear effect of any of the tested compounds on the amount of DNA in the cells. Moreover, the proportion of cells in G1 and G2/M phases did not change over a 24 h incubation time, with gated cells 60-70% in G1 and 20-30% in G2/M phases. As shown in Figure 4.12, an example figure, there were no significant differences between control and treated cells with T1 at any concentration, at up to 24 h. The results of the highest concentration (10 μM) showed that the ratio of trypanosomes in G1 or G2 phase did not change during the incubation time. Furthermore, none of the 24 h incubations with choline analogues caused a significant proportion of cells that had accumulated more than 2K2N amount of DNA.

Zoids, cells that had <1K1N DNA content, were excluded from the calculation of the 1K1N /2K2N ratios. Yet it is clear from Figure 4.12 that the proportion of cells in the zoids category increased with incubation time and/or concentration of the test compound. In our opinion, this phenomenon may represent dead or terminally damaged cells with extensive DNA degradation but a sufficiently intact cellular structure to be counted in the flow cytometer. DNA damage was also particularly pronounced after 24 h incubation with 5 or 7.5 μM M38, but not with any of the other choline compounds (see Figure 4.13 and appendix III). Similar DNA degradation has been observed after treatment of *T. b. brucei* with specific prostaglandins (Figarella *et al*, 2006).

This raises the possibility that some of the choline analogues cause DNA degradation and/or fragmentation prior to cell death. Further investigations will be required to establish whether this is part of the mechanism of action or a downstream effect of the primary cellular effect of these compounds.

From Table 4.1 it can be noted that choline compounds did not have a specific effect on the cell cycle and did not cause cell cycle arrest - there is no clear accumulation of cells in a particular stage of the cell cycle.

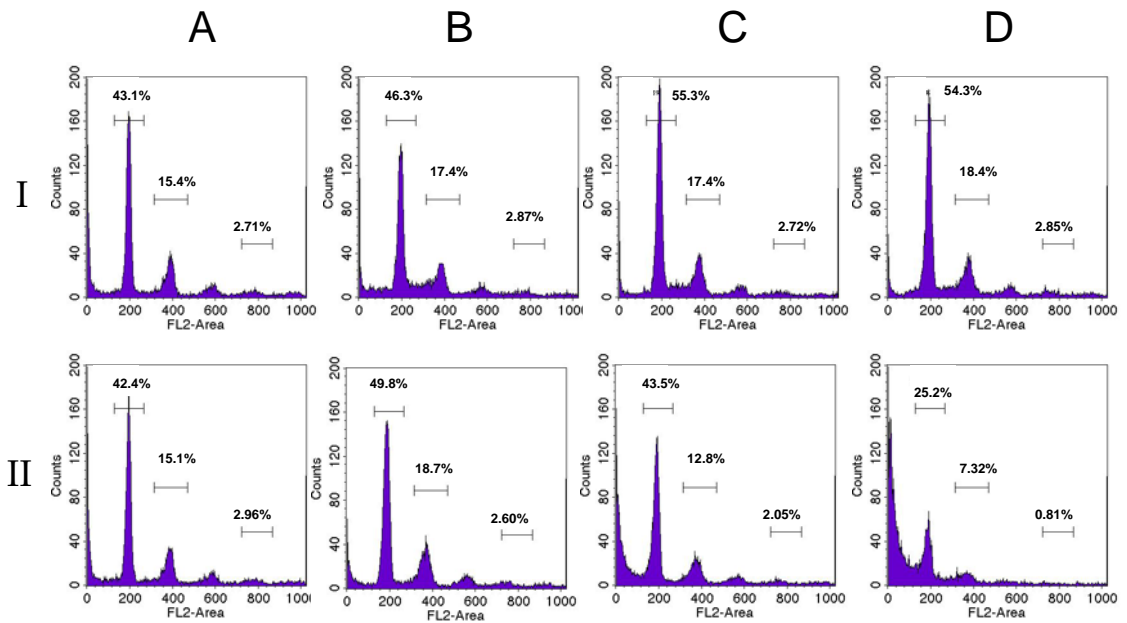


Figure 4.12. Analysis of DNA content of *T. brucei* bloodstream forms incubated with compound T1.

Cells were cultured in HMI-9 medium containing (A) no drug; (B) 5 μ M, (C) 7.5 μ M; and (D) 10 μ M, and samples were withdrawn after (I) 8 h and (II) 24 h and analyzed by flow cytometry as described in section 2.10.1. 10,000 events were analyzed and the percentage of cells in each cell cycle phase was determined using CELLQuest software.

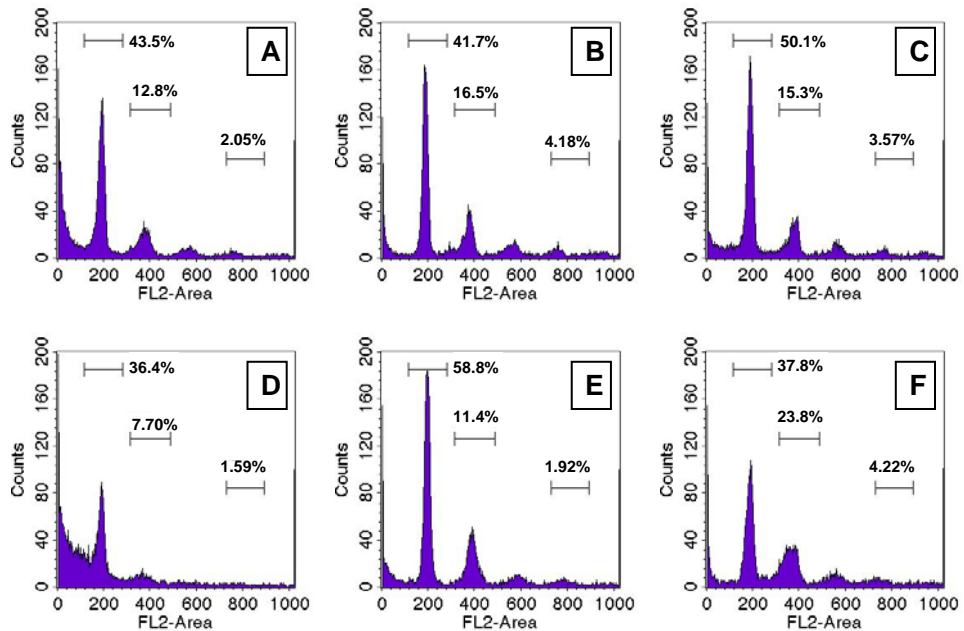


Figure 4.13. DNA degradation in *T. brucei* bloodstream forms incubated for 24 h with 7.5 μ M choline compounds. T1 (A); T3 (B); T4 (C); M38 (D); G25 (E) and M53 (F).

Table 4.1 FACS results of DNA Percentages of *T. brucei* bloodstream forms in presence and absence of three different concentrations of choline analogues after 8 h and 24 h.

ND: not determined because too few cells with intact DNA were observed.

compounds		8 h			24 h		
		1K1N (%)	2K2N (%)	4K4N (%)	1K1N (%)	2K2N (%)	4K4N (%)
Drug free		70.4	25.2	4.4	70.1	25.0	4.9
T3	5 μM	61.0	31.7	7.3	70.9	23.7	5.4
	7.5 μM	64.2	29.8	6.1	66.8	26.5	6.7
	10 μM	63.3	30.8	5.9	65.8	26.9	7.3
T4	5 μM	63.2	29.9	6.9	73.9	21.7	4.5
	7.5 μM	60.4	31.6	7.9	72.6	22.2	5.2
	10 μM	72.2	24.3	3.5	74.1	21.1	4.8
M38	5 μM	76.0	20.2	3.8	83.5	14.4	2.1
	7.5 μM	77.3	19.0	3.7	79.7	16.9	3.5
	10 μM	76.0	20.4	3.6	ND	ND	ND
G25	5 μM	76.4	20.0	3.6	82.5	15.4	2.1
	7.5 μM	77.7	18.7	3.6	81.5	15.8	2.7
	10 μM	75.8	19.6	4.6	ND	ND	ND
MS1	5 μM	75.6	21.1	3.3	ND	ND	ND
	7.5 μM	75.3	21.3	3.5	ND	ND	ND
	10 μM	ND	ND	ND	ND	ND	ND
M53	5 μM	60.3	32.5	7.3	64.0	29.6	6.4
	7.5 μM	65.6	28.3	6.1	57.4	36.2	6.4
	10 μM	76.0	20.6	3.4	60.1	33.5	6.4

4.4 Some choline compounds reduce the mitochondrial membrane potential

To determine the changes of the mitochondrial membrane potential Ψ_m , either as an apoptosis marker or as indicator of mitochondrial targeting, bloodstream forms of *T. brucei* were cultivated for up to 24 h in the presence and absence of 2 or 5 μ M choline compounds. The samples were stained with 25 nM tetramethylrhodamine (TMRE) for 30 min at 37 °C and analyzed by flow cytometry. Valinomycin was used as control for mitochondrial membrane depolarisation and troglitazone as a control for mitochondrial membrane hyperpolarisation as described for *T. b. brucei* by Denninger *et al.* (2007). Figure 4.14 depicts histograms of TMRE fluorescence with panels I-A and II-A displaying the control cells, incubated without test compound for 8 h and 16 h, respectively. Values are given as the percentage of cells with fluorescence above 100 arbitrary units, which was

approximately 50% for the controls. A shift to higher fluorescence signifies an increased Ψ_m , whereas a shift to lower fluorescence signifies a depolarization of the mitochondrial membrane (Denninger *et al*, 2007).

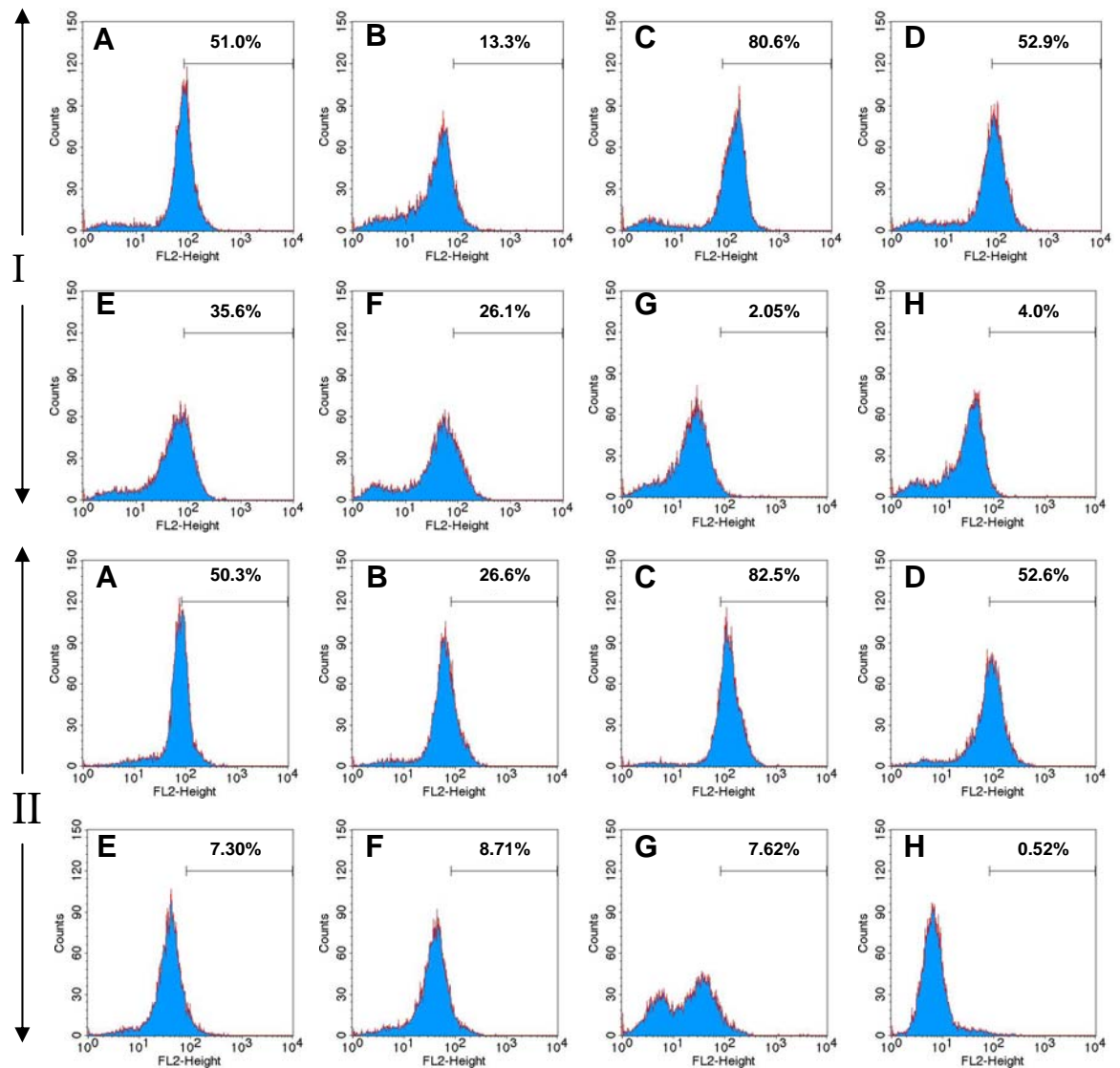


Figure 4.14. Effect of choline analogues on mitochondrial membrane potential as measured by flow cytometric analysis of *T. brucei* bloodstream forms stained with 25 nM of tetra-methylrhodamine (TMRE).

Control (A) and treated cells: (D) 5 μ M T1; (E) 5 μ M T4; (F) 2 μ M M38; (G) 5 μ M G25 and (H) 2 μ M MS1, were harvested at (I) 8 and (II) 16 h and prepared for mitochondrial membrane potential analysis as described in chapter 2. The positive controls were performed with 100 nM valinomycin (B) and 10 μ M troglitazone (C) for decreasing and increasing the mitochondrial membrane potential, respectively.

Compared with the drug free control at 8 h (51.0%) and at 16 h (50.3%), the results showed that there was no effect on mitochondrial membrane potential with 5 μM of compounds T1, T3 and M53 for up to 24 h (Figure 4.15). At 8 h the percentages of these compounds were 52.9, 49.8 and 52.3%, whereas, at 16 h they were 52.6, 50.4 and 51.6% respectively. In contrast, 5 μM T4, 2 μM M38, 5 μM G25, and 2 μM MS1 caused a clear decrease in mitochondrial membrane potential occurred within 8 h cultivation (Figures 4.14 and 4.15). These compounds clearly decreased Ψ_m , showing a reduction of cells with fluorescence >100 AU, from 50% at $t = 0$ h to 35.6, 26.1, 2.1, and 4.0%, respectively at 8 h. The mitochondrial membrane was completely depolarised after 16 h in T4 (7.30%) and M38 (8.71%). Cells incubated with G25 for 16 h displayed two fluorescence peaks, the one at approximately 150AU representing cells with a highly depolarised mitochondrial membrane and those displaying fluorescence below 100 AU possibly represented dead cells. This interpretation means that 16 h of incubation with compound MS1 leaves all cells dead, and this is in agreement with results presented above (e.g. Figure 4.11).

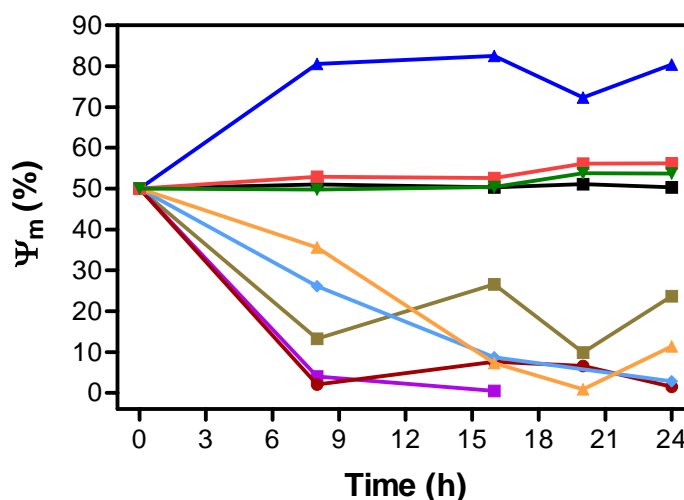


Figure 4.15. Effect of choline compounds on the mitochondrial membrane potential of *T. brucei* bloodstream forms as measured by fluorescence of TMRE.

(■) 5 μM T1; (▼) 5 μM T3; (▲) 5 μM T4; (◆) 2 μM M38; (●) 5 μM G25; (■) 2 μM MS1; (■) 100 nM valinomycin; (▲) 10 μM troglitazone and (■) drug free control.

4.5 Effects of choline analogues on the generation of Reactive Oxygen Species (ROS) in *T. b. brucei*.

In order to assess the production of reactive oxygen species (ROS) in *T. brucei* bloodstream forms, the ROS-sensitive fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DH) (Figarella *et al*, 2006), was used to measure the increase of fluorescence using fluorimetry at an excitation wavelength of 485 nm and 520 nm for emissions. Cells in the stationary phase at 2×10^6 cells/ml in PBS were seeded in 96 well plates and exposed to serial doubling dilution of choline compounds. The dye was then directly added to all the plate wells at 10 μ M as a final concentration and the fluorescence signals was measured over time. Four wells were included at each plate as controls: (1) 2×10^6 cells/ml in PBS without test compound, (2) 2×10^6 cells/ml in PBS incubated with 100 μ M hydrogen peroxide, (3) PBS without cells or test compound and (4) 100 μ M H_2O_2 in PBS without cells.

The results show that trypanosomes in culture generate substantial amounts of reactive oxygen species over time, which is greatly enhanced in the presence of H_2O_2 . The rate at which ROS were generated was dose-dependently reduced by incubation with T1 and MS1 (Figure 4.16 A, B). The same trends continued for up to 12 h (data not shown), with the no drug control showing a higher production of ROS than cells incubated with MS1 or T1.

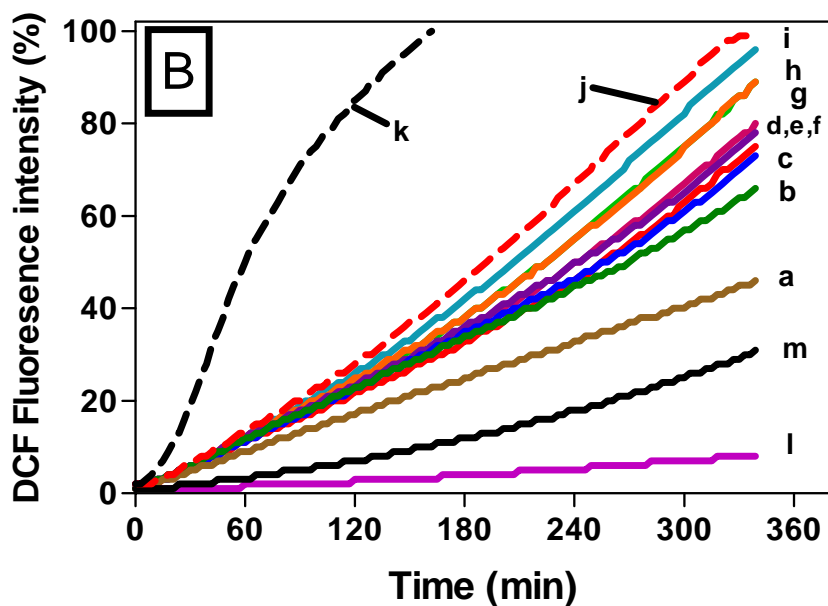
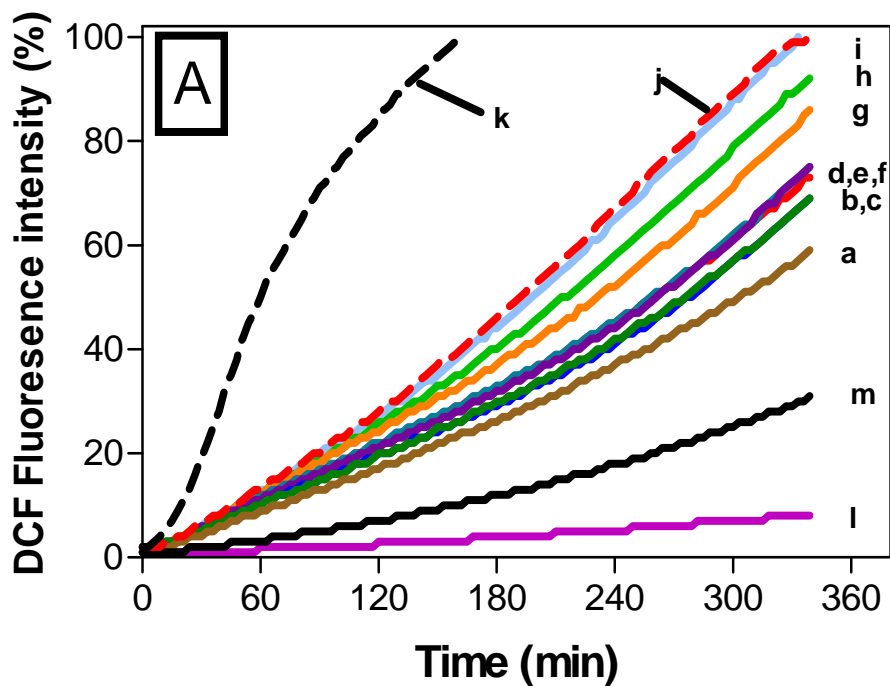


Figure 4.16. Effect of serial dilutions of choline analogues on reactive oxygen species production in bloodstream forms of *T. brucei*.

Cells at 2×10^6 cells per ml PBS were cultured in 96 well plate in presence and absence of H_2O_2 and some choline compounds, T1 (A) and MS1 (B), and the DCFH-DH was added. Drug dilutions were at 50 μM (a); 25 μM (b); 12.5 μM (c); 6.25 μM (d); 3.13 μM (e); 1.56 μM (f); 0.78 μM (g); 0.39 μM (h) and 0.195 μM (i). Hydrogen peroxide at 100 μM (k), drug free cells (j), H_2O_2 without cells (m) and PBS without cells (l) were used as controls. This experiment is representative of two identical experiments performed independently.

4.6 Effects of choline analogues on cellular lipid content.

The ES-MS of the lipid extracts of the untreated cells, and cells treated with choline analogues for 8 h was employed to characterise the major *T. brucei* phospholipid classes and their molecular species from total lipid extracts using nano-electrospray tandem mass spectrometry (ESI-MS). Cymelarsan was used as a control trypanocidal compound that does not act through the lipid metabolism (Figure 4.17F).

The positive ion survey spectrum (300-1000m/z) of wild-type cells (Figure 4-17A) shows multiple molecular species between 650-950m/z, as a result of multiple different fatty acid side chains for the phospholipids. The corresponding positive ion survey spectra of the cells treated with the test compounds T1, M38, G25 and MS1 (Figure 4-17B-E, respectively) initially look different from the untreated cells (Fig 4-17A). However most of these differences can be attributed to the presence of molecular species in the lipid extract samples, which ionise better than the phospholipids, resulting in the suppression of the phospholipid ionisation/signal.

This is particularly true for Figure 4-17B, where the positively charged molecular ion of T1 can clearly be observed at 312m/z, Figure 4.17C, whereas the positively charged molecular ion of M38 can clearly be observed at 434m/z, and Figure 4.17D, where the positively charged molecular ion of G25 can be observed at 395m/z, very close to the background non-related ion at 393m/z (observed in all samples). No positively charged ions are observed for MS1 and cymelarsan (Fig 4.17E and F respectively).

As the choline analogues were suspected to possibly interfere with metabolism of choline containing phospholipids, i.e. phosphatidylcholine and sphingomyelin, these were analysed. A positive ion precursor scan for m/z 184 (phosphocholine) detects all PC and SM $[M+H]^+$ ions, and their natural isotopes, each represented as a m/z signal in a spectrum. Colliding ions from the monoisotopic signals produces specific daughter ion fragments, which were used to assign the m/z signal to a molecular species that contains a certain number of FA carbons and associated degree of unsaturation. There are numerous PC and SM molecular species in total lipid extracts of bloodstream form wild type *T. brucei* (Fig. 4.18A). Annotation of these species is provided in a separate table (Table 4.2).

The composition of the choline containing lipid species in untreated cells and those treated with potential inhibitors were remarkably similar indicating that there are no major defects

in choline phospholipid metabolism upon treatment either the various potential inhibitors at their respective concentrations (compare Fig. 4.18A, with frames B to F).

Negative ion survey spectra (600-1000m/z) of wild-type cells and cells treated with the potential inhibitors were compared, but no significant differences were observed (data not shown). ES-MS-MS analysis of all of the other major phospholipids classes, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and phosphatidylglycerol were conducted in both untreated and treated cells, but no major differences were observed (data not shown). These mass spectrometry analyses suggest that phospholipids metabolism is not the target of the anti-trypanocidal activity observed by these compounds.

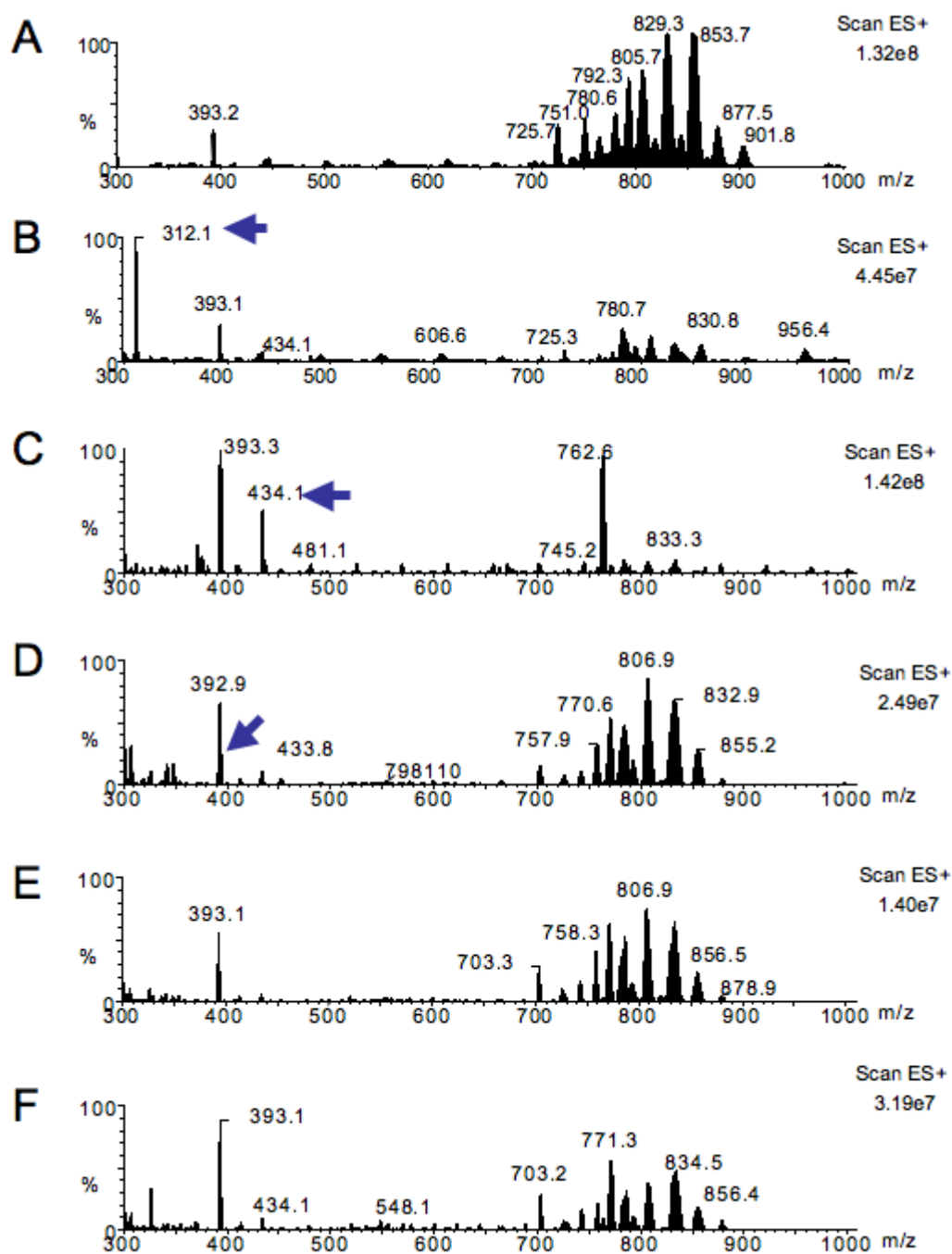


Figure 4.17. Positive ES-MS survey scans of lipid fraction of untreated and treated *T. b. brucei*. Lipids extracted from untreated *T. b. brucei* (A) or treated with 7.5 μM T1 (B), 10 μM M38 (C), 10 μM G25 (D), 5 μM MS1 (E) or 0.05 μM Cymelarsan (F). The samples were analysed by positive ion mode ES-MS (300-1000 m/z) as described in section 2.12. The arrows indicate the presence of the potential inhibitor that has been co-extracted with the lipids.

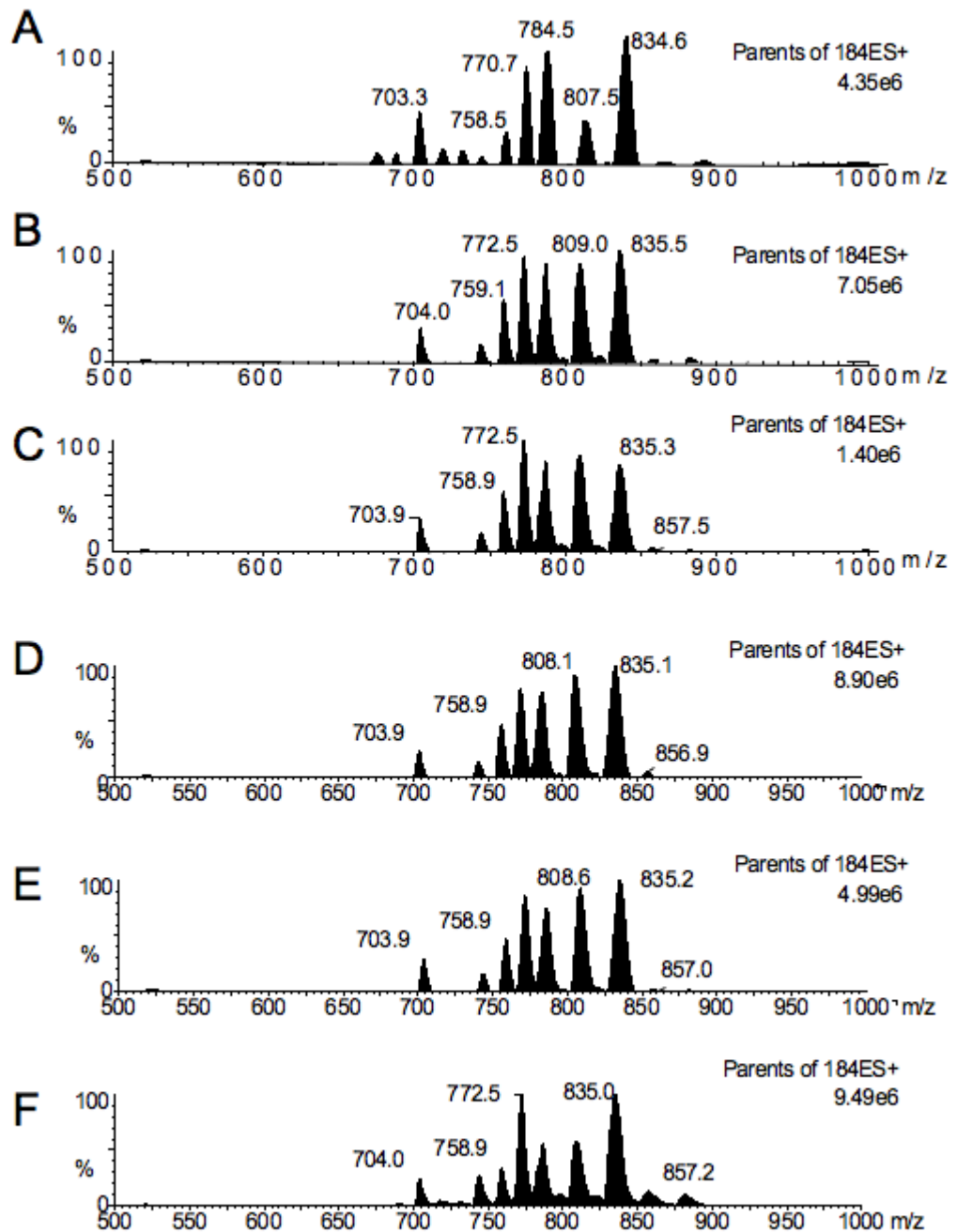


Figure 4.18. ES-MS-MS scans of untreated and treated *T. b. brucei*. Lipids extracted from *T. brucei* wild type (A) or treated with 7.5 μ M T1 (B), 10 μ M M38 (C), 10 μ M G25 (D), 5 μ M MS1 (E) or 0.05 μ M Cymelarsan (F). The samples were analysed by parent ion scanning of 184m/z ES-MS-MS for Gro-P-Cho and SM in positive ion mode (600-1000 m/z), parent-ion scanning of m/z 184 as described in section 2.12.

Table 4.2. Composition of Choline-containing phospholipids in extracts of bloodstream *T. b. brucei* not treated with test compounds.

	Peak ^b	%	PC Series ^c	m/z ^d range	Principal component ^e	m/z ^d
	-	0.3	lysoPC	520-572	see Fig. 4.18	
di- acyl	A	0.2	28:2 - 28:0	674-678	28:1 (14:0/14:1)	676
	C	1.3	30:4 - 30:0 and SM(16:0)	698-706	SM(16:0)	703
	E	1.2	32:3 - 32:0 and SM(18:0)	728-734	SM(18:0)	731
	G	4.8	34:4 - 34:0 and SM(20:0)	754-762	SM(20:0)	759
	I	19.1	36:6 - 36:0 and SM(22:0)	778-790	36:2 (18:0/18:2) (18:1/18:1)	786
	K	13.6	38:6 - 38:0	806-818	38:4 (18:0/20:4) (16:0/22:4)	810
	M	34.4	40:8 - 40:0	830-846	40:5 (18:0/22:5)	836
	N	3.8	42:11 - 42:0 a/o e-42:3 - a-42:0	852-874	42:8 a/o e-42:0 a/o a-42:1	858
	O	3.0	44:12 - 44:0 a/o e-44:4 - a-44:0	878-902	44:9 a/o e-44:1 a/o a-44:2	884
ether	B	0.2	e-30:4 - a-30:0	682-692	e-30:0 a/o a-30:1	690
	D	0.4	e-32:2 - a-32:0	714-720	e-32:0 a/o a-32:1	718
	F	1.8	e-34:4 - a-34:0	738-748	e-34:1 a/o a-34:2	744
	H	13.0	e-36:4 - a-36:0	766-776	e-36:1 a/o a-36:2 (a-18:0/18:2)	772
	J	1.4	e-38:6 - a-38:0	790-804	e-38:2 a/o a-38:3	798
	L	1.6	e-40:6 - a-40:2	818-828	e-40:4 a/o a-40:5	822

^a Precursor scanning for the m/z 184 ion detects PC and sphingomyelin (SM).

^b Peak identities refer to the spectrum peaks in Fig. Y. The peak series containing the most abundant Gro-P-Cho species (>10% of total Gro-P-Cho) are in bold.

^c All of the molecular species detected within the area of the peak are contained within the lowest and highest mass range outliers listed, the degree of unsaturation decreases by one from the lowest mass in each series until the highest mass is met. e = plasmenyl (alkenylacyl); a = plasmany (alkylacyl); a/o = and/or

^d $[M+H]^+$ ions over charge.

^e Defined here as the $[M+H]^+$ ion within the series with the greatest intensity. Precise fatty acyl constituents and their positions are not discernable in positive ion mode; however, where possible, the most likely fatty acid candidates (in parentheses) for the *sn*-1 and *sn*-2 constituents of the molecular species presented were deduced from the available literature.

PC^a Composition in bloodstream *T. brucei*

4.7 Discussion

The effect of choline compounds on cellular growth of *T. brucei* bloodstream forms was reported in this study under *in vitro* culture conditions.

Various techniques were employed and indeed developed to assess the effects of the choline analogues on trypanosome growth, viability, cell cycle and proliferation. It was clear that all these compounds act rapidly to inhibit trypanosome proliferation at concentrations as low as 3x EC₅₀, and gradually reduce the actual cell density in a population over a number of hours. The rate at which a population declines is clearly concentration dependent and varies (depending on the choline analogue and concentration) from a few hours to few days)

The outcomes of the cell counts, spectrophotometric lysis assays and PI fluorescence were all in good agreement, showing that at concentrations of 10 or 20 µM, MS1 induces cell death within hours (e.g. figures 4.3B, 4.4 and 4.7), although cell death is variously defined as a decline in trypanosome population, reduced light scatter and breach of plasma membrane. Compound T1 displayed similar effects at this concentration (e.g. figures 4.3B, 4.5) but whereas 20 µM constitutes 150xEC₅₀ for MS1, it represents only 11xEC₅₀ for T1. The EC₅₀ values were determined using the Alamar Blue assay over 72 h. It would therefore appear that T1 is the quickest-acting compound of the test series and it is possible to speculate that this is related to its structure as monocation whereas the other test compounds are symmetrical di-cations at neutral pH, with two quaternary ammonium head groups connected by a long flexible linker (see figure 3.1). This structural difference gives rise to a hypothesis that the action of T1 is faster than for the di-cationic analogues, possibly reflecting a difference in uptake rates.

Transport of the choline analogues was briefly addressed in Chapter 3. In *Leishmania* species and *Plasmodium* (Lehane *et al*, 2004; Zufferey & Mamoun, 2002), these or similar choline analogues are potent inhibitors of choline transporters, but no such transporters are expressed by bloodstream *T. b. brucei* and our studies have not yet identified a definitive route of uptake for this class of compounds. It was concluded that the HAPT and LAPT transporters were unlikely to play a significant role in their uptake at pharmacologically relevant concentrations, because the IC₅₀ values were very high. The observation that up to 100 µM pentamidine was unable to delay cell lysis induced by T1 and MS1 (Figs 4.5 and 4.6) shows that at least HAPT and TbAT1/P2 do not mediate (most of) the uptake these compounds, as both are fully inhibited by even 1 µM of pentamidine (De Koning, 2001b;

Matovu *et al.*, 2003). Indeed the low affinity transporter LAPT would also be mostly inhibited by 100 μM pentamidine, as it displays a K_m value of 56 μM (De Koning, 2001b). A full inhibition of MS1-induced trypanosome lysis was observed in the presence of 1 mM pentamidine (figure 4.6) but at this concentration pentamidine blocks many processes at the plasma membrane (De Koning, unpublished observation) and this action cannot be considered specific without further investigation.

The conclusion that the choline analogues act slowly on trypanosomes prompted the important question whether a population of trypanosomes needs to be constantly exposed to high concentrations of these potential drugs until all parasites are dead. Considering the evidence presented in Figure 4.7 this does seem to be the case: washing 20 μM of either T1 or MS1 out after a 2-hour incubation (i.e. almost up to the time where the first cells would start to die) completely prevented subsequent cell death. This seems to suggest that the compounds are not, like pentamidine, rapidly accumulated in the cell and subsequently kill the cell by a slow but irreversible process (Delespaux & De Koning, 2007). Rather, it suggests that uptake is indeed slow, and probably equilibrative, which means that the compounds will leave the cells as soon as the extracellular concentration becomes lower than the intracellular concentration.

While the diamidines are known to induce cell death in trypanosomes after an exposure time measured in days (e. g. Gould *et al.*, 2008) the melamine arsenical drugs, such as melarsoprol and cymelarsan, as well as simple aromatic arsenicals such as phenyl arsine oxide (PAO) rapidly kill trypanosomes and lyse the cells as can be easily monitored by any of the assays employed in the current study (e.g. Carter and Fairlamb, 1993; Matovu *et al.*, 2003; Gould *et al.*, 2008). However, we found no evidence, using flow cytometry with propidium iodide, that the choline analogues used in this study cause plasma membrane defects as a cause of cell death: even when a large proportion of trypanosomes had died after exposure to 1 μM G25, the remaining cells were non-permeable to propidium iodide (compare figure 4.10, frames III-E and IV-E).

Monitoring the DNA content with flow cytometry did reveal that M38, and possibly T1, appear to induce extensive DNA fragmentation (Figure 4.13), probably before the integrity of the cell is compromised. DNA degradation has been linked with programmed cell death in many species but it is unclear whether the observation reported here should be categorised as evidence for apoptosis. Another marker for apoptosis would be a depolarisation of the mitochondrial membrane potential Ψ_m . Several choline compounds,

including M38, G25, T4 and MS1, but not T1, did have very pronounced effects on Ψ_m as early as 8 h and at concentrations that do not affect cell viability so quickly. Rather than indicating the induction of apoptosis as a mechanism of action for these compounds, we believe this suggests that the mitochondria may be the main target of these compounds, especially the dicationic ones. This would not be entirely surprising as the dicationic diamidines are also known to predominantly accumulate in the mitochondria, driven by the Ψ_m . While the mechanism of action of diamidines on trypanosomes has never been satisfactorily defined and may be multi-factorial, it is believed that the mitochondria are the main target for this class of drugs (Lanteri *et al*, 2008). For instance, pentamidine resistance in *L. donovani* has been linked to exclusion of pentamidine from the mitochondrion (Basselin *et al*, 2002; Mukherjee *et al*, 2006). Indeed, fluorescent diamidines can be detected within the *T. b. brucei* mitochondria within one minute, but this fluorescence is much-delayed in diamidine-resistant trypanosomes (Stewart *et al*, 2005).

The fact that all the dicationic choline analogues tested rapidly depolarised the mitochondrial membrane, but only one of these compounds (M38) affected DNA integrity, leads to the conclusion that the choline compounds, in general, do not induce apoptosis in trypanosomes, as reported for some prostaglandins, which caused both mitochondrial membrane depolarisation and DNA degradation (Figarella *et al*, 2005; Figarella *et al*, 2006). In addition, the programmed cell death (PCD) induced by the prostaglandins was found to be associated with the production of reactive oxygen species (Figarella *et al*, 2006) as was PCD in *Trypanosoma cruzi* (Piacenza *et al*, 2007). However, we found that the choline lipid analogues actually reduced the production of ROS.

Finally, it has been shown that some of these choline analogues disrupt the biosynthesis of choline-containing phospholipids in *Plasmodium falciparum* and *Saccharomyces cerevisiae* (Roggero *et al*, 2004). The analysis of lipid content in section 4.6, clearly show that no change of lipid content is evident in the membrane of *T. b. brucei*. For this experiment we used combinations of incubation times and test compound concentration that would take the trypanosome population close to the onset of cell death, so as to maximise our chances to observe any alterations to the lipid composition and we must conclude that none of the choline analogues tested acts through disruption of lipid metabolism. The straightforward conclusions thus that these choline analogues do not inhibit or alter choline metabolism in *T. b. brucei*. Yet, it may not be possible to exclude alternative explanations altogether at this point. As the choline analogues rapidly halt proliferation, this in itself could prevent further lipid metabolism and the alteration of lipid

composition in the plasma membrane. Indeed, it could be argued that the compounds inhibit proliferation as a result of inhibiting lipid metabolism. Further investigations may be required to arrive at a definitive answer.

In conclusion, it appears that the dicationic choline lipid analogues, like the structurally related dicationic diamidines pentamidine, DB75 and diminazene (Lanteri *et al*, 2008), collapse the mitochondrial membrane potential but do not induce PCD, and are thus highly likely to act on an intra-mitochondrial target, although this in itself does not yet prove that the mitochondrion is the pharmacologically relevant target for this class of drugs. It is not unlikely that these compounds interfere with mitochondrial fatty acid metabolism and thus the lipid composition of the mitochondrial rather than plasma membrane, as mitochondrial fatty acid metabolism was recently shown to be essential for normal mitochondrial function, including proper maintenance of Ψ_m (Guler *et al*, 2008). For now, the exact nature of this target remains to be identified and will be the subject of further investigation.

Chapter five

5 Analogues of the natural spice curcumin as antiprotozoal agents.

5.1 Introduction

Human African trypanosomes and leishmaniasis are two of the most important world wide diseases caused by protozoan parasites. All the drugs used currently to treat these diseases exhibit many side effects. Curcumin, a natural yellow pigment extracted from *curcuma longa* rhizomes, has been traditionally used to treat a large number of infectious and non-infectious diseases (see section 1.4), but is safe enough to be used as a natural food additive and spice.

Curcumin has been reported to display ED₅₀ values between 7.8 μ M (Rasmussen *et al*, 2000c) and 46.5 μ M (Koide *et al*, 2002) on the growth of promastigotes of *L. major* *in vitro*. Several derivatives of curcumin including diarylheptanoids; diarylpentanoids; 1,7-bis-(4-propargyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione and 1,7-bis-(2-hydroxy-4-methoxyphenyl)-1,6-heptadiene-3,5-dione were tested against *L. amazonensis* promastigotes and found to have better activity than the original curcumin (Araujo *et al*, 1999; Gomes *et al*, 2002a; Gomes *et al*, 2002b). Furthermore, the first two derivatives had a very good anti-leishmanial activity against *L. braziliensis* and *L. chagasi* promastigotes, and against *L. amazonensis* promastigotes and amastigotes (Alves *et al*, 2003). Curcumin also displayed an average IC₅₀ of 5.3 μ M against promastigotes of three species of *Leishmania*, *L. major*; *L. tropica* and *L. infantum*, and was observed to have better efficacy than pentamidine against *L. major* amastigotes (Saleheen *et al*, 2002).

In the light of the promising antileishmanial effects of the curcuminoides, it is surprising that no effort to systematically screen curcumin and its analogues for their anti-trypanosomal activity has been undertaken. Only one study has tested curcumin on African trypanosomes (Nose *et al*, 1998). In this study, curcumin was reported to show potent *in-vitro* activity against *Trypanosoma brucei brucei* with IC₅₀ values of 4.77 μ M for bloodstream forms and 46.5 μ M for procyclic forms.

In this chapter we describe the synthesis and anti-parasitic screening of a series of curcuminoid analogues. Compared to the parent compound, curcumin (AS-HK01), many of the new compounds displayed a much-enhanced activity against kinetoplastid parasites that cause human and veterinary disease, while displaying limited if any toxicity against a human cell line. In addition, we investigated the effect of some of more potent analogues on freshly isolated murine hepatocytes. We propose that analogues of curcuminoids are

promising new lead compounds against leishmaniasis and African trypanosomiasis that warrant further investigation.

The hepatocytes were used in this study as a model system to assess early cytotoxic effects of the curcuminoids or their metabolites including hepatocytes viability and glutathione (GSH) depletion. There are many reasons for choosing this kind of cells. The first reason is that the liver is the primary source of GSH, and hepatocytes express several enzymes which are utilized in GSH metabolism (Kretzschmar, 1996). Another reason is that hepatocytes are more resistant to GSH depletion than the target cells due to their high metabolic activity (Freidig *et al*, 2001). GSH is considered to play an important role in the cell living by protecting the cells from oxidative damage caused by reactive oxygen species, producing coenzymes and maintaining the oxidation status of intracellular thiols (Amir *et al*, 1998; Lu, 1999). It is a tripeptide consisting of glutamate, glycine and cysteine (Figure 5.1) found in all mammalian and prokaryotic cells (Anderson, 1998).

The finding that none of the curcuminoids affected hepatocytes viability or protein content, while only some of them affected GSH levels, is only a very preliminary indication that safe curcuminoid antiprotozoals may be feasible, and needs a much more extensive toxicological evaluation of selected lead compounds that goes well beyond the aims of the current study. However, as almost no information on the safety and metabolism of curcuminoids exists our preliminary experiments at least have not identified significant safety concerns.

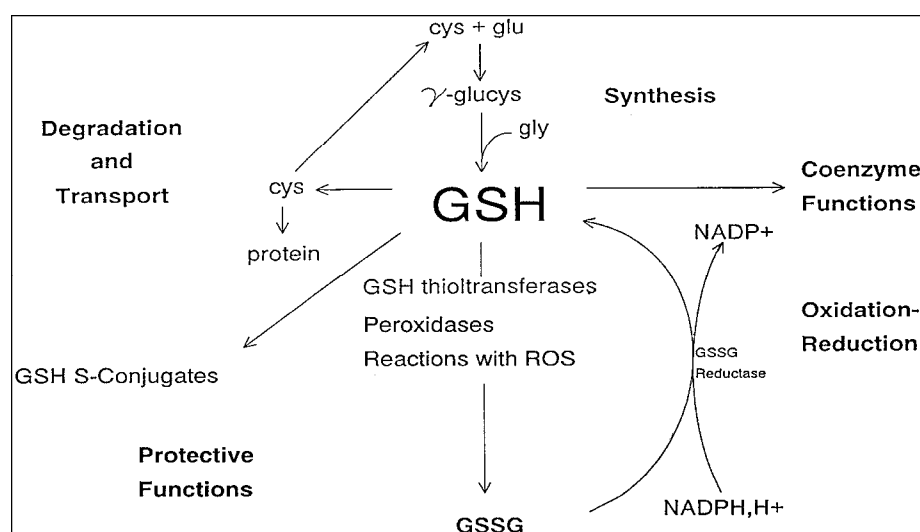


Figure 5.1 Glutathione (GSH) metabolism.

GSSG, oxidized glutathione; glu, glutamate; cys, cysteine; gly, glycine; and ROS, reactive oxygen species. Reproduced from Anderson (1998).

5.2 *In vitro* activity of curcuminoids on trypanosomes

Curcumin has a broad range of biological activities, especially against protozoan parasites. Based on these properties and using the Alamar Blue assay as described in chapter 2, curcumin and all its synthesized analogues were tested on trypanosomes to determine the EC_{50} values of these compounds. All the *in vitro* Alamar Blue assays were performed at least three times for each test compound, and the mean values and standard error (SE) were calculated. Drug free incubations and diminazene were utilized as negative and positive controls, respectively. The no-drug control was considered to represent a 100% value for fluorescence, typically measuring 100-250 arbitrary units out of a scale of 1000. Typical background fluorescence was observed to be between 25 – 30 units.

5.2.1 *Trypanosoma brucei brucei*

The three parent curcuminoids (compounds AS-HK001 - AS-HK003) and 95 curcuminoid analogues (compounds AS-HK004 - AS-HK020, AS-HK022 - AS-HK053, AS-HK055 - AS-HK062, AS-HK065, AS-HK066 and AS-HK073 - AS-HK098) were assessed for *in vitro* trypanocidal properties on the standard drug-sensitive laboratory strain 427 (Table 5.1). Eight of these compounds (018, 034, 044, 048, 086, 095, 097 and 098) are pairs of 1:1 (a:b) isomeric mixtures. Our value for compound AS-HK001 against bloodstream forms of the monomorphic strain 427 was $2.5 \pm 0.4 \mu\text{M}$ ($n = 3$) which is in agreement with a previously published value (Nose *et al*, 1998). The two other parent curcuminoids, demethoxycurcumin (AS-HK002) and bisdemethoxycurcumin (AS-HK003) were approximately 2- and 3-fold less active than compound AS-HK001 with EC_{50} values of 4.6 ± 0.8 and $7.7 \pm 1 \mu\text{M}$, respectively.

The results of the Alamar Blue assay of curcumin analogues are summarized in Table 5.1 and an example of this assay is shown in Figure 5.2A. About 35 out of 98 of the curcumin analogues were observed to exhibit trypanocidal activity better than the original curcumin AS-HK001 against *T. brucei* 427 strain. Furthermore, 15 of these compounds have EC_{50} values below $1 \mu\text{M}$ including 4 compounds; AS-HK014, AS-HK093, AS-HK096 and AS-HK097 that display EC_{50} values below $100 \mu\text{M}$ with EC_{50} values of 0.053 ± 0.007 , 0.087 ± 0.03 , 0.052 ± 0.01 and 0.089 ± 0.03 , respectively.

5.2.2 Assessment of cross-resistance with current treatments

The efficacy of curcumin compound was also tested on two additional clonal lines, derived from *T. b. brucei* strain 427: TbAT1-KO (Figure 5.2B) and KO-B48 (Figure 5.2C). Whereas TbAT1-KO was almost 25-fold resistant to diminazene aceturate in this series of experiments (0.10 ± 0.01 (n = 13) vs 2.4 ± 0.3 μ M (n = 14); $P < 0.001$, Student T-test), there was no significant resistance to curcuminoid analogues in this line (Wilcoxon's signed ranks test for two groups, paired observations; $P > 0.05$). The activity of most of the compounds appeared to be within a 2-fold difference in sensitivity against the two lines and at most 2.7-fold (for compound AS-HK019).

Out of 98 compounds tested (Table 5.1), only six displayed significantly less activity against this line: compounds AS-HK033 (3.1-fold), AS-HK044 (5-fold), AS-HK045 (3.6-fold), AS-HK080 (3.1-fold), AS-HK006 (3-fold) and AS-HK089 (>4.3-fold). The first four compounds are close analogues of AS-HK001, with conjugated di-keto linkers, differing only in the substitutions at the aromatic rings, in particular allyl ether substitutions (AS-HK044, AS-HK045). Unexpectedly, the B48 line proved to be significantly more sensitive to some of the most active curcuminoids (Table 5.1), including compounds AS-HK014, AS-HK034, AS-HK036, AS-HK048, AS-HK049, AS-HK052, AS-HK051, AS-HK057 and AS-HK059. Moreover, compounds AS-HK036 and AS-HK048 displayed a highly significant ($P < 0.001$, Student's T-test) 83- and 60-fold higher activity against the B48 strain.

Considering that the only known differences between B48 and WT cells are the loss of the TbAT1 and HAPT transporters in the former, it is difficult to find an explanation for this phenomenon. Apparently, the adaptation to high levels of pentamidine that lead to the development of the B48 clonal line produced further changes of unknown identity.

5.2.3 Evaluation of activity against veterinary trypanosomes: *T. evansi* and *T. equiperdum*.

A sub-set of the analogues was tested against *Trypanosoma evansi*. Most of the analogues were equally, or more potent against this species than against the *T. b. brucei* reference strain 427 (Table 5.2), but there was no statistical significant difference in overall curcuminoid sensitivity between the two species ($P > 0.05$; Wilcoxon's signed ranks test for two groups, paired observations). Although the *T. evansi* strain was significantly more

sensitive to diminazene than *T. b. brucei* WT ($0.018 \pm 0.003 \mu\text{M}$, $n=5$; $P<0.001$; unpaired Student's T-test) and compound AS-HK044 ($P<0.05$), it was less sensitive to compound AS-HK014 ($P<0.02$) and compound AS-HK045 ($P<0.01$). Figure 5.3 illustrates the results obtained for *T. b. brucei* WT and *T. evansi*.

Another sub-set of the analogues (AS-HK065, AS-HK066 and AS-HK073-AS-HK098) was tested *in-vitro* against *Trypanosoma equiperdum*. Most of these compounds had a similar activity to those EC_{50} values which have been obtained from *T. b. brucei* reference strain 427 with no significant difference in overall curcuminoid sensitivity between the two tested species (Table 5.3). For four compounds, the sensitivity of *T. b. brucei* WT was significantly higher than the sensitivity of *T. equiperdum* (Figure 5.4): AS-HK093 ($P<0.05$), AS-HK096 ($P<0.01$), AS-HK097 ($P<0.05$) and AS-HK098 ($P<0.02$). Furthermore, similar to *T. evansi*, *T. equiperdum* was significantly more sensitive to diminazene than *T. b. brucei* WT ($0.013 \pm 0.002 \mu\text{M}$, $n=3$; $P<0.001$; unpaired Student's T-test).

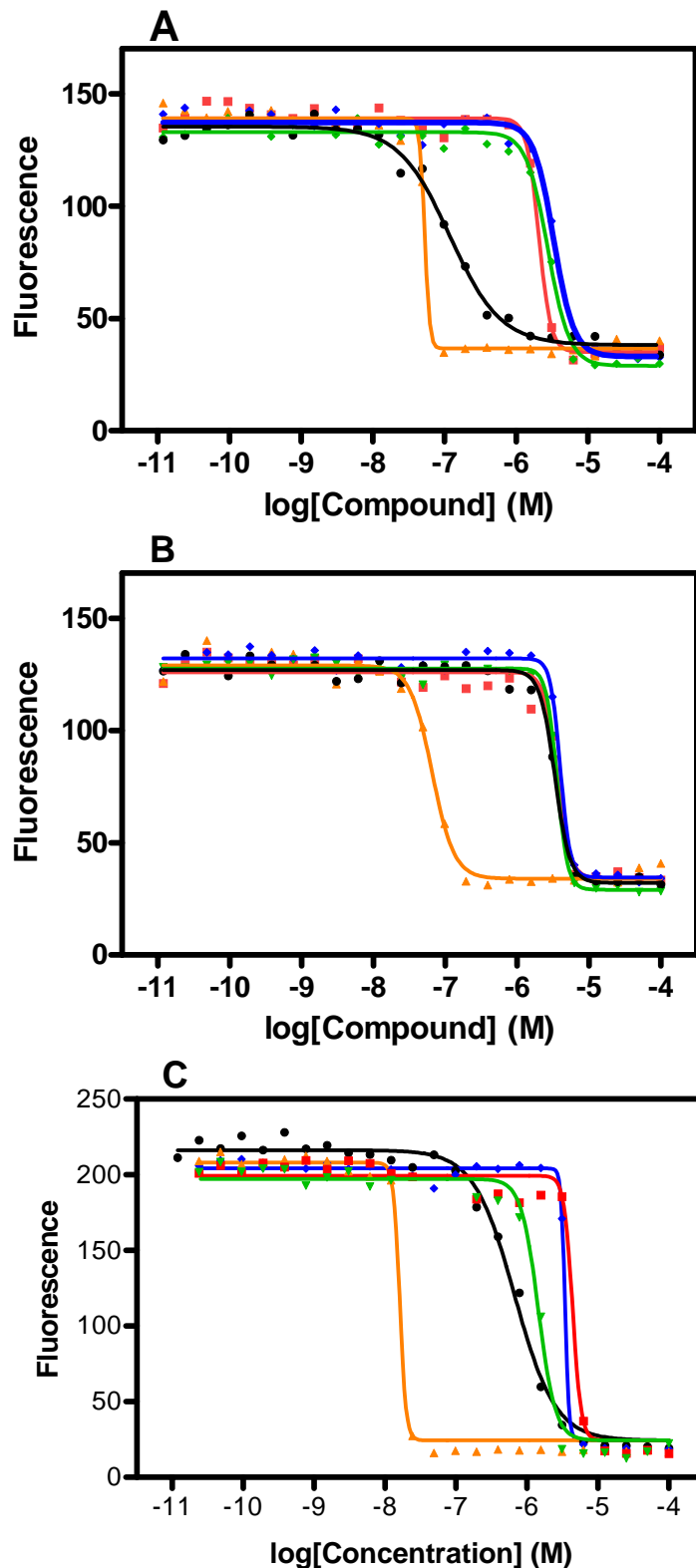


Figure 5.2 Efficacy of some curcumin analogues against bloodstream forms of three *T. brucei* clones.

Tb427 (A), TbAT1-KO (B), and KO-B48 (C). The test compounds were: AS-HK009 (■), AS-HK014 (▲), AS-HK027 (◆) and AS-HK028 (▼). Diminazene (•) was used as a positive control. The data shown is a representative experiment using Alamar Blue method as described in section 1.3.1. As described (Matovu *et al*, 2003d) TbAT1-KO and B48 (Bridges *et al*, 2007c) displayed considerable resistance to diminazene.

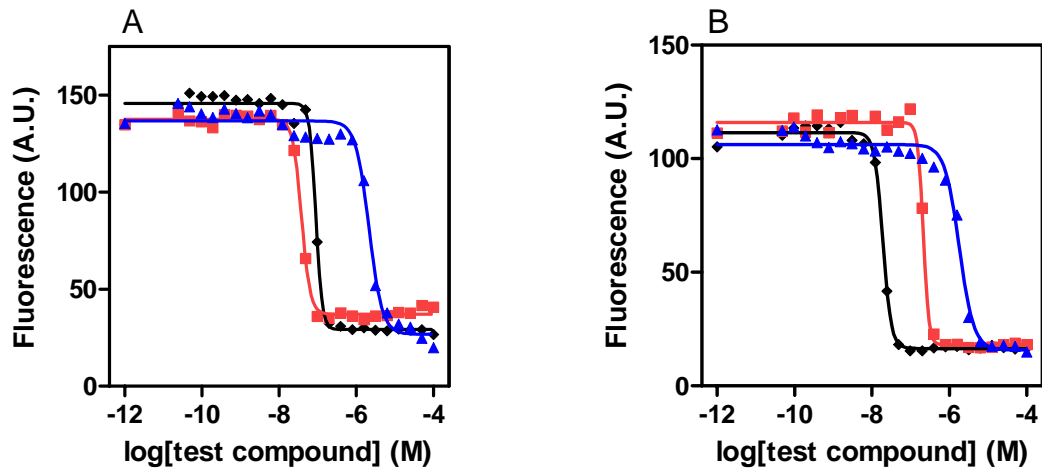


Figure 5.3 Effect of some curcuminoid analogues on *Trypanosoma brucei* and *Trypanosoma evansi* bloodstream forms culture.

Curcuminoid analogues and a control drug (diminazene aceturate) were tested for effect against the two trypanosome species *in vitro*, using the Alamar Blue protocol described in the Materials and Methods section. A: *T. b. brucei* WT. B: *T. evansi*. (♦) diminazene aceturate; (■) AS-HK014; (▲) AS-HK001. Data are representative of 3 – 4 independent experiments.

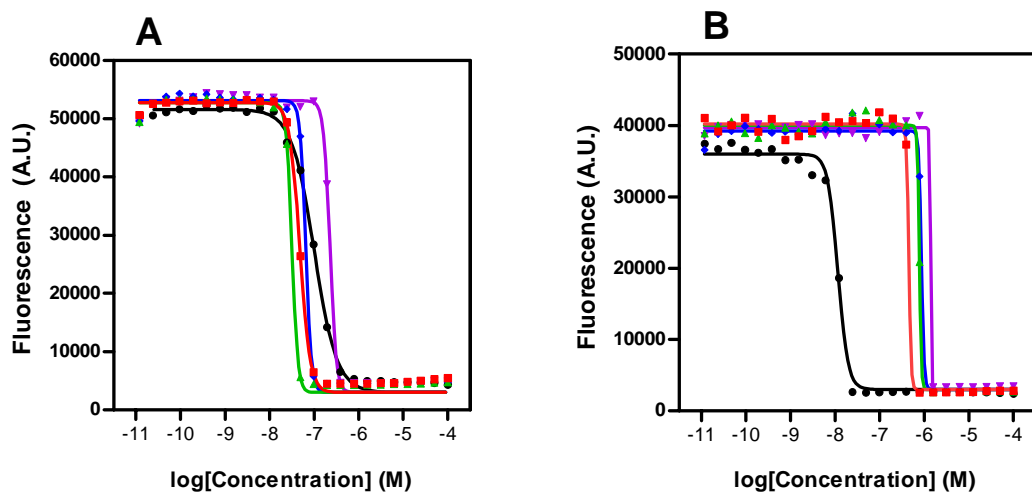


Figure 5.4 shows the susceptibility of bloodstream forms of *T. brucei* and *T. equiperdum* to some curcumin compounds using Alamar Blue method.

T. brucei 427 WT (A) and *T. equiperdum* (B). Compounds: (●) diminazene aceturate; (■) AS-HK093; (▲) AS-HK096, (♦) AS-HK097; (▼) AS-HK098. Data are representative of 3 independent experiments.

5.3 *In vitro* activity of curcumin analogues on *Leishmania* spp

5.3.1 Promastigotes of *Leishmania major* and *L. mexicana*

The same curcuminoids and analogues were tested on promastigotes of *Leishmania major* (Figure 5.5A) and *L. mexicana* (Figure 5.5B). The results are listed in Table 5.1. The parent compound AS-HK001 exhibited poor activity against the promastigotes of both species (*L. major* and *L. mexicana*) with EC₅₀ values of 33 ± 4 and 26 ± 0.6 μM , respectively. No significant differences were found in the overall sensitivity to curcuminoids, between the promastigotes of *L. major* and *L. mexicana* ($P > 0.05$).

For *L. major* promastigotes, five curcumin analogues, AS-HK016, AS-HK033, AS-HK073, AS-HK093 and AS-HK095, exhibited antileishmanial activity with EC₅₀ values of less than 5 μM , whereas ten curcuminoid analogues, AS-HK014, AS-HK017, AS-HK028, AS-HK044, AS-HK051, AS-HK077, AS-HK081, AS-HK094, AS-HK096, and AS-HK097, showed an activity within the EC₅₀ range of 5-10 μM . The rest of the analogues displayed EC₅₀ values in excess of activity of more than 10 μM . The most active compounds, AS-HK016, and AS-HK073, exhibited activity of 2.7 ± 0.7 μM and 2.8 ± 0.4 in *L. major*, respectively (Table 5.1).

For *L. mexicana* promastigotes, the general trend was that the activity of the curcuminoids was less than against *L. major*, and the two lowest EC₅₀ values were obtained with compounds AS-HK033 and AS-HK093 (6.2 ± 0.2 and 12 ± 0.5 μM , respectively). About 25% of the compounds (24 out of 98) shows significantly higher activity against *L. major* than against *L. mexicana* promastigotes with P value < 0.001 for: AS-HK018, AS-HK036, AS-HK041, AS-HK044, AS-HK046, AS-HK051, AS-HK055, AS-HK057, AS-HK094 and AS-HK096; $P < 0.01$ for: AS-HK014, AS-HK017, AS-HK028, AS-HK081, AS-HK093, AS-HK095 and AS-HK097; $P < 0.02$ for: AS-HK016, AS-HK048, AS-HK077 and AS-HK098; and $P < 0.05$ for: AS-HK045, AS-HK075 and AS-HK079. Moreover, only one compound (AS-HK061) exhibited significantly less activity against *L. major* than against *L. mexicana* promastigotes ($P < 0.01$).

5.3.2 Amastigotes of *Leishmania mexicana*

The compounds were also assessed against axenic *L. mexicana* amastigotes (Figure 5.5C). Compounds AS-HK033, AS-HK073, AS-HK016, AS-HK017 showed highest antileishmanial activity and had also displayed submicromolar activity against *T. b. brucei* (see section 5.2). This suggests that these compounds act on a well-conserved target in the kinetoplasts. However, the best performing compound against *T. b. brucei*, the enone AS-HK014, was observed to perform relatively poorly against *L. mexicana* amastigotes, with an EC_{50} value of $17 \pm 2 \mu\text{M}$. The general trend was that the curcuminoids were, on average, very slightly less active against the axenic amastigotes than against promastigotes (see Table 5.1), but there was not statistically significant ($n = 3$, $P < 0.2$, Wilcoxon's signed ranks test for two groups, paired observations). Fifteen compounds, including curcumin itself, displayed *in vitro* activity against the amastigote stage that was equal or superior to the clinically used drug pentamidine ($EC_{50} = 16 \pm 2 \mu\text{M}$; $n=6$).

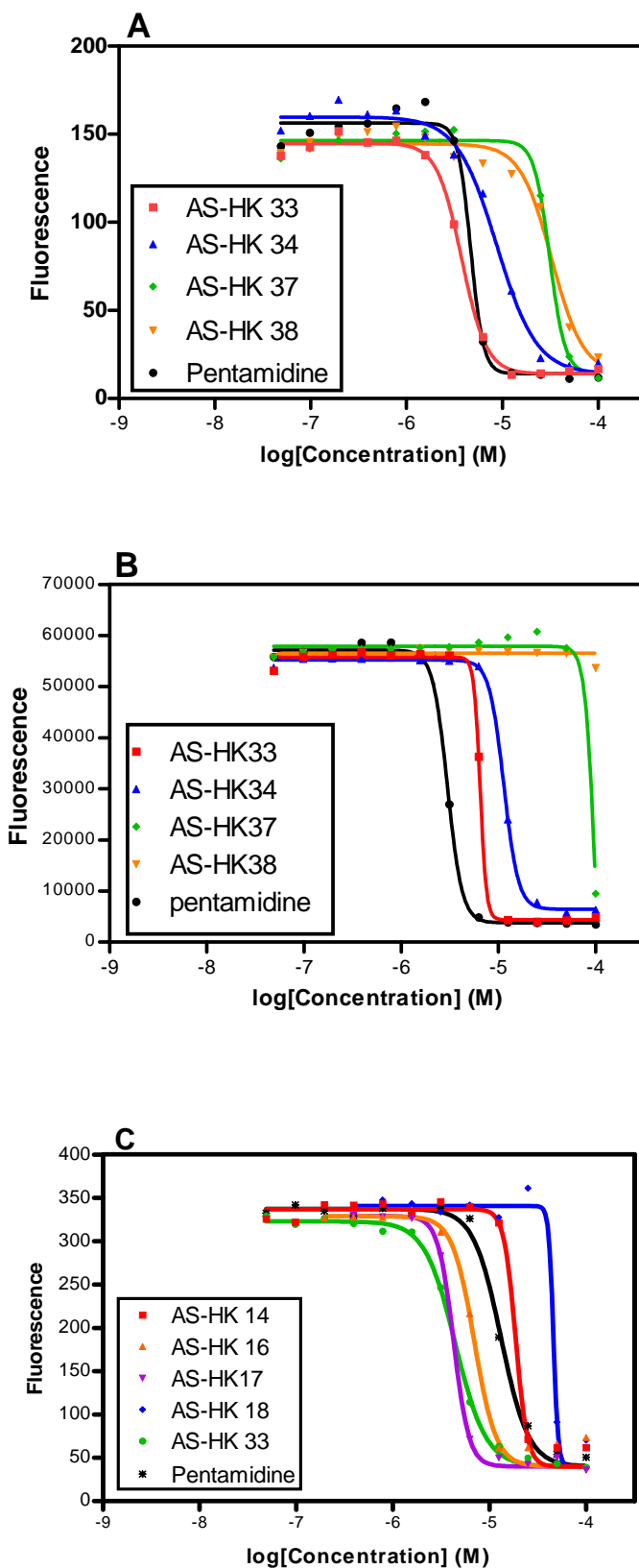


Figure 5.5 shows the effect of some curcumin analogues on promastigotes of *L. major* and *L. mexicana* and axenic *L. mexicana* amastigotes.

The Alamar Blue assay was used as described in chapter 2. (A) *L. major*, (B) *L. mexicana* and (C) axenic *L. mexicana* amastigotes. Pentamidine was used as a positive drug control. Data are representative of 3 independent experiments.

Table 5.1 Antitrypanosomal, antileishmanial and cytotoxic activities of curcuminoids

AS-HK	TbAT1 WT	TbAT1 KO	TbAT1 B48	<i>L. major</i> Pro-mastigote	<i>L. mexicana</i> Pro-mastigote	<i>L. mexicana</i> amastigot	HEK
001	2.5 ± 0.4	4.7 ± 0.3	2.9 ± 1	33 ± 4	26 ± 0.6	16 ± 3	37 ± 6
002	4.6 ± 0.8	5.9 ± 0.5	2.2 ± 0.2	37 ± 1	46 ± 0.5	37 ± 4	40 ± 5
003	7.7 ± 1	9.5 ± 0.3	4.5 ± 0.5	72 ± 3	>100	63 ± 3	200 ± 40
004	9.2 ± 2	16 ± 2	8.2 ± 0.2	20 ± 0.8	20 ± 0.3	18 ± 3	22 ± 3
005	37 ± 7	64 ± 1	39 ± 5	61 ± 3	>100	54 ± 8	ND
006	25 ± 6	>100	76 ± 8	>100	81 ± 30	>100	ND
007	21 ± 10	37 ± 7	12 ± 2	90 ± 20	59	43 ± 9	120 ± 10
008	22 ± 8	34 ± 1	18 ± 1	>100	>100	>50 ¹	ND
009	2.5 ± 0.4	4.0 ± 0.3	5.1 ± 0.9	80 ± 10	>100	64 ± 10	350 ± 90
010	51 ± 10	76 ± 2	50 ± 5	82 ± 5	99 ± 1	32.2	ND
011	93 ± 10	99 ± 1	55 ± 6	>100	>100	67	ND
012	15 ± 0.2	22 ± 0.3	13 ± 0.7	19 ± 2	18 ± 1	27 ± 2	50 ± 4
013	34 ± 4	36 ± 0.2	26 ± 2	36 ± 1	>100	35 ± 8	ND
014	0.053 ± 0.007	0.082 ± 0.005	0.023 ± 0.004 ²	8.9 ± 0.8	34 ± 1	17 ± 2	24 ± 2
015	4.1 ± 2	9.7 ± 2	2.2 ± 0.4	30 ± 2	48 ± 1	22 ± 7	76 ± 3
016	0.22 ± 0.09	0.22 ± 0.02	0.10 ± 0.03	2.7 ± 0.7	14 ± 2	4.6 ± 0.7	8.1 ± 1
017	1.1 ± 0.3	1.4 ± 0.2	0.58 ± 0.08	7.4 ± 0.1	21 ± 2	4.8 ± 0.6	22 ± 1
018	0.14 ± 0.05	0.29 ± 0.1	0.032 ± 0.004	13 ± 1	38 ± 1	28 ± 6	20 ± 2
019	0.65 ± 0.02	1.8 ± 0.1	1.0 ± 0.1	22 ± 2	26 ± 5	21 ± 2	30 ± 3
020	8.9 ± 1	8.8 ± 0.2	2.9 ± 0.4 ²	87 ± 5	73 ± 20	32 ± 2	400 ± 0
022	14 ± 1	19 ± 2	10 ± 0.7	16 ± 0.2	16 ± 0.8	8.1 ± 2	61 ± 2
023	1.8 ± 0.8	2.3 ± 0.1	1.6 ± 0.5	33 ± 8	27 ± 4	37 ± 5	20 ± 1
024	2.9 ± 1	2.9 ± 0.3	1.8 ± 0.4	22 ± 4	33 ± 3	30 ± 4	25 ± 3
025	1.6 ± 0.5	2.2 ± 0.2	1.2 ± 0.2	73 ± 20	>100	34 ± 4	80 ± 20
026	1.5 ± 0.3	2.0 ± 0.1	0.92 ± 0.08	>100	>100	43 ± 7	49 ± 6
027	3.2 ± 0.4	3.9 ± 0.1	3.2 ± 0.3	66 ± 10	32 ± 10	7.1 ± 1	>500
028	3.1 ± 0.7	3.8 ± 0.2	2.1 ± 0.5	9.3 ± 1	>100	76 ± 10	94 ± 11
029	3.7 ± 0.4	7.8 ± 0.8	7.8 ± 0.8	20 ± 1	>100	>100	>500
030	17 ± 3	38 ± 3	5.9 ± 0.9 ²	>100	>100	>100	370 ± 60
031	78 ± 5	61 ± 5	>100	>100	>100	>100	ND
032	1.8 ± 0.1	2.5 ± 0.2	3.4 ± 0.1	26 ± 0.1	29 ± 2	12 ± 3	39 ± 7
033	0.87 ± 0.06	1.1 ± 0.1	2.7 ± 0.5 ²	4.3 ± 0.5	6.2 ± 0.2	3.2 ± 0.5	36 ± 4
034	0.75 ± 0.1	0.73 ± 0.08	0.30 ± 0.06 ²	10 ± 2	19 ± 6	7.4 ± 1	63 ± 9
035	2.5 ± 0.5	3.2 ± 0.1	ND	>100	>100	17 ± 5	220 ± 60
036	1.9 ± 0.2	1.6 ± 0.1	0.023 ± 0.003 ⁴	12 ± 1	42 ± 1	28 ± 8	26 ± 2
037	22 ± 0.8	19 ± 0.6	39 ± 4	38 ± 3	66 ± 0.1	72	130 ± 20
038	32 ± 0.4	28 ± 1	27 ± 2	37 ± 3	>100	>50	140 ± 10
039	29 ± 2	30 ± 0.6	12 ± 1	76 ± 9	>100	43 ± 20	110 ± 10
040	30 ± 2	29 ± 0.7	25 ± 4	40 ± 2	64 ± 4	23 ± 7	100 ± 7
041	15 ± 2	18 ± 0.2	25 ± 8	28 ± 3	>100	14 ± 4	82 ± 5
042	43 ± 3	46 ± 1	37 ± 3	77 ± 30	>100	>100	130 ± 20
043	7.9 ± 0	9.6 ± 0	12 ± 1	19 ± 5	27 ± 0.3	10 ± 4	61 ± 20
044	2.4 ± 0.1	2.2 ± 0.1	12 ± 2 ³	5.7 ± 0.7	35 ± 0.8	12 ± 4	270 ± 100
045	1.6 ± 0.1	1.9 ± 0.1	5.8 ± 0.6 ³	25 ± 9	>100	>100	>500
046	13 ± 0.6	14 ± 0.2	24 ± 2	13 ± 0.7	>100	20 ± 5	19 ± 1
047	80 ± 6	81 ± 4	ND	75 ± 30	ND	ND	239 ± 0
048	3.0 ± 0.3	2.1 ± 0.2	0.049 ± 0.008 ⁴	15 ± 2	85 ± 30	23 ± 4	40 ± 5

049	45 ± 1	48 ± 2	9.0 ± 3 ⁴	55 ± 3	>100	>100	200 ± 2
050	40 ± 3	42 ± 4	6.3 ± 0.6 ³	50 ± 4	82 ± 20	64 ± 20	130 ± 2
051	2.6 ± 0.5	1.9 ± 0.1	0.12 ± 0.02 ²	9.9 ± 1	46 ± 3	21 ± 8	45 ± 3
052	23 ± 4	40 ± 0.7	4.7 ± 0.3	97 ± 1	>100	>100	220 ± 20
053	47 ± 0.2	48 ± 1.1	33 ± 5	86 ± 20	>100	>100	220 ± 10
054	>100	>100	ND	>100	>100	>100	220 ± 10
055	3.6 ± 0.9	3.2 ± 0.3	3.8 ± 0.5	13 ± 1	>100	13 ± 3	90 ± 10
056	15 ± 0.7	18 ± 0.6	7.7 ± 0.5	42 ± 2	46 ± 2	29 ± 0.3	120 ± 10
057	2.8 ± 0.8	1.9 ± 0.1	0.6 ± 0.1	8.9 ± 2	30 ± 0.1	12 ± 2	45 ± 8
058	44 ± 2	45 ± 1	45 ± 4	92 ± 20	>100	>100	>500
059	14 ± 2	14 ± 0.6	5.1 ± 0.8	76 ± 20	>100	>100	230 ± 7
060	35 ± 0.8	33 ± 2	40 ± 5	71 ± 6	74 ± 20	>50	140 ± 20
061	33 ± 2	35 ± 1	30 ± 2	83 ± 4	41 ± 3	>100	150 ± 10
062	34 ± 3	38 ± 2		69 ± 20	>100	>100	>500
065	1.2 ± 0.3	1.6 ± 0.2	4.7 ± 0.4	42 ± 10	>100	>100	370 ± 100
066	4.1 ± 2	10 ± 2	12 ± 2	60 ± 10	>100	>100	270 ¹
073	0.45 ± 0.07	0.62 ± 0.07	0.77 ± 0.02	2.8 ± 0.4	18 ± 5	10 ± 1	200 ± 50
074	1.6 ± 0.4	1.9 ± 0.2	1.9 ± 0.1	23 ± 6	65 ± 20	61 ± 3	690 ± 200
075	1.7 ± 0.7	2.0 ± 0.2	2.1 ± 0.1	23 ± 3	50 ± 3	38 ± 6	79 ± 8
076	1.2 ± 0.2	1.6 ± 0.2	1.3 ± 0.1	>100	>100	27 ± 0.8	>1000
077	1.4 ± 0.2	1.7 ± 0.1	2.0 ± 0.2	7.6 ± 0.7	27 ± 4	21 ± 4	>500
078	1.9 ± 0.3	2.9 ± 0.3	3.1 ± 0.1	48 ± 9	55 ± 0.4	27 ± 7	>500
079	1.3 ± 0.4	1.8 ± 0.1	1.6 ± 0.1	28 ± 2	60 ± 30	34 ± 0.2	>500
080	0.75 ± 0.5	1.7 ± 0.8	2.3 ± 2	>100	>100	>100	840 ± 80
081	0.7 ± 0.3	0.85 ± 0.1	1.1 ± 0.1	9.7 ± 2	29 ± 1	32 ± 0.7	46 ± 3
082	33 ± 10	22 ± 5	19 ± 4	41 ± 10	>100	>100	370 ± 20
083	15 ± 5	13 ± 2	19 ± 0.6	>100	57 ± 30	82 ± 7	158 ± 10
084	64 ± 10	71 ± 1	91 ± 30	>100	>100	>100	630
085	0.50 ± 0.10	0.52 ± 0.10	0.43 ± 0.01	11 ± 1	14 ± 0.7	16 ± 3	130 ± 20
086	3.0 ± 0.7	2.9 ± 0.5	2.5 ± 0.4	>100	55 ± 3	40 ± 5	77 ± 2
087	1.9 ± 0.6	1.5 ± 0.2	1.7 ± 0.1	>100	>100	53 ± 3	150 ± 8
088	17 ± 5	22 ± 2	32 ± 2	75 ± 0.8	89 ± 4	43 ± 6	180 ± 10
089	23 ± 8	28 ± 2	>100	70 ± 30	56 ± 30	>100	240 ± 9
090	26 ± 8.1	27 ± 1	45 ± 4	>100	>100	>100	240 ± 10
091	13 ± 5	20 ± 3	30 ± 0.8	66 ± 20	>100	45 ± 8	180 ± 4
092	1.4 ± 0.4	1.0 ± 0.3	1.5 ± 0.4	14 ± 0.7	25 ± 0.1	50 ± 30	230 ± 7
093	0.087 ± 0.03	0.041 ± 0.02	0.039 ± 0.01	4.5 ± 0.7	12 ± 0.5	25 ± 4	21 ± 2
094	1.1 ± 0.3	1.2 ± 0.2	1.8 ± 0.1	6.6 ± 1.0	51 ± 0.8	53 ± 8	43 ± 2
095	0.86 ± 0.3	0.62 ± 0.1	0.89 ± 0.2	4.0 ± 1.3	27 ± 0.8	19 ± 4	30 ± 0.3
096	0.052 ± 0.01	0.042 ± 0.02	0.021 ± 0.01	7.8 ± 1.0	31 ± 0.8	35 ± 1	30 ± 2
097	0.089 ± 0.03	0.078 ± 0.02	0.045 ± 0.02	7.5 ± 0.8	33 ± 0.8	29 ± 0.8	25 ± 1
098	0.27 ± 0.06	0.27 ± 0.07	0.11 ± 0.04	15 ± 0.1	38 ± 4	41 ± 4	26 ± 1

Data are EC₅₀ values in μM ± standard errors and are the average and SE of 3 – 6 independent experiments except where indicated. ND = Not done. INS = Insoluble. ¹, n = 1; ², P<0.05 relative to strain 427; ³, P<0.01 relative to strain 427; ⁴, P<0.001 relative to strain 427.

Table 5.2 Comparing the activity of curcuminoids on *T. b. brucei* WT and *T. evansi*.

compound	<i>T. b. brucei</i> WT	<i>T. evansi</i>	Student's T-tetst
AS-HK 001	2.5 ± 0.4	2.0 ± 0.3	NS
AS-HK 012	15 ± 0.2	14 ± 0.5	NS
AS-HK 014	0.053 ± 0.007	0.17 ± 0.02	P<0.02
AS-HK 016	0.22 ± 0.09	0.21 ± 0.02	NS
AS-HK 018	0.14 ± 0.05	0.29 ± 0.04	NS
AS-HK 022	14 ± 1	7.9 ± 0.6	P<0.05
AS-HK 027	3.2 ± 0.4	2.5 ± 0.6	NS
AS-HK 028	3.1 ± 0.7	1.5 ± 0.1	NS
AS-HK 029	3.7 ± 0.4	2.9 ± 0.2	NS
AS-HK 033	0.87 ± 0.06	1.1 ± 0.2	NS
AS-HK 034	0.75 ± 0.10	0.99 ± 0.2	NS
AS-HK 044	2.4 ± 0.1	1.6 ± 0.2	P<0.05
AS-HK 045	1.6 ± 0.1	5.6 ± 0.7	P<0.01

Table 5.3 Comparing the activity of curcuminoids on *T. b. b.* WT and *T. equiperdum*

compound	<i>T. b. brucei</i> WT	<i>T. equiperdum</i>	Student's T-tetst
AS-HK065	1.2 ± 0.3	1.6 ± 0.3	NS
AS-HK 066	4.1 ± 2	12 ± 5	NS
AS-HK 073	0.45 ± 0.07	0.45 ± 0.1	NS
AS-HK 074	1.6 ± 0.4	1.5 ± 0.3	NS
AS-HK 075	1.7 ± 0.7	1.8 ± 0.3	NS
AS-HK 076	1.2 ± 0.2	1.0 ± 0.1	NS
AS-HK 077	1.4 ± 0.2	1.5 ± 0.3	NS
AS-HK 078	1.9 ± 0.3	2.2 ± 0.1	NS
AS-HK 079	1.3 ± 0.4	1.4 ± 0.2	NS
AS-HK 080	0.75 ± 0.5	1.3 ± 0.5	NS
AS-HK 081	0.7 ± 0.3	0.75 ± 0.2	NS
AS-HK 082	33 ± 10	44 ± 9	NS
AS-HK 083	15 ± 5	17 ± 5	NS
AS-HK 084	64 ± 10	91 ± 30	NS
AS-HK 085	0.50 ± 0.1	0.60 ± 0.1	NS
AS-HK 086	3.0 ± 0.7	5.1 ± 1	NS
AS-HK 087	1.9 ± 0.6	2.1 ± 0.4	NS
AS-HK 088	17 ± 5	13 ± 2	NS
AS-HK 089	23 ± 8	22 ± 5	NS
AS-HK 090	26 ± 8	24 ± 4	NS
AS-HK 091	13 ± 5	15 ± 3	NS
AS-HK 092	1.4 ± 0.4	2.5 ± 1	NS
AS-HK 093	0.087 ± 0.03	0.42 ± 0.1	P<0.05
AS-HK 094	1.1 ± 0.3	1.1 ± 0.2	NS
AS-HK 095	0.86 ± 0.3	1.3 ± 0.3	NS
AS-HK 096	0.052 ± 0.01	0.67 ± 0.2	P<0.01
AS-HK 097	0.089 ± 0.03	0.78 ± 0.3	P<0.05
AS-HK 098	0.27 ± 0.06	1.1 ± 0.2	P<0.02

Data in the two tables are the average of 3 – 4 independent experiments, given as EC₅₀ values in µM ± SE. NS = Not Significant.

5.4 Preliminary toxicology of curcuminoids

5.4.1 *In vitro* effect on Human Embryonic Kidney cells.

To assess whether the antiprotozoal activity described above should be attributed to general toxicity, rather than specific antiprotozoal activity, the analogues were also tested for their effect on human embryonic kidney (HEK) cells (see Table 1). Without exception, the toxicity to HEK cells was lower than to *T. b. brucei* WT bloodstream forms. Out of 98 analogues, only one compound, AS-HK016, had significantly higher activity against HEK cells than curcumin (AS-HK001) itself ($EC_{50} = 37 \pm 6 \mu\text{M}$) and was the only compound that displayed an EC_{50} value below $20 \mu\text{M}$. The ratio of EC_{50} (HEK)/ EC_{50} (*T. b. brucei*) could be described as the *in vitro* selectivity index and ranged from ~3-fold for compound AS-HK050 to >1,000-fold for compound AS-HK076 and 480-fold for the most active analogue, AS-HK014.

As the leishmanicidal activity of most analogues was less than their trypanocidal activity, the therapeutic index calculated from the *L. major* EC_{50} values was correspondingly lower. The most promising compound, by this measure, was AS-HK073, with a selective index of just over 70-fold.

5.4.2 Effects on hepatocyte viability and protein content

Some curcumin compounds were tested for their effects on rat hepatocytes. Three compounds were tested: AS-HK001 (curcumin); AS-HK009; and AS-HK014. All the experiments were performed in triplicate, and the viability, glutathione (GSH), and protein content were calculated separately and adjusted to percentages. The percentage viability of control and cells treated for up to 120 min was not affected by the three tested compounds: AS-HK001, AS-HK009 and AS-HK014 which are shown in Figure 5.6 A, B, and C, respectively. In all these experiments, the cell viability at T=0 min was found to be about 100% in all three groups, whereas this percentage was about 71% at the end of the experiment. There were no statistically significant differences between the control and treated cells ($P > 0.05$), and the viability of all groups reduced similarly over time.

The total protein content was determined by Lowry assay, and it is usually used to estimate the number of cells and would also highlight significant protein loss through reduced cell integrity. These values were calculated as mg protein/ml at various points during two hours

of incubation of the drugs with hepatocytes, and also adjusted to percentages. No clear differences between treated and control groups were observed over the period of incubation (Figure 5.7), confirming the results of the viability measurements performed in parallel. The results would indicate that the curcuminoids studied do not have significant hepatotoxic effects, at least not over the length of the incubation.

5.4.3 Effects on hepatocyte glutathione content

The effect of some curcumin analogues on glutathione (GSH) was also conducted using rat hepatocytes. The compounds tested were: AS-HK001 (curcumin), AS-HK009 and AS-HK014. The GSH levels of the cells were calculated and expressed per 10^6 /cells, as expressed per mg of protein as percentages. As shown in Figure 5.6. at T=0 min, the GSH content of the cell with curcumin, AS-HK 09 and AS-HK 14 were 17, 15.1, and 17.9 nmol/ 10^6 cells, respectively, and taken as 100% value. The results showed there was a GSH depletion in all experimental cells at 100 μ M drug concentration with no significant difference between the treated and control cells ($P>0.05$). At T = 120 min, GSH levels in cells treated with curcumin (Figure 5.8A) and AS-HK 09 (Figure 5.8B) were down to 30% and 60%, respectively when compared with 65% in the controls. This trend was more sharply within the first 15 min of treatment with AS-HK 14 (Figure 5.8C), which rapidly reduced GSH content on the hepatocytes at 100 μ M concentration used in this experiment.

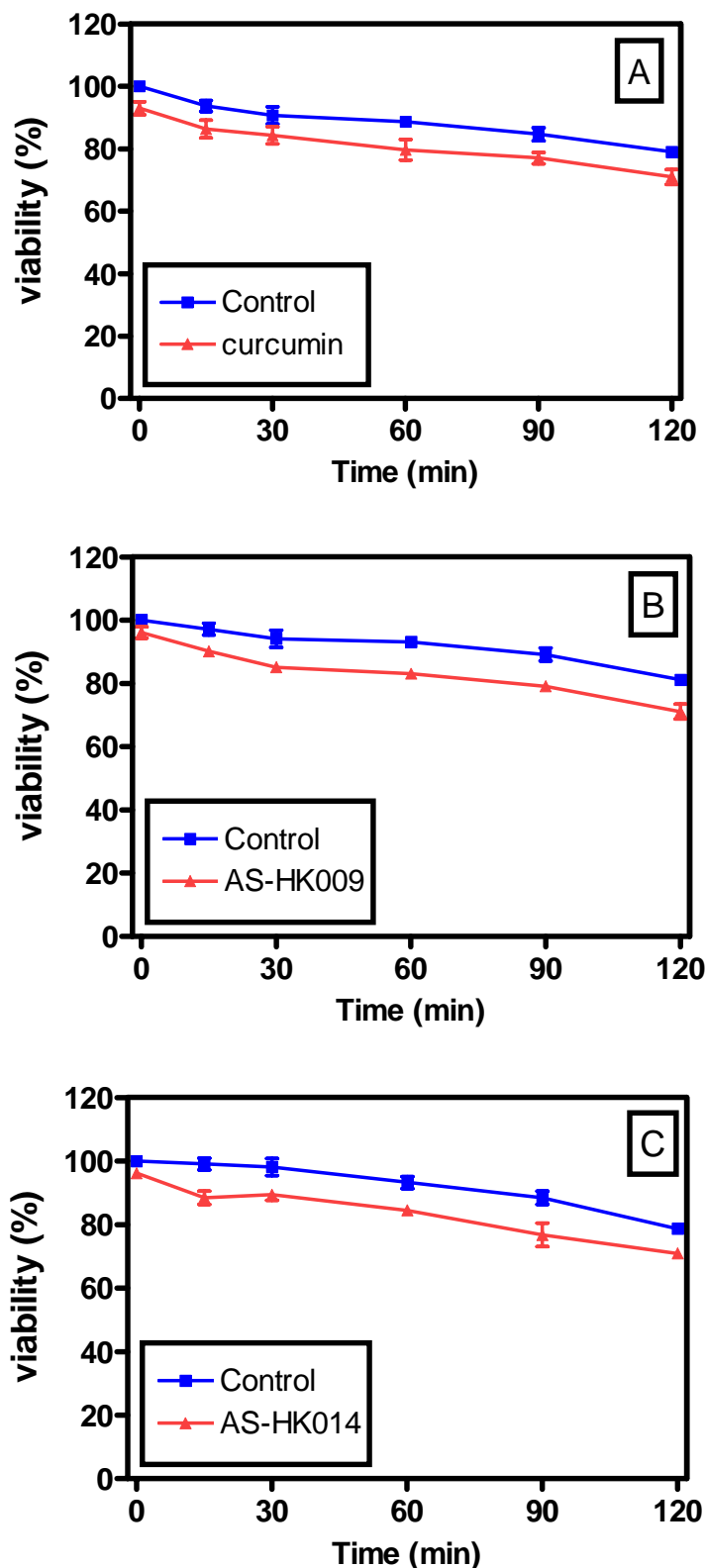


Figure 5.6 Effects of exposure of freshly isolated rat hepatocytes to 100 μ M of curcumin analogues on cell viability.

(A) AS-HK001 (curcumin), (B) AS-HK009, and (C) AS-HK014. Hepatocytes were exposed during two hours. Data shown are the average and SE percentages of three independent experiments.

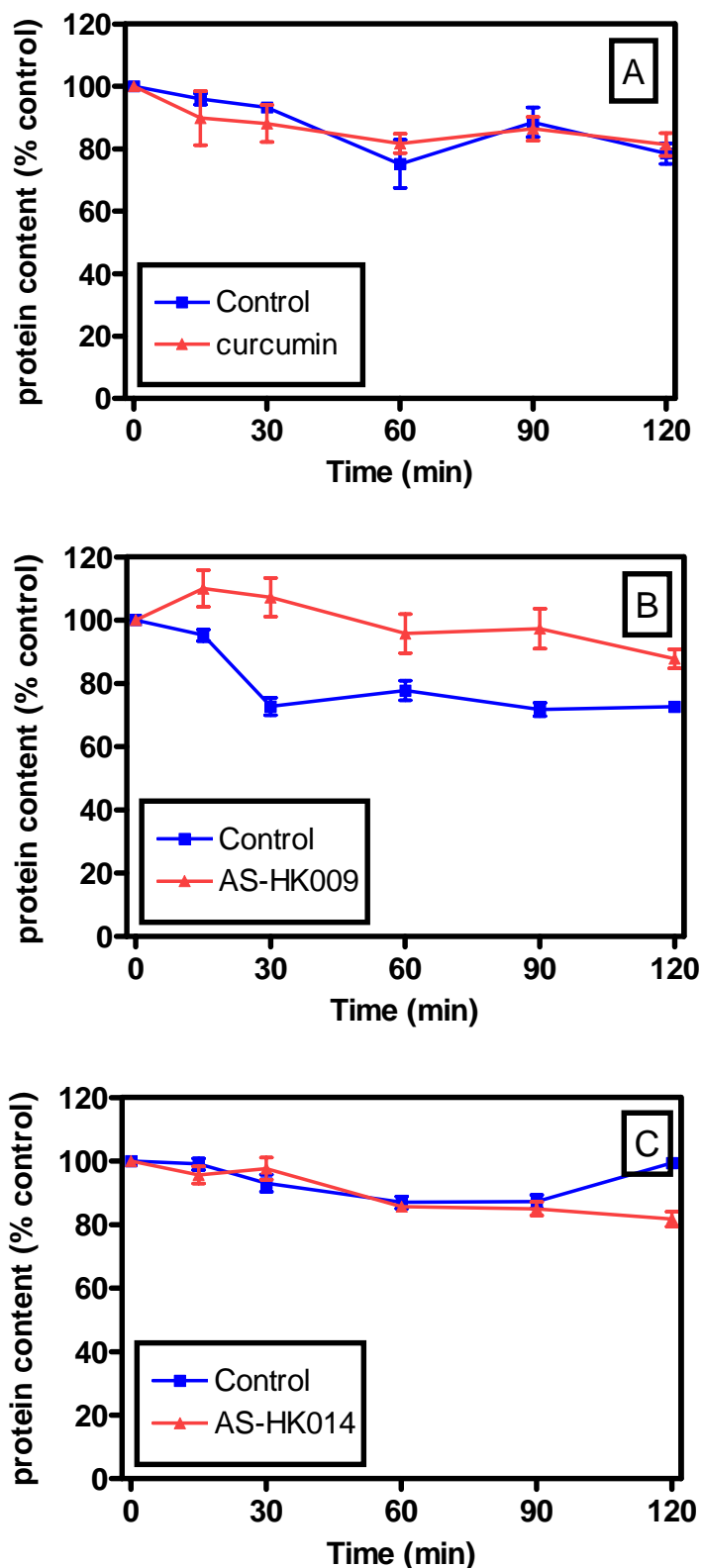


Figure 5.7 Percentages of protein content reduced by hepatocytes during two hours incubation with 100 μ M of curcumin analogues.

(A) AS-HK001 (curcumin), (B) AS-HK009, and (C) AS-HK014. Data shown are the average and SE of three independent experiments.

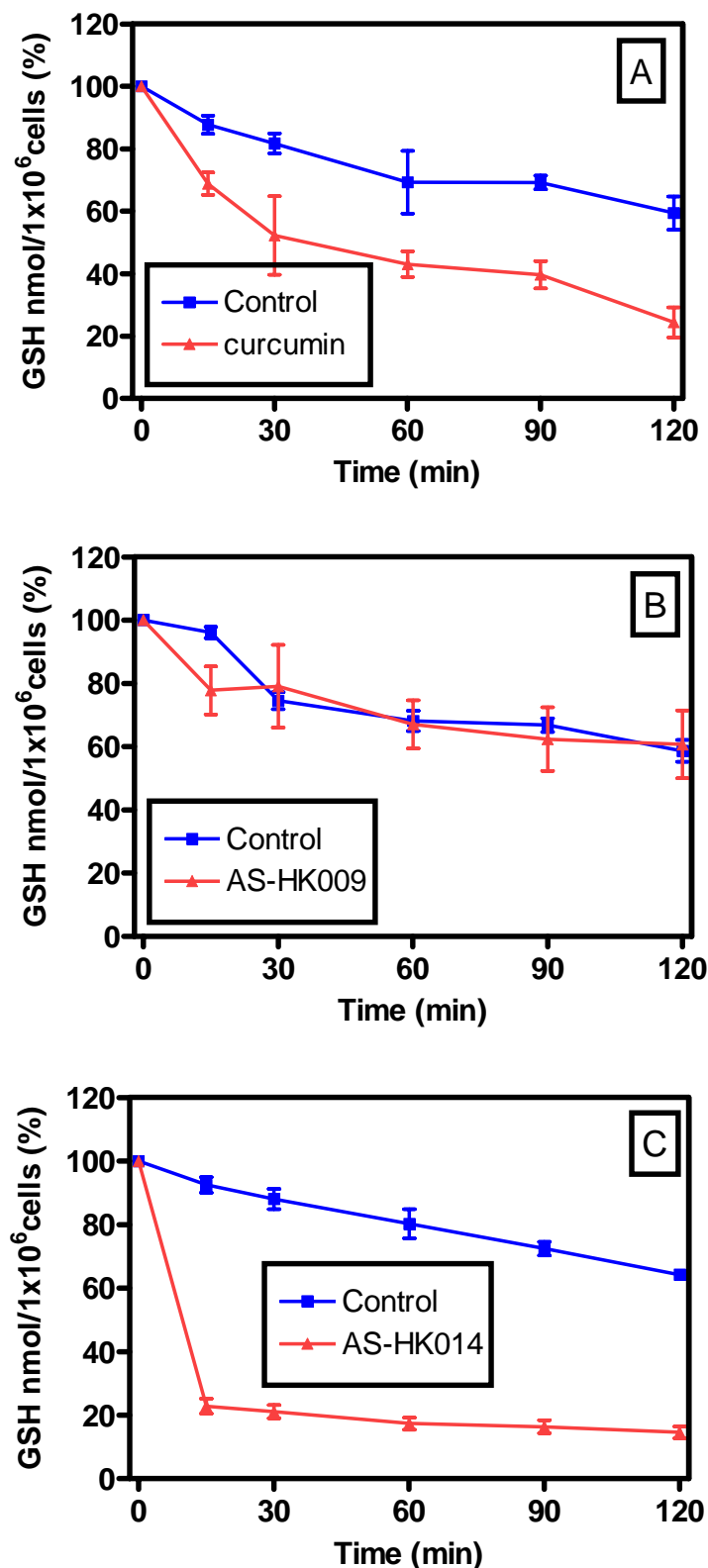


Figure 5.8 Effect of some curcumin compounds on the glutathione content of rat hepatocytes during two hours of incubation time.

The content was calculated per 10⁶ hepatocytes. All compounds tested at 100 μ M: (A) AS-HK001 (curcumin), (B) AS-HK009, and (C) AS-HK014. Data shown are the average and SE of three independent experiments.

5.5 Discussion

5.5.1 *Trypanosoma spp.*

Ninety eight new curcumin analogues were tested against bloodstream forms of *Trypanosoma brucei brucei* 427 (TbAT1-WT, TbAT1-KO and TbAT1-KO-B48), promastigotes of *Leishmania major* and *L. mexicana* and axenic *Leishmania mexicana* amastigotes. The test was conducted *in vitro* using the Alamar Blue method. The EC₅₀ value of $2.5 \pm 0.4 \mu\text{M}$ on the TbAT1-WT strain seems to be in agreement with a previously published value that showed curcumin (AS-HK001) has distinct trypanocidal properties, with an *in vitro* EC₅₀ value of $4.8 \pm 0.9 \mu\text{M}$ for bloodstream forms of the polymorphic strain GUTat 3.1 (Nose *et al*, 1998). Some of the curcumin analogues were found to display substantial and indeed promising activities against bloodstream forms of *Trypanosoma brucei*. More than 35% of these compounds were equal or more active than the original compound (AS-HK001). Four analogues exhibited a highly promising activity better than the control drug diminazene. These four compounds have similar structures and they are alkyl ethers and acetate derivatives with an enone linker. The structure activity relationship of the curcumin analogues will be discussed in detail in chapter 7.

As it is vital that any new drug developed against African trypanosomiasis is not cross-resistant with the diamidine and arsenic-based drugs currently in use, we tested the activity of all the analogues on two additional clonal lines, derived from *T. b. brucei* strain 427. The first line, TbAT1-KO, was derived by the disruption of both alleles coding for the TbAT1/P2 transporter (Matovu *et al*, 2003) and is highly resistant to diminazene aceturate as well as slightly resistant to pentamidine and melaminophenyl arsenicals. The second line, B48, was derived from TbAT1-KO by incremental exposure to pentamidine *in vitro* and is highly resistant to diminazene, pentamidine and melaminophenyl arsenicals (Bridges *et al*, 2007). The curcuminoids and analogues exhibited antitrypanosomal activity against these two clonal lines in similar manner to that of the wild-type strain and it should be emphasized that no resistance was observed for any of the highly active analogues, such as AS-HK014, AS-HK016, AS-HK093, AS-HK096, AS-HK097 and the AS-HK098. No significant differences were observed between the multi-drug resistant TbAT1-KO strain and its parental line, indicating that the P2 transporter is not involved in the internalisation of these compounds, at least not to a significant extent, though a trend towards slightly higher EC₅₀ values in the TbAT1-KO line was noticeable.

B48 is a multi-drug resistant line, with *in vitro* resistance to pentamidine and melarsen oxide of 128 and 11-fold, respectively (Bridges *et al*, 2007). Unexpectedly, the B48 line proved to be the most sensitive to the curcuminoids (Table 5.1). In particular compounds AS-HK014, AS-HK034, AS-HK036, AS-HK048, AS-HK039, AS-HK052, AS-HK051, AS-HK057 and AS-HK059 displayed significantly higher activity to this line. Some of these compounds share the enone motif of the most active compound for WT trypanosomes, AS-HK014.

A very significant finding was that the curcuminoids were active against several different *Trypanosoma* spp that cause infectious in livestock (Nagana). Animal African trypanosomiasis (AAT) is one of the most serious veterinary problems in the world. This disease caused by different species of trypanosomes *T. vivax*, *T. congolense*, *T. equiperdum*, *T. evansi*, *T. simiae*, *T. brucei* (Usman *et al*, 2008). Animal trypanosomiasis caused by these species have a greater impact by denying livestock over vast areas, and also affecting agricultural production (Schofield & Kabayo, 2008). Furthermore, the economic cost of this disease in Africa has been estimated at US\$4.75 billion every year (<http://www.fao.org/ag/againfo/programmes/en/paat/disease.html>).

Drug control of animal trypanosomiasis relies on three essential drugs, namely: Diminazine aceturate (Berenil), Isometamidium chloride and Homidium (Homidium chloride - Novidium; and Homidium bromide - Ethidium). One of the factors which lead to increase this disease is the emerging parasite strain that became resistance to the available drugs (Anene *et al*, 2001; Geerts *et al*, 2001; Matovu *et al*, 2001). In addition to *T. brucei*, we just tested the effect of curcuminoids on two species of those cause animal trypanosomiasis namely, *T. equiperdum*, *T. evansi*, and the finding that these parasites are sensitive to these compounds lead to a promising hope to use these compounds in future instead of those that became less active.

5.5.2 *Leishmania* spp.

The published EC₅₀ value for curcumin against promastigotes of *L. major* is 37.6 ± 3.5 µM (Koide *et al.*, 2002). However, another publication (Saleheen *et al.*, 2002) reported a higher level of activity against promastigotes of *L. major*, *L. tropica* and *L. infantum* (EC₅₀ values ~5 µM for all three species). These values were based on parasite numbers at the end of 72 h incubation with the drug, whereas Koide *et al.* (2002) used an incubation time of 24 h.

This difference is thus consistent with a slow action of curcumin, especially at lower concentrations. Yet it should be noted that our own observations, based on Alamar Blue metabolism after 72 h incubation with the test compound, agree with the 24 h incubations. Differences in technique and parasite strain may contribute to this apparent discrepancy but it must be remembered that both Saleheen and Koide assessed only the anti-leishmanial effects of curcumin, a too small sample size to base general conclusions on.

Most of the analogues tested in the current study displayed higher antileishmanial activity than curcumin. However, the activity was generally well below that displayed for *T. brucei*, *T. evansi* or *T. equiperdum*, using very similar Alamar Blue-based assessment protocols for all species. The activity against *L. mexicana* and *L. major* promastigotes was very similar ($P > 0.05$), confirming the conclusion of Saleheen *et al.* (2002) that curcumin does not appear to be selective for any *Leishmania* species – a great advantage for any potential lead compound against leishmaniasis.

While the structural determinations for antitrypanosomal activity are discussed in chapter 7, a few observations on structure activity relationship (SAR) for leishmanicidal activity are appropriate here. The general structures of the most active antileishmanocidal curcuminoids are showed in Figure 5.9. It seemed that influence of polarity from the oxygen function on the aromatic ring(s) has varying effects on antileishmanial activity. This was exemplified in the case of the parent curcuminoid AS-HK001 which exhibited antileishmanial activity (against promastigotes) of $33 \pm 4 \mu\text{M}$, whereas its mono-*O*-demethylated analogue AS-HK033 was almost 8-fold and 4-fold more active against *L. major* and *L. mexicana*, respectively. However, going from AS-HK033 to the more polar analogue AS-HK032, the di-*O*-demethylated analogue of AS-HK001, resulted in a 6-fold and 5-fold decreases in activities, respectively. The increase in lipophilicity in going from AS-HK001 to the di-*O*-methylated analogue AS-HK0073 resulted in ~12-fold and 1.5-fold increases in activities, respectively (see Table 5.1). A larger *O*-alkyl group which increased the lipophilicity of the molecule also improved the antileishmanial activity of the analogues. For example in *L. major*, an increase of 5.7- and 4.3-fold in activity was noted for the mono-*O*-alkyl analogues AS-HK044 and AS-HK077, respectively, compared to the parent curcuminoid AS-HK001. However, addition of the second alkyl group to the analogues AS-HK044 and AS-HK077 to give the corresponding dialkyl analogues AS-HK045 and AS-HK076 resulted in a sharp decrease in activity.

The conjugated keto system is also required for a curcuminoid analogue to exhibit high antileishmanial activity. However, analogues with a conjugated di-keto system (e.g. AS-HK033, AS-HK073), as well as analogues with the conjugated mono-keto system (enones, AS-HK016, AS-HK017, AS-HK094 – AS-HK098), displayed similarly high antileishmanial activity.

The cytotoxicity curcumin compounds was assessed by screening these compounds for cellular cytotoxicity in human kidney (HEK293) cells. The toxicity results using HEK cell line T293 revealed that the most tested compounds have a toxicity lower than curcumin itself (AS-HK001) which has been found to be safe and not toxic in all previous studies (Messner *et al*, 2009; <http://www.turmeric-curcumin.com>) and is widely used in large quantities as a food additive. Furthermore, the toxicological and pharmacological results on viability, GSH and protein contents exposed the absence of significant differences between the controls and treated hepatocytes with AS-HK001, AS-HK009, and AS-HK014. While AS-HK014 rapidly reduced hepatocytes GSH content to 20% of control, this did not affect the cell viability, suggesting that the lower intracellular concentration of GSH remained sufficient to protect these cells against oxidative damage. This result is similar to a previous result by (Carvalho *et al*, 2004), who found that cell necrosis *in vitro* and *in vivo* occurs only if the level of intracellular GSH decreases below 10-15% of the initial level. Furthermore, this effect of AS-HK014 is most probably linked to the use of the high drug concentration at 100 μM , which is 2000x higher than the actual EC_{50} value on trypanosomes. Di Monte and colleagues determined the status of hepatocyte thiols exposed to 200 μM of a compound called menadione and found that 85% of depleted glutathione is recovered in medium in the oxidized form (GSSG) (Di Monte *et al*, 1984).

It can be concluded that compounds: AS-HK001 and AS-HK009; are not toxic to hepatocytes, while AS-HK014 rapidly depletes hepatocytes of GSH, which may not necessarily be linked to hepatotoxicity. The strong antitrypanosomal activity of curcuminoids, particularly against multi-drug resistant strains, demands a more in depth analysis of their mode of action and structure activity relationships. These issues were addressed in chapters 6 and 7, respectively.

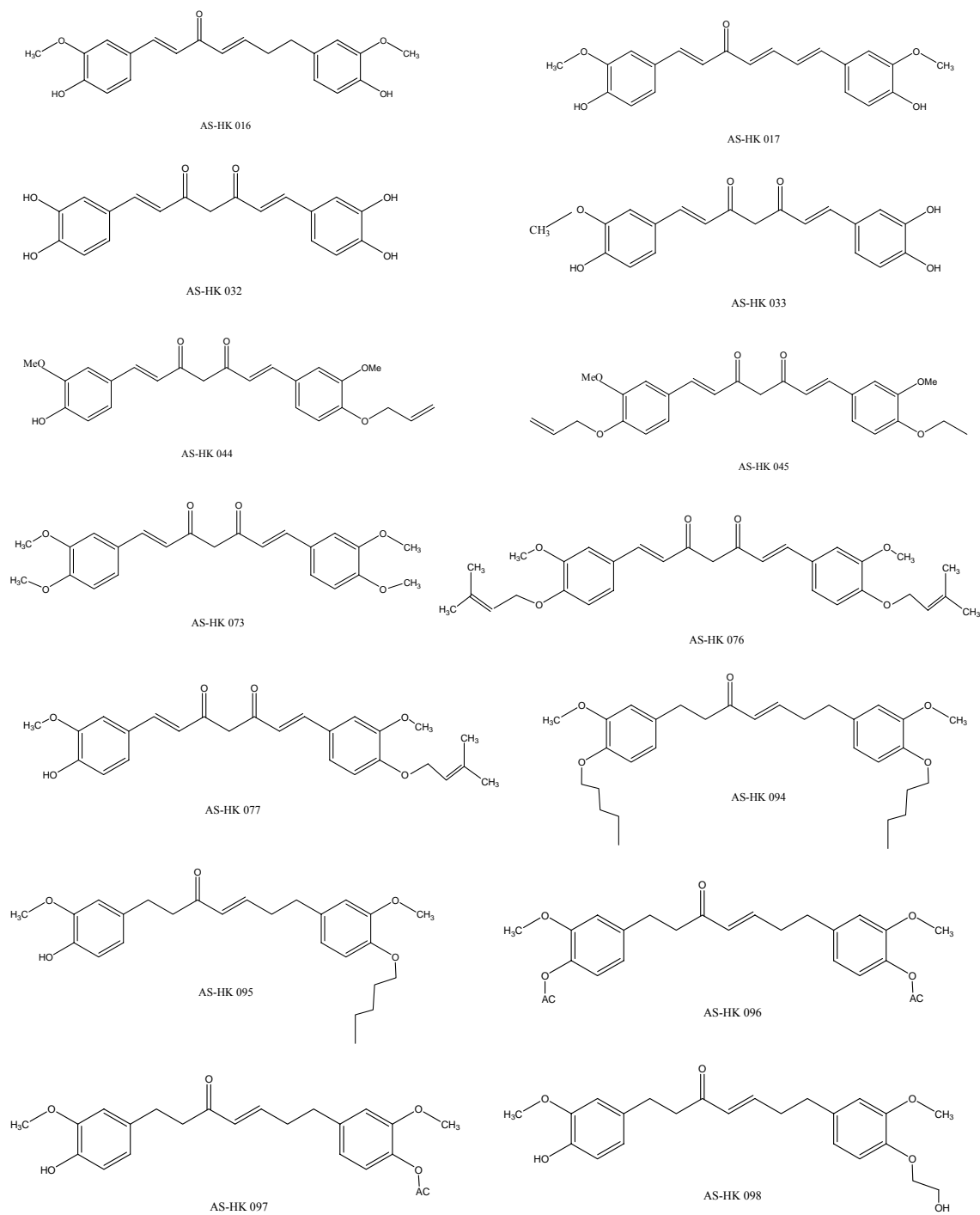


Figure 5.9 Chemical structures of some curcumin analogues that have shown antileishmanial activities.

Chapter six

6 Assessment of the mechanism of action of curcuminoid compounds on *Trypanosoma brucei*

6.1 Introduction

Trypanosoma brucei is a parasitic protozoan and the causative agent of Human African trypanosomiasis. Curcuminoids have been reported to possess antiparasitic activity against the causative agents of several diseases (Nose *et al*, 1998; Perez-Arriaga *et al*, 2006; Koide *et al*, 2002; Rasmussen *et al*, 2000). Although the activity of Curcumin against trypanosomes has been reported previously, its target in this parasite or mechanism of antiparasitic action is not well understood.

Curcumin has previously been reported to possess both anti-oxidant and pro-oxidant properties. On the one hand, curcumin has been shown to prevent lipid peroxidation and to scavenge radicals (Priyadarsini, 1997), to protect endothelial cells against oxidative stress (Motterlini *et al*, 2000b) and protect against oxidative stress induced by the carcinogen ferric nitrilotriacetate (Iqbal *et al*, 2009). Similarly it showed concentration dependant protection of human erythrocytes (Banerjee *et al*, 2008) and brain tissue (Rosello *et al*, 2008) against oxidative agents. On the other hand, curcumin has been implicated in DNA damage and apoptosis (Yoshino *et al*, 2004; Bhaumik *et al*, 1999), leading to the conclusion that curcumin displays both pro-oxidant and anti-oxidant properties depending on cell type, concentration and environment (Ahsan *et al*, 1999; Banerjee *et al*, 2008).

A large body of scientific literature has been generated in the last few years on the interactions of curcumin and curcuminoids with the oxidative stress responses in various cells and tissues. For instance, Kunwar *et al*. (2009) very recently reported that expression of oxidative stress protective enzymes glutathione peroxidase, catalase, Cu,Zn-superoxide dismutase and haeme oxygenase were dose-dependently increased on incubation of macrophages with curcumin. Yet, the situation remains complex and dependent on the exact cell type: the same paper reports a suppression of Mn-superoxide dismutase expression by curcumin. An emerging hypothesis is that curcumin acts as an antioxidant in normal cells and tissues (Kunwar *et al*, 2009; Motterlini *et al*, 2000; Aggarwal *et al*, 2007), partly through inhibition of the transcription factor NF-kappa B (Weber *et al*, 2006; Shishodia *et al*, 2007; Kunnumakkara *et al*, 2007), while damaging DNA, leading to apoptosis in cancer cells (Ahsan *et al*, 1999; Bhaumik *et al*, 1999; Hsu & Cheng, 2007) and broad chemopreventive properties (Goel *et al*, 2008). Indeed, the actions of curcumin on oxidative stress may be a function of concentration in some cases: 5 μ M curcumin protected murine macrophages from γ -irradiation-induced reactive oxygen species (ROS),

while 25 μM curcumin actually increased ROS formation under these conditions (Kunwar *et al*, 2009).

Very little is known about the effects of curcumin and its analogues on protozoan cells, but, considering the above, it is conceivable that this is the result of DNA damage and/or increased oxidative stress – either through the direct generation of ROS or through the inhibition of the mechanisms preventing oxidative damage.

During the normal metabolic process, all organisms produce a range of ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$) and superoxide ($\cdot\text{O}_2$) (Maxwell *et al*, 1999). These products should be immediately removed from the cells by enzymatic action, otherwise they can damage the cell macromolecules and cause cell death (Maxwell *et al*, 1999).

As observed by Sharma *et al*. (2005), curcumin's effect on the cell cycle and proliferation, like its effect on ROS, are not straightforward and likely to be cell type specific. For instance, curcumin inhibited proliferation and accumulation in the G2/M phase of the cell cycle in three different colon cancer cell lines [e.g.(Chen *et al*, 1999)] and similar effects have been observed in tumour cells from other tissues (reviewed by Sharma *et al*., 2005) including the a human breast cancer cell line (Simon *et al*, 1998). There have been reports of direct binding of curcumin to DNA, particularly to the minor groove (Stockert *et al*, 1990; Stockert *et al*, 1989; Zsila *et al*, 2004), preferring AT-rich regions (Bera *et al*, 2008), but it is unclear whether the effects on cell cycle could be attributed directly to this interaction.

Thus, a possible antiparasitic action of curcuminoids could be that it interferes in the synthesis of the parasite's DNA and thereby prevent parasite growth and proliferation. A trypanosome cell has four organelles that play critical roles in cell division: nucleus, single mitochondrion, the kinetoplast and the flagellum (Hammarton *et al*, 2003). There is a requirement for these cells to replicate and segregate these single organelles and interference in any of these processes could lead to cessation of parasite growth.

Using biochemical methods, this study investigated whether curcumin compounds are trypanocidal or trypanostatic. Specifically, it was investigated in detail whether curcumin or its trypanocidal analogues has any specific effect on the cell cycle, causing the parasites to accumulate in a particular stage of the cell cycle as described for the kinase inhibitor Hesperadin (blocks nuclear division and cytokinesis; (Jetton *et al*, 2009) and for

dihydroxyacetone (cell cycle arrest in G(2)/M phase; (Uzcategui *et al*, 2007). Cell cycle progression in the presence or absence of the test compounds was followed using flow cytometry for DNA content with propidium iodide. To investigate any effects on ROS production or oxidative stress defenses in trypanosomes, the fluorescent dye 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DH) was used to measure the redox state of the cells. DCFH-DH is a nonpolar compound which is rapidly taken up by the cell and converted to a nonfluorescent polar agent H₂DCF-DA. In presence of H₂O₂ or other peroxidises, H₂DCF-DA is rapidly oxidized to a highly fluorescent agent DCF (Zhu *et al*, 1994). Because this dye is very sensitive to the changes in redox state of cell, it was used in this study to follow changes in ROS levels over a period.

Since the compound need to be taken into the cell before an action, it is equally important to study the method of transport into the cell. We report here the kinetics of curcuminoids uptake into trypanosomes.

6.2 Effects of curcuminoids on *T. b. brucei* cell growth

6.2.1 Monitored by cell counts

Cell cultures were set up at 1×10^5 cells/ml for 24 h in the presence or absence of different concentrations of some lead curcumin analogues (AS-HK009, AS-HK014 and AS-HK027). Selection of these compounds was based on their activities against *T. brucei* BSF from the Alamar Blue assay (see chapter 5). The cells were incubated with the indicated compounds at 20 μ M and cell numbers were monitored by counting the cells with a haemocytometer under phase contrast microscope. At this concentration, all test compounds appeared to kill the trypanosomes within 24 hours, although the rate at which this happened varied. Compound AS-HK014 in particular induced rapid cell death, with over half the parasite population dead at 0.5 h and no live trypanosomes detectable after 2 hours (Figure 6.1). The onset of cell death was slower for the other test compounds, but exposure to any of them seems to have prevented further cell growth. The no-drug control cells continued to grow during the test period. Phenylarsine oxide (PAO), which is known to induce rapid lysis in *T. b. brucei* (Matovu *et al*, 2003) served as a positive control. The experiment was repeated with lower concentrations of test compound, set at 10 times the experimental EC₅₀ value for each compound, being 0.5 μ M for AS-HK014. The results were very similar to those obtained at 20 μ M, with near-complete cell death at 2 hours

exposure to AS-HK014 (data not shown). These results demonstrated that AS-HK014 is a fast acting drug and it rapidly kills the cells in about 2 h even with the onset of lysis in the first 15 minutes.

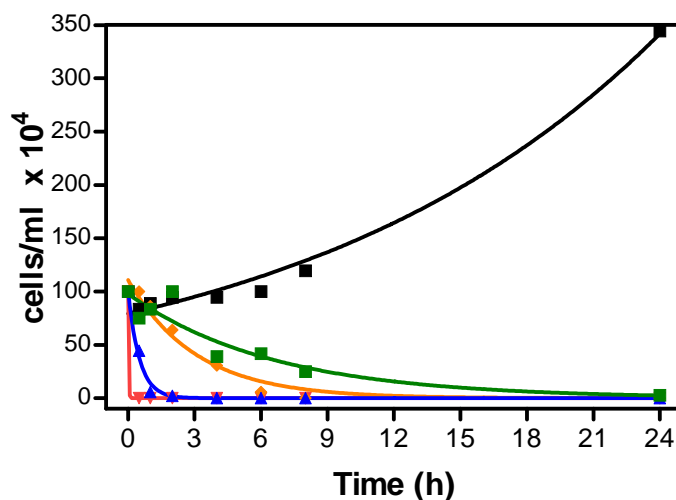


Figure 6.1 Effects of curcumin compounds on proliferation of *T. brucei* bloodstream forms.

Cells were cultured in HMI-9 medium at 37° C and 20 μM of AS-HK009 (■), AS-HK014 (▲), AS-HK027 (◆) and 0.5 μM of phenylarsene oxide (▼) were added and cell numbers counted at the indicated time intervals. The data were analyzed and compared with the untreated control cells (■).

6.2.2 Monitored by spectrophotometric lysis assay

Lysis assays monitoring absorbance at 750 nm with a Hewlett-Packard HP8453 was used to study the effect of curcumin analogues on trypanosome viability. Cells at $\sim 1 \times 10^8$ cells/ml were incubated with different concentrations of drugs (5, 10, 15 and 20 μM) and the effect of these compounds on cell viability was monitored. Using this assay, no clear activity of up to 20 μM of compounds AS-HK009, AS-HK027, AS-HK029 and AS-HK085 was detected over 8 hours of incubation time (Figures 6.2 and 6.3). In contrast, AS-HK014 was very active within the first two hours even at a low concentration of 5 μM (Figure 6.2). Cell lysis in the presence of this compound (at 15 μM) started at 1 hour and was complete at 3 hours. Compounds AS-HK093, AS-HK096, AS-HK097 and AS-HK098 were also observed to be very active causing cell lysis after 45 minutes which was complete at approximately 90 minutes. With AS-HK073, cell lysis started after 2 hours and was finished by 5 hours (Figure 6.3).

It was also investigated whether the co-administration of 20 μM of AS-HK014 with serial concentrations of pentamidine has an effect on the cell lysis. The activity of AS-HK014 at 20 μM was tested in the presence and absence of pentamidine at 1, 10, 100 μM and 1 mM. The results revealed that the activity of this compound was slightly decreased when pentamidine was added up to 100 μM , whereas the cell lysis was completely stopped and the drug activity was inhibited when 1 mM of pentamidine was added (Figure 6.4). It was concluded that, as up to 100 μM pentamidine does not antagonise AS-HK014, the activity of this compound is not dependent on the TbAT1 or HAPT transporters (Matovu *et al*, 2003). The involvement of LAPT, which can be inhibited by one mol pentamidine (De Koning, 2001a) can not be excluded.

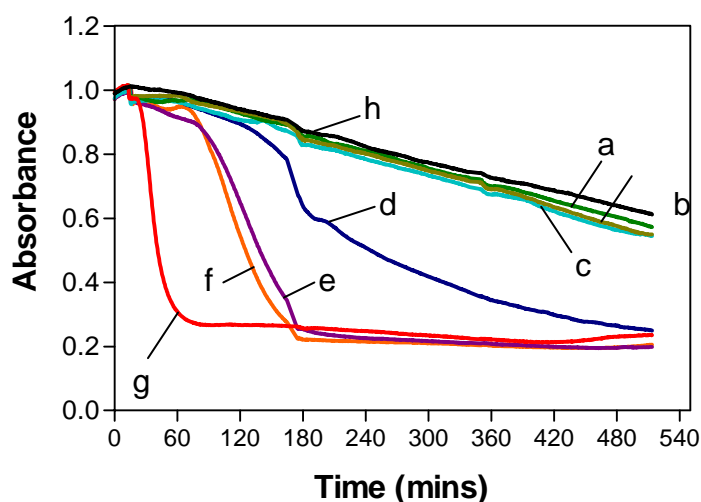


Figure 6.2 Inhibition curves of *T. brucei* BSF treated with different concentrations of curcumin compounds.

AS-HK009: 5 μM (a), 10 μM (b) and 20 μM (c); AS-HK014: 5 μM (d), 10 μM (e) and 20 μM (f). 0.5 μM Phenylarsine oxide (g) and drug free (h) were used as positive and negative controls, respectively.

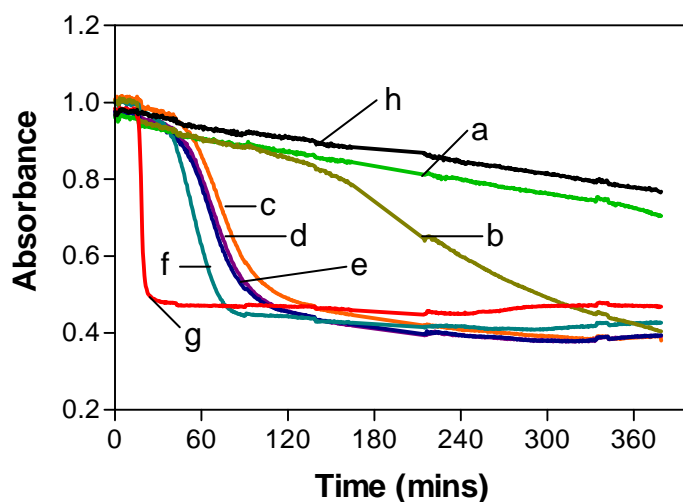


Figure 6.3 Inhibition curves of *T. brucei* BSF incubated with 20 μM of some curcumin compounds.

AS-HK085 (a), AS-HK073 (b), AS-HK098 (c), AS-HK097 (d), AS-HK096 (e) and AS-HK093 (f). 0.5 μM Phenylarsine oxide (g) and drug free (h) were used as positive and negative controls, respectively.

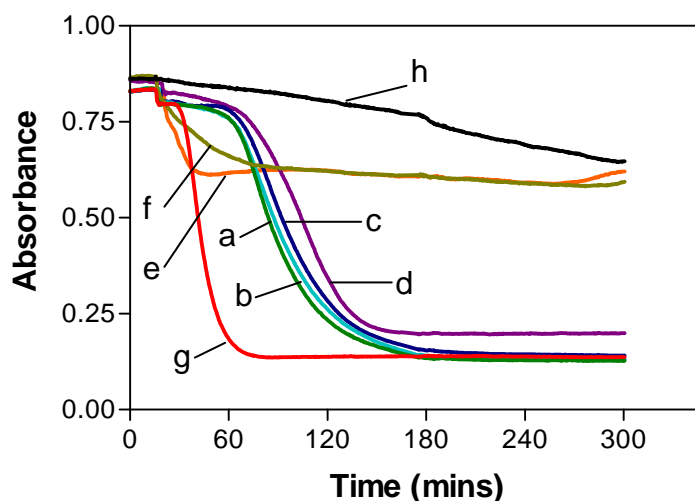


Figure 6.4 Effect of serial dilutions of pentamidine on the activity of 20 μM of AS-HK014 for lysing *T. brucei*, measured at 750 nm by light absorbance.

Traces: (a) 20 μM AS-HK014; (b) 20 μM AS-HK014 plus 1 μM pentamidine; (c) 20 μM AS-HK014 plus 10 μM pentamidine; (d) 20 μM AS-HK014 plus 100 μM pentamidine; (e) 20 μM AS-HK014 plus 1 mM pentamidine; (f) 1 mM pentamidine; (g) 0.5 μM Phenylarsine oxide; (h) drug free control. The experiment is representative of two similar repeats.

6.2.3 Monitored by propidium iodide fluorimetry

Since propidium iodide (PI) can not cross intact cell membranes but could penetrate only permeable membranes, it was used in this study to investigate the effect of serial dilutions of curcumin compounds on the cell viability, following a protocol recently developed in our laboratory (Gould *et al*, 2008). PI becomes strongly fluorescent upon entering the cell and binding to nucleic acids. This method is more accurate and high-throughput than the spectroscopic lysis assay (section 6.2.2) that it was designed to replace and allows the real-time monitoring of trypanosome viability in a 96-well plate format under a CO₂ atmosphere. Trypanosomes at 5x10⁶ cells/ml were exposed to various drug concentrations and fluorescence was measured for up to 8 hours in the appropriate conditions (37 °C with 5% CO₂ atmosphere).

The time-dose responses were investigated for the most promising curcumin analogues. In general, the results are consistent with the spectrophotometric lysis assays and cell counts. At a drug concentration of 3 µM, compounds AS-HK096, AS-HK093, AS-HK095, and AS-HK034 killed trypanosomes within 1, 2, 3 and 6 hours, respectively (Figures 6.5 and 6.6), whereas, 0.8 µM of compounds AS-HK097 and AS-HK014 killed the cells within 30 minutes and 6 h , respectively (Figures 6.5 and 6.6). Drugs AS-HK009, AS-HK029 and AS-HK080 at 50 µM killed the trypanosomes within 4, 6 and 8 hours, respectively (data not shown). The above results show that at least some of the curcuminoids, including AS-HK014, AS-HK034, AS-HK093, AS-HK095, AS-HK096 and AS-HK097, rapidly kill trypanosomes and must therefore be tentatively classified as trypanocidal rather than trypanostatic. Other compounds, such as AS-HK029 and AS-HK080, seem to act much more slowly – even at high concentrations – possibly reflecting a different mechanism of action. It is worth emphasizing that AS-HK080, AS-HK034 and AS-HK095 displayed very similar EC₅₀ values in the 72 hour Alamar Blue assay (see chapter 5).

6.2.4 Reversibility of trypanocidal effects

A further important question is whether the parasites need to be continuously exposed to the drug until cell death is complete, or whether a relatively brief exposure to the drug is sufficient to ‘condemn’ the parasites even if the test compound is withdrawn after a period. Using the spectrophotometric assay described in section 2.6.2, this was investigated for AS-HK014. The reversibility of this compound was tested by incubating the bloodstream forms of *T. brucei* cells with 20 µM of the drug and the absorbance was monitored for 30

minutes. After the 30 minutes incubation, the cells were spun at 2500 rpm for 5 minutes and washed twice in fresh HMI-9 medium and incubated again in the same conditions, or without drug. The results shown in Figure 6.7 indicate that 30 minutes exposure of the parasite to 20 μ M AS-HK014 is sufficient for irreversible cell lysis. A similar experiment was performed where a culture of trypanosomes was exposed to 20 μ M AS-HK014 or AS-HK027 for 30 minutes, after which the cells were washed into fresh media. After 24 hours no trypanosomes were detected. In contrast, exposure of up to 8 hours to 20 μ M AS-HK029 failed to clear all trypanosomes at the 24 hour point. This could be taken as further evidence for diversity in mechanism of action of the curcuminoid trypanocides. However, these results confirm that at least some curcumin compounds are trypanocidal rather than trypanostatic.

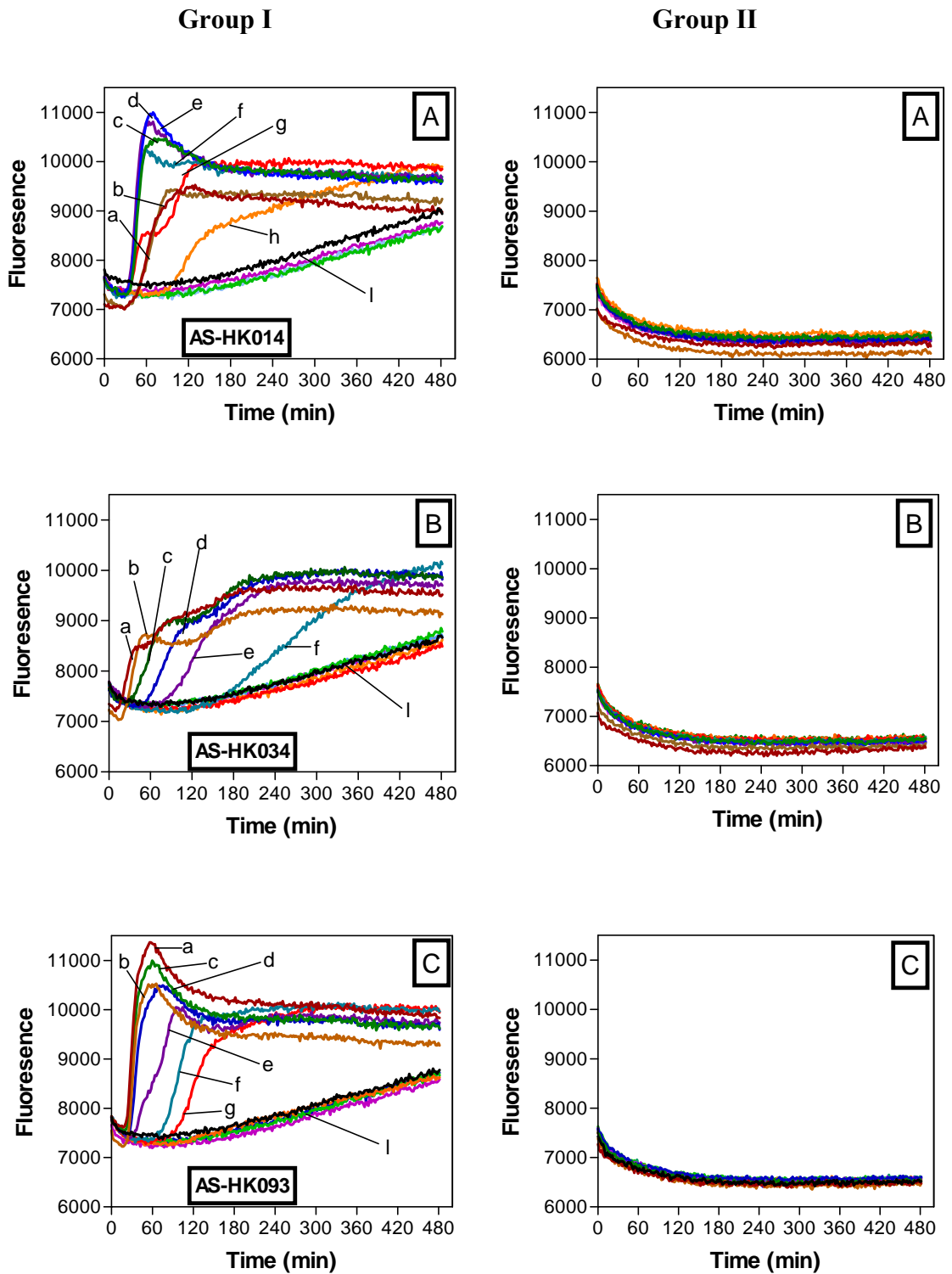


Figure 6.5 Dose response curves over time of some curcumin compounds.

AS-HK014 (A), AS-HK034 (B) and AS-HK093 (C). The drugs were incubated with (group I) and without (group II) *T. brucei* bloodstream forms in HMI-9 medium supplemented with 10% FCS under CO₂ atmosphere. The drugs were tested at different concentrations: 100 μM (a), 50 μM (b), 25 μM (c), 12.5 μM (d), 6.25 μM (e), 3.13 μM (f), 1.56 μM (g), 0.78 μM (h), 0.39 μM (i), 0.19 μM (j), 0.09 μM (k) and drug free (l). Propidium iodide at 9 μM was added and the fluorescence was monitored over 8 hours at 544 nm excitation and 620 nm emission.

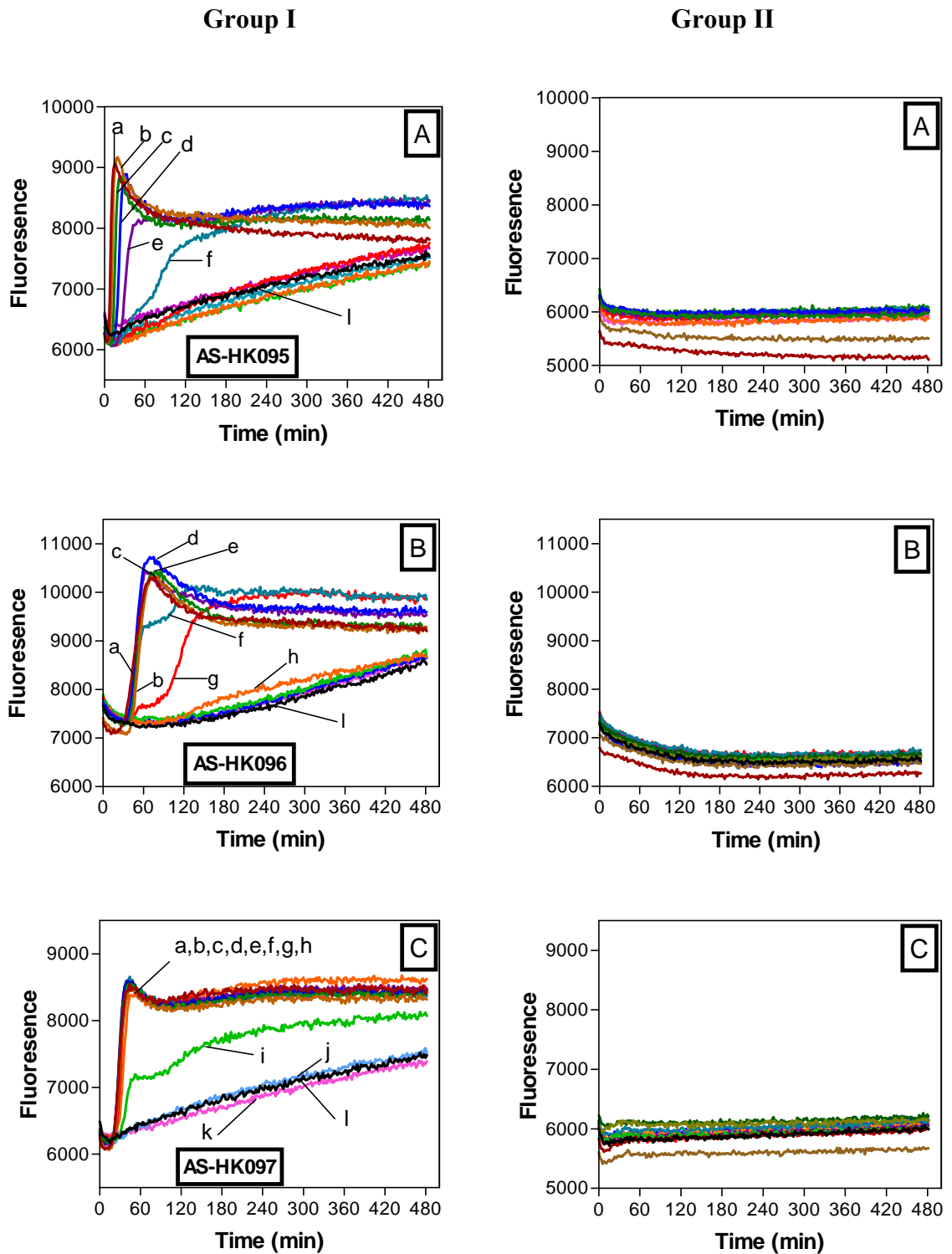


Figure 6.6 Dose response curves of some curcumin compounds.

AS-HK095 (A), AS-HK096 (B) and AS-HK097 (C). The drugs were incubated with (group I) and without (group II) *T. brucei* bloodstream forms in HMI-9 medium supplemented with 10% FCS under CO₂ atmosphere. The drugs were tested at different concentrations: 100 μM (a), 50 μM (b), 25 μM (c), 12.5 μM (d), 6.25 μM (e), 3.13 μM (f), 1.56 μM (g), 0.78 μM (h), 0.39 μM (i), 0.19 μM (j), 0.09 μM (k) and drug free (l). Propidium iodide at 9 μM was added and the fluorescence was monitored over 8 hours at 544 nm excitation and 620 nm emission.

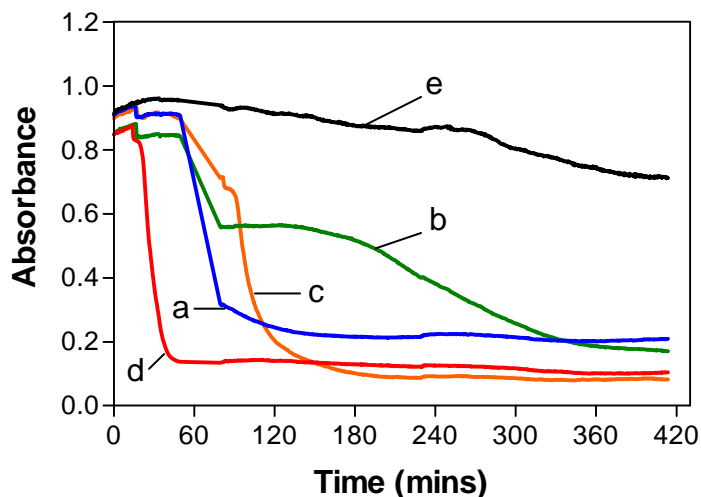


Figure 6.7 Reversibility curves of *T. brucei* s427 bloodstream forms with 20 μM AS-HK014.

Drug was added to cells ($\sim 5 \times 10^7$ cells/ml) in HMI-9 medium after 15 min of recording. Monitoring continued for 30 minutes at 750 nm and after which cells were washed twice and re-suspend in fresh medium and monitored again for 6 h . Traces: (a) No wash, (b) wash after 30 minutes and re-suspend in fresh medium, (c) wash after 30 minutes and resuspended in the same medium with 20 μM AS-HK014, (d) 0.5 μM Phenylarsine oxide , and (e) no drug control.

6.3 Curcumin analogues induce cell lysis rather than cell cycle abnormalities

The membrane permeability of treated and untreated bloodstream forms of *T. brucei* was determined using flow cytometry and the propidium iodide dye. The cells were treated for pre-determined times with different concentrations of some of the most active curcumin analogues. After the incubation time, cells at $\sim 2 \times 10^6$ cells/ml were prepared as described in section 2.10.2.

In a first experiment, the cells were analyzed at 8 and 20 h with the FACSCalibur and CellQuest software. This experiment showed only a minor effect of AS-HK014 (0.4 μM), AS-HK092 (1.5 μM), AS-HK095 (1.5 μM), AS-HK096 (0.3 μM), AS-HK097 (0.1 μM) and AS-HK098 (0.2 μM) on the cellular permeability. At 20 h , the percentages of the cellular permeability with these compounds were 4.1%, 3.5%, 6.2%, 3.9%, 5.1% and 3.7%, respectively, compared with 2.7% for the drug free control. However, compound

AS-HK014 had clear effect at 0.8 μM on the cellular permeability. At 8 h more than 90% of cells were permeable to PI (data not shown), resulting in cell lysis.

When the drug concentrations were increased to 1, 2 and 5 μM and the incubation time was decreased to 4 hours, untreated control trypanosomes showed only 2.6% cell lysis after 4 h of incubation (Figure 6.8). The cells exposed to AS-HK014, AS-HK096 and AS-HK097 at the various concentrations showed a rapid increase in PI permeability, which reached between 80% and 90% at 4 hours. The permeability was strongly increased at 2 and 5 μM for compounds AS-HK034 (>80%, Figure 6.8B) and AS-HK098 (>75%, Figure 6.8G) and at 5 μM of compound AS-HK095 (>70%, Figure 6.8D). However, there was no significant increase in the permeability even at 5 μM with compound AS-HK009, AS-HK029, AS-HK033, AS-HK045 (Figure 6.8H) and AS-HK073 (Figure 6.8C). For all the tested compounds, it was noted that the cellular permeability increased with increasing concentration of all drugs or the exposure time. This trend is evident in Figure 6.9 where the effects of 1 μM compound AS-HK096 on PI permeability started at 1 h (22.5%), continued at 2 h (27.3%) and reached 77% at 4 h. At 2 μM , the effect was approximately 49% at 1 h, increasing to 71.9% at 2 h, and almost completed at 4 h (87.2%). At 5 μM , the PI permeability was very clear after the first hour (72.8%) and nearly completed at 2 h (86.9%) with little further increase at 4 h (89.3%) (Appendix III).

To determine whether curcumin analogues arrest division of *T. brucei* bloodstream forms at any particular phase in the cell cycle, the DNA content of the treated trypanosomes was determined by flow cytometry. Untreated and treated cells with selected curcumin compounds (AS-HK014 and AS-HK034 at 0.4 μM ; AS-HK073, AS-HK092 and AS-HK095 at 1.5 μM ; AS-HK096 at 0.3 μM ; AS-HK097 at 0.1 μM and AS-HK098 at 0.2 μM) were lysed at 8, 12, 16 and 20 h in 70% methanol, 30% PBS. The samples were then kept in the fridge overnight or until the analysis. Cells were stained with 10 $\mu\text{g/ml}$ propidium iodide solution containing RNase at 10 $\mu\text{g/ml}$, and incubated in the dark at 37°C for 45 minutes before being analysed by flow cytometry. A summary of the results is shown in Figure 6.10 and indicate that there was no effect of any of the tested compounds on the overall DNA content of trypanosomes. Specifically the proportion of cells in G1 and G2/M phases did not change over a 20 h incubation period, with gated cells 45-60% in G1 phase and 15-21% in G2/M phase (Appendix III). For instance, there were no significant differences between untreated and treated cells with the most two potent compounds, AS-HK014 and AS-HK096, at G1 and G2/M phases up to 20 h.

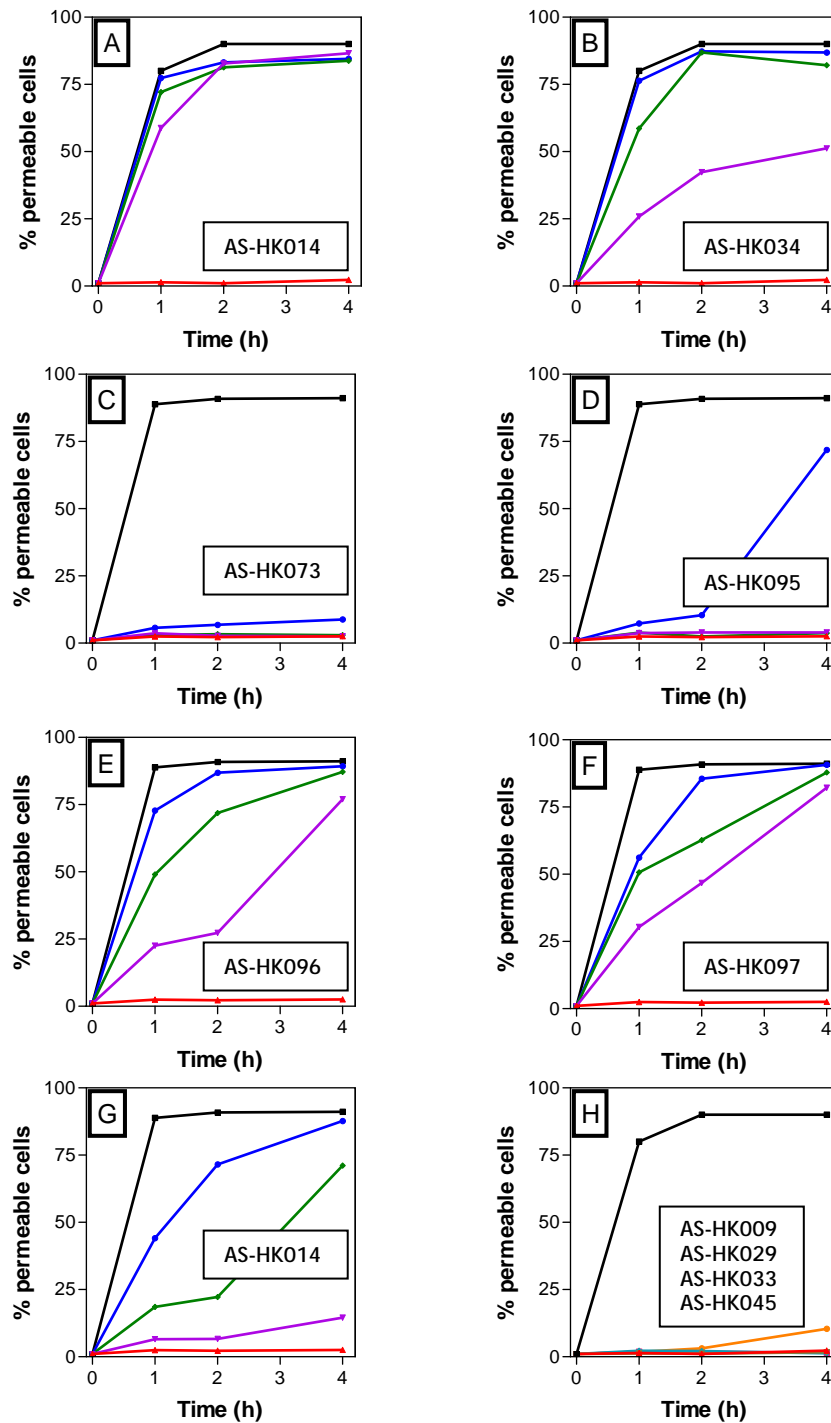


Figure 6.8 Flow cytometric analysis of *T. brucei* s427 bloodstream forms treated with some curcumin compounds.

Untreated cells, (\blacktriangle), concentrations at: 1 (\blacktriangledown), 2 (\blacklozenge), and 5 (\bullet) μM of compounds AS-HK014 (A), AS-HK034 (B), AS-HK073 (C), AS-HK095 (D), AS-HK096 (E), AS-HK097 (F), AS-HK098 (G), and with 5 μM of compounds AS-HK009, AS-HK029, AS-HK033, and AS-HK045 (H). Digitonin (\blacksquare) at 6 μM was used as a positive control. Cells were taken at 1, 2, and 4 h and stained with 5 $\mu\text{g/ml}$ propidium iodide and analyzed by flow cytometry for cellular permeability as described in section 2.10.2.

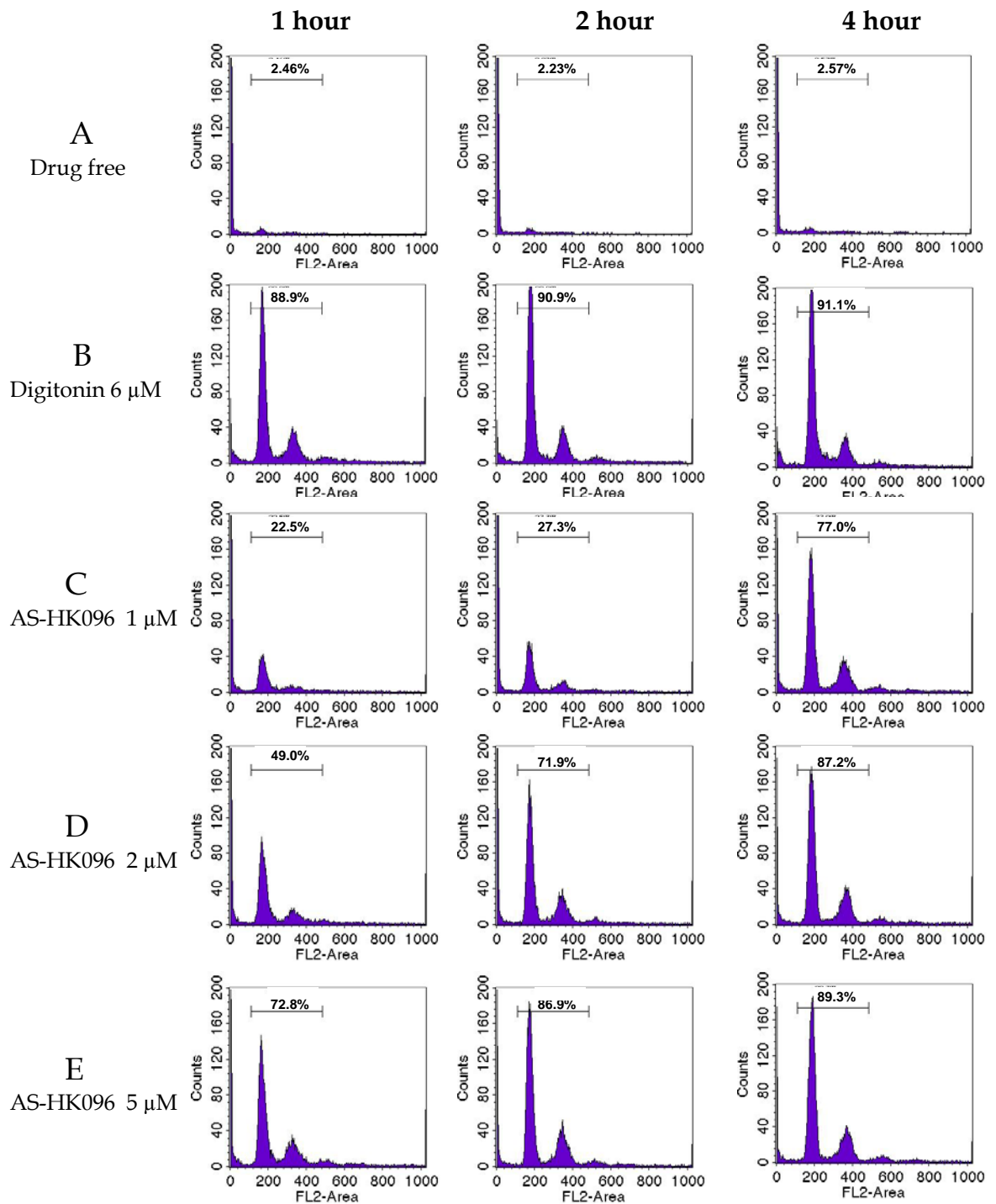


Figure 6.9 Propidium iodide staining of *T. brucei* bloodstream forms in FACS analysis.

Cells were treated for 1 h (I), 2 h (II) and 4 h (III) with three concentrations of AS-HK096: 1 μ M (C), 2 μ M (D) and 5 μ M (E) and prepared for analysis of cellular permeability. Drug free cells (A) and 6 μ M digitonin were used as negative and positive controls, respectively. The data shown are representative three independent experiments of three different concentrations of some curcumin compounds.

The results of DNA content also showed that the proportion of cells in G1 and G2/M phases were 53.6% and 16.9%, for AS-HK014 and 45.7% and 15.4%, for AS-HK096, compared with drug free control cells: 59.1% and 19.2% in G1 and G2/M phases, respectively. Furthermore, no significant changes were observed in the proportion of cells that contained 2K2N DNA or more, which did not exceed 5% on any of the samples. Zoids (cells that had < 1K1N DNA content) were excluded for the calculation of the percentages.

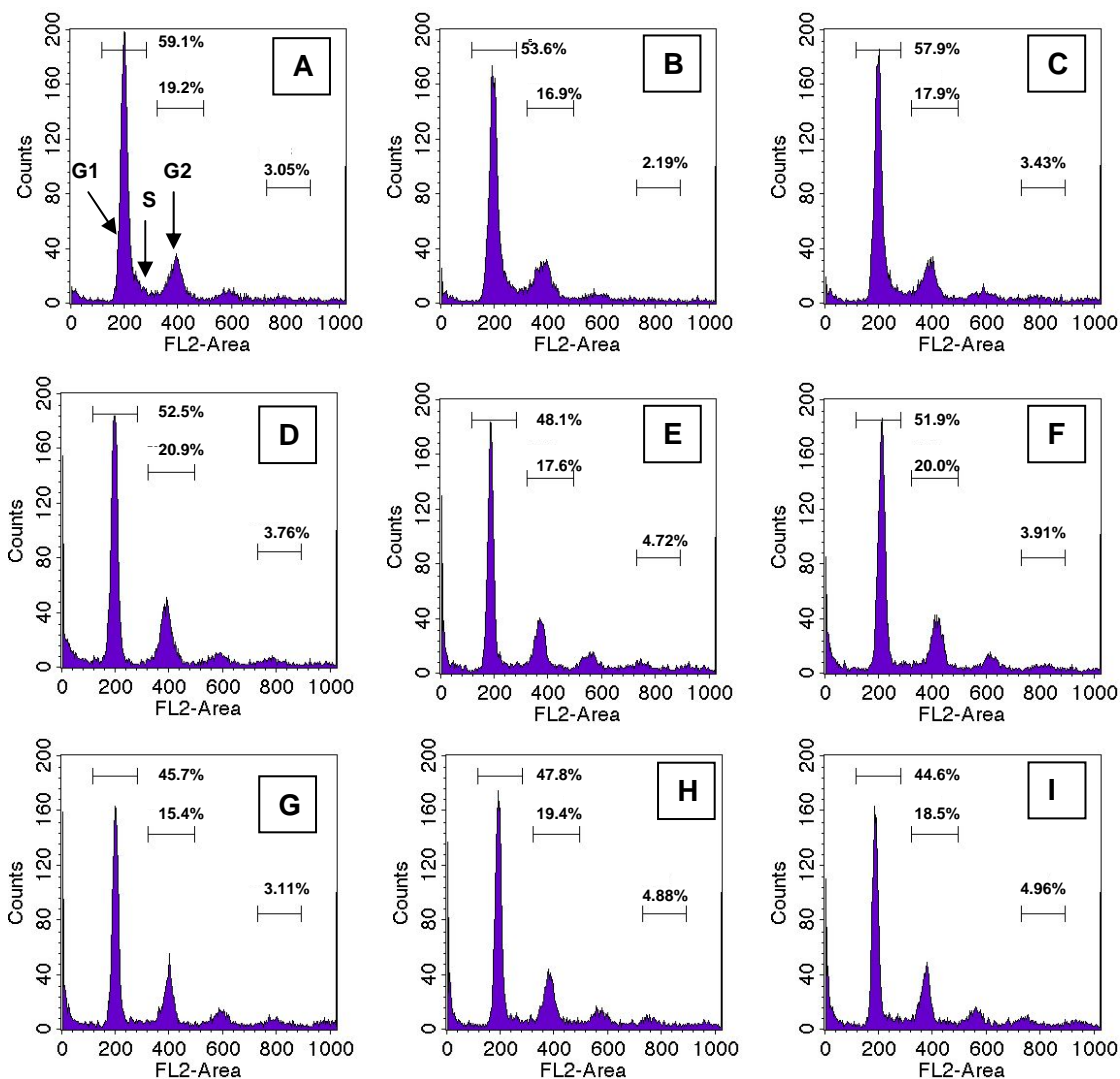


Figure 6.10 Analysis of DNA content of *T. brucei* bloodstream forms incubated with some curcumin analogues.

Cells were withdrawn after 20 h, fixed in methanol and stained with PI, after which the DNA was analyzed by flow cytometry as described in section 2.10.1. The graphs shown are histograms of the DNA content evaluated by collecting 10 000 events for each sample. Each histogram represents the amount of DNA for (A) untreated control; (B) AS-HK014 at 0.4 μ M; (C) AS-HK034 at 0.4 μ M; (D) AS-HK073 at 1.5 μ M; (E) AS-HK092 at 1.5 μ M; (F) AS-HK095 at 1.5 μ M; (G) AS-HK096 at 0.3 μ M; (H) AS-HK097 at 0.1 μ M and (I) AS-HK098 at 0.2 μ M.

6.4 Effects of curcuminoids on the *T. b. brucei* mitochondrial membrane potential

One of the cellular effects observed in tumour cell lines treated with curcumin is a reduction of the mitochondrial membrane potential Ψ_m or activation of mitochondria-mediated apoptosis pathways (Kellner & Zunino, 2004; Su *et al*, 2006; Karmakar *et al*, 2006) by promoting the opening of the mitochondrial permeability transition pore (Ligeret *et al*, 2004). On the other hand, curcumin attenuates mitochondrial dysfunction in diabetic rats (Rastogi *et al*, 2008).

To investigate whether the single mitochondrion of *T. b. brucei* might be a target for curcumin and its analogues, Ψ_m was determined in trypanosomes incubated in the presence or absence of test compounds, using the fluorescent dye tetramethylrhodamine (TMRE). The bloodstream forms of *T. brucei* were cultivated for up to 16 h in the presence or absence of different concentrations of curcumin lead compounds. Samples of the cultures were taken at 8 and 16 h and stained with 25 nM of TMRE and analyzed after 30 min at 37 °C by flow cytometry. The effects on Ψ_m of three control drugs were also determined.

Results show that addition of valinomycin (100 nM) or pentamidine (2 μ M) resulted in a maximal decrease of TMRE fluorescence from 50 % at 0 time to 2.3 and 23.5% at 8 h; and to 6.9 and 9.9% at 16 h, respectively. These decreases in fluorescence intensity indicate a reduction of the mitochondrial membrane potential (Figures 6.11B and 6.11D). In contrast, the potential was sharply increased with 10 μ M troglitazone with percentages of 80.6 and 82.5% at 8 and 16 h, respectively (Figure 6.11C). However, the fluorescence intensity in the drug free control was 49.7% at 16 h.

The results showed that no effect occurred up to 16 h in mitochondrial membrane potential of untreated cells (Figure 6.11A), nor with cells treated with: 5 μ M of AS-HK009, AS-HK027, AS-HK029 and AS-HK045; 1.5 μ M of AS-HK073, AS-HK092 and AS-HK095; 1 μ M of AS-HK033, AS-HK034 and AS-HK044. Similarly, the fluorescence did not change over time with 100, 200, 400 nM of compound AS-HK014; AS-HK097 at 0.1 μ M and AS-HK098 at 0.2 μ M. However, compounds AS-HK014 at 0.8 μ M and AS-HK096 at 0.3 μ M exhibited a clear decrease in mitochondrial potential from 50 % at 0 times to 24.7% and 37.3% at 8 h, and 21.7% and 31.9% at 16 h, respectively (Figures 6.11F and 6.11G). Even though these two compounds reduced the potential, yet the decreases were associated with cell death measured in parallel using FACS and propidium iodide stain. These results suggest that cell death was occurred by increased cellular permeability, which preceded

cell disintegration or extensive DNA fragmentation, and that the mitochondrion is not a primary target for curcuminoids in *T. brucei*.

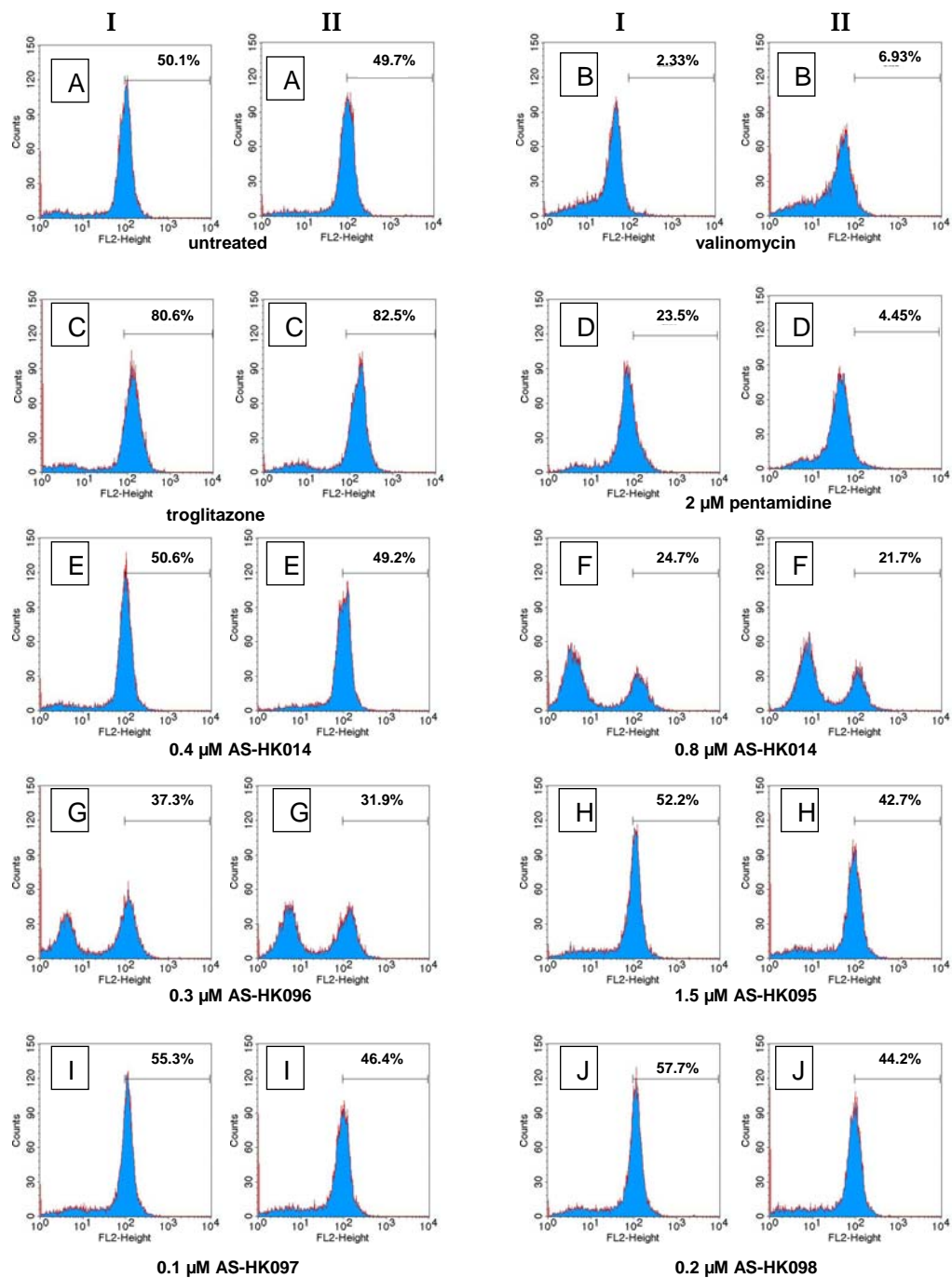


Figure 6.11 Flow cytometry analysis of *T. brucei* bloodstream forms stained with 25 nM of tetra-methylrhodamine (TMRE).

Untreated control (A) and cells treated with 2 μ M pentamidine (D); 400 nM AS-HK014 (E); 800 nM AS-HK014 (F); 0.3 μ M AS-HK096 (G); 1.5 μ M AS-HK095 (H); 0.1 μ M AS-HK097 (I) and 0.2 μ M AS-HK098 (J) were harvested at (I) 8 and (II) 16 h and prepared for mitochondrial membrane potential analysis as described in chapter 2. The positive controls were performed with 100 nM valinomycin (B) and 10 μ M troglitazone (C) for decreasing and increasing the mitochondrial membrane potential, respectively.

6.5 Curcuminoids do not increase the production of reactive oxygen species in trypanosomes

In order to assess whether curcumin compounds induce a rise in reactive oxygen species (ROS) in *T. brucei* bloodstream forms, dichlorofluorescein (DCF) fluorescence was monitored using a fluorimeter set at 485 nm excitation and 520 nm emission wavelengths. Three lead compounds, AS-HK014, AS-HK034 and AS-HK096, were used in this analysis. Cells in the stationary phase (2×10^6 cells/ml) in PBS were seeded into 96-well plate and exposed to serial doubling dilution of the curcumin compounds. The dye (10 μ M) was then added directly to all the wells and the fluorescence signal was measured over time. Drug free conditions, hydrogen peroxide, PBS without cells and H₂O₂ without cells were used as controls.

The results showed that there was no effect of any of the curcumin compounds tested on the generation of reactive oxygen species by *T. brucei* when compared with the no-drug control (100%) over 6 hours incubation. As shown in Figure 6.12, doubling dilutions (20-0.01 μ M) resulted in a fast dose dependent protection: increasing the drug concentration lead to a decrease in the level of ROS. For instance, the fluorescence intensity DCF percentage with 0.01 μ M AS-HK014 was 93.6% at 6 h, and was reduced to 21.9% when the drug concentration was increased to 20 μ M. However, all the treated cells had a lower ROS than the drug free control which did not exceeded 100% at the end of the experiment.

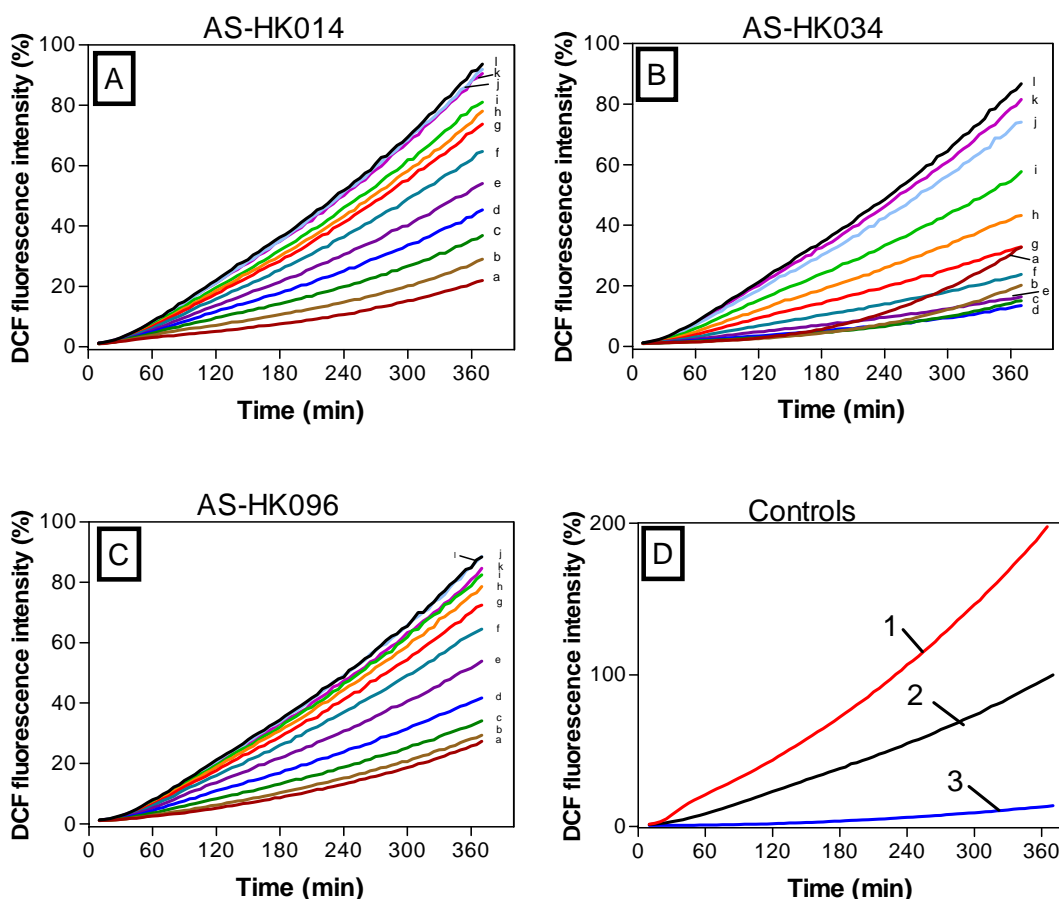


Figure 6.12 Curcumin analogues dose-dependently inhibit intracellular ROS generation bloodstream forms of *T. brucei*.

2×10^6 *T. b. brucei* were incubated in PBS for 6 hours in the presence of $10 \mu\text{M}$ of DCF at room temperature. The intensity of DCF fluorescence was monitored in the absence and presence of serial doubling dilutions of AS-HK014 (A), AS-HK034 (B) and AS-HK096 (C). Drug dilutions were at $20 \mu\text{M}$ (a); $10 \mu\text{M}$ (b); $5 \mu\text{M}$ (c); $2.5 \mu\text{M}$ (d); $1.25 \mu\text{M}$ (e); $0.63 \mu\text{M}$ (f); $0.33 \mu\text{M}$ (g); $0.16 \mu\text{M}$ (h); $0.08 \mu\text{M}$ (i); $0.04 \mu\text{M}$ (j); $0.02 \mu\text{M}$ (k) and $0.01 \mu\text{M}$ (l). Hydrogen peroxide at $100 \mu\text{M}$ (D1), drug free cells (D2) and PBS without cells (D3) were used as controls. This experiment is a representative of four separate experiments. The fluorescence level of the drug-free control was set as 100% in order to simplify comparisons between treated and non-treated cells.

6.6 Curcumin transport by *T. b. brucei* BSF

6.6.1 Uptake of [^3H]-curcumin

In order to understand the mechanism of action of curcumin analogues, the uptake of these compounds into the bloodstream forms of *Trypanosoma brucei brucei* was investigated using standard uptake techniques. It was investigated whether the test curcumin compounds are taken up by the parasite through a specific transporter. While a vast

literature on the pharmacology of curcumin has emerged over the last few years, virtually nothing is known about curcumin uptake into cells, although it is now known that curcumin can inhibit ABC-transporters in various tissues (Ishikawa, 2009) but these are efflux transporters and do not mediate uptake into cells.

Uptake of [^3H]-curcumin into *Trypanosoma brucei brucei* bloodstream forms was performed using a rapid oil-stop protocol as described in section 2.9 and exactly as published previously (Wallace *et al*, 2002). All assay points were performed in triplicate and in three different experiments. Radioactivity in the cell pellets was determined using liquid scintillation counting.

The time-course assessment of curcumin uptake by *T. brucei* bloodstream forms showed that the transport of 1 μM [^3H]-curcumin was slow, and inhibited by 100 μM of unlabeled curcumin over 60s (Figure 6.13). [^3H]-curcumin uptake was also inhibited by 100 μM of AS-HK014, over 120s (Figure 6.14). Overall, the uptake rate of [^3H]-curcumin by *Trypanosoma brucei brucei* bloodstream forms was very low $0.088 \pm 0.06 \text{ pmol}(10^7 \text{ cells})^{-1} \text{ s}^{-1}$ (n=4).

No radiolabel of any of the curcumin analogues was available and the only [^3H]-curcumin commercially available was of insufficient specific activity to conduct detailed kinetic studies.

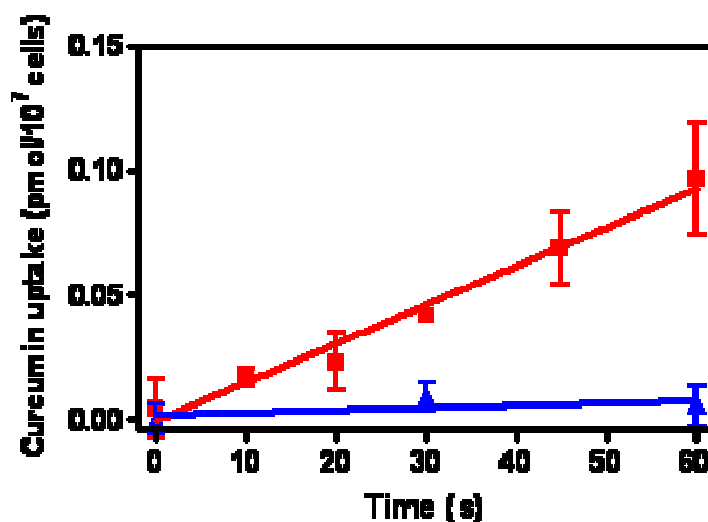


Figure 6.13 Time course of 1 μM [^3H]-curcumin uptake by bloodstream forms of *T. brucei* 427.

Figure shows upake in the presence (▲) or absence (■) of 100 μM unlabelled curcumin over 60 seconds.

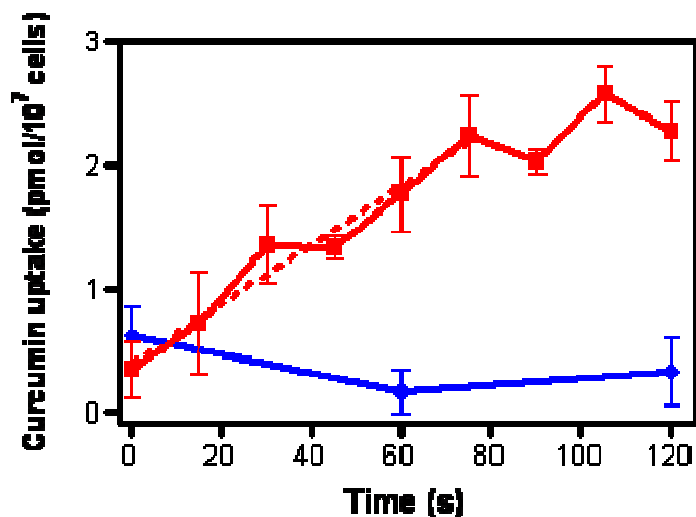


Figure 6.14 Time course of 1 μM [^3H]-curcumin uptake by bloodstream forms of *T. brucei* 427.

Uptake in the presence (◆) or absence (■) of 100 μM AS-HK014 over 120 seconds.

6.6.2 Inhibition of pentamidine transport by curcumin analogues

To investigate whether curcumin analogues inhibit the activity of pentamidine transporters in *T. brucei* bloodstream forms and thus might be substrates for these transporters, inhibition of pentamidine uptake was assessed in bloodstream forms. Inhibition by curcumin and AS-HK014 of the uptake of 1 μM and 40 nM [^3H]-pentamidine through low affinity pentamidine transporter (LAPT1) and high affinity pentamidine transporter (HAPT1), respectively was investigated. These assays were determined in three independent experiments, and each experiment was performed in triplicate. The results indicated that there was no effect of these two compounds on [^3H]-pentamidine uptake through LAPT1 (Figure 6.15). However, both compounds inhibited HAPT at high concentrations. (Figure 6.16), with K_i values of 568 (n=4) and 398 (n=3), respectively. As these concentrations are several orders of magnitude above the level at which the compounds kill trypanosomes, it is highly improbable that HAPT contributes significantly to the uptake of these compounds.

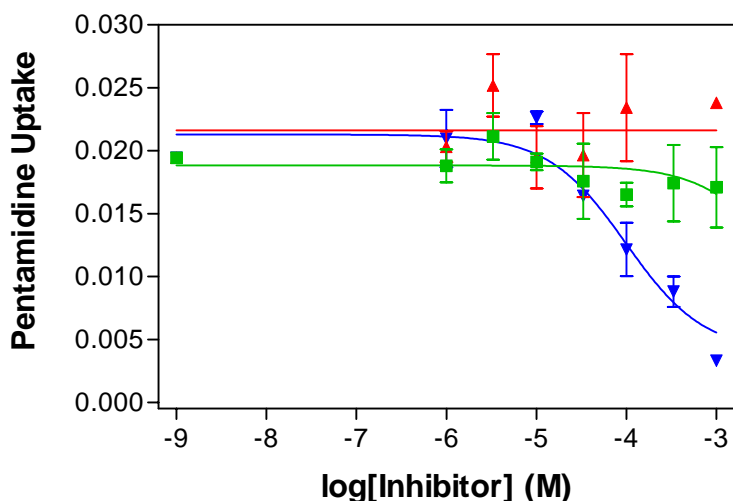


Figure 6.15 Inhibition of 1 μM [^3H]-pentamidine uptake (low affinity pentamidine transporter) in bloodstream forms of *T. brucei* 427 by various concentrations of curcumin analogues.

Curcumin (■), AS-HK014 (▲), and unlabelled pentamidine (▼). Only pentamidine inhibited uptake of radiolabel with IC_{50} values of 99 μM .

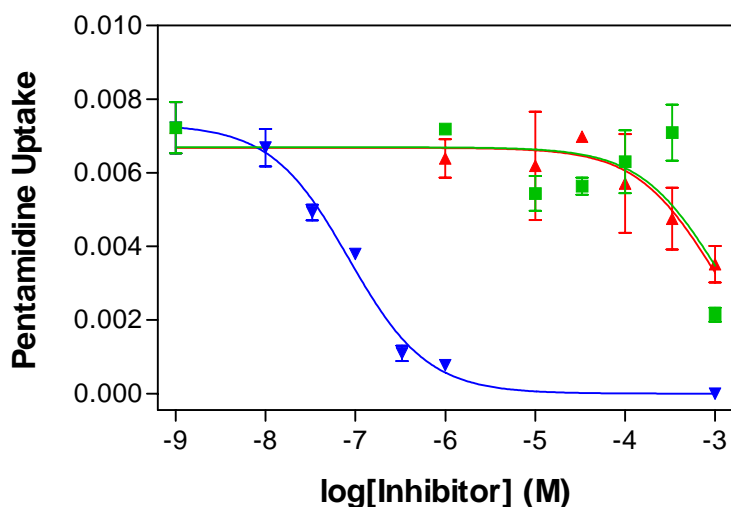


Figure 6.16 Inhibition of 0.04 μM [^3H]-pentamidine uptake (high affinity pentamidine transporter) in *T. brucei* 427 bloodstream forms by various concentrations of curcumin analogues.

Curcumin (■), AS-HK014 (▲), and unlabelled pentamidine (▼), with IC_{50} values of 1.1 mM, 1.0 mM and 0.085 μM , respectively. The values for curcumin and AS-HK014 were extrapolated from the data using a value of -1 for the high slope and a maximum inhibition to zero uptake (level of the uptake in the presence of 1 mM unlabeled pentamidine).

6.7 Preliminary assessment of *in vivo* toxicity and trypanocidal activity of curcumin analogues

Preliminary results of determining the acute toxicity of curcumin drugs showed no toxic effects of AS-HK009, AS-HK014, and AS-HK027 when they were injected intraperitoneally into mice model (ICR strain) at 50 mg/kg BW. Therefore, a series of experiments was conducted to determine whether these compounds have an activity against trypanosomiasis in a mouse model.

In the first experiment, two doses of drugs were injected i.p. into ICR mice, 6 and 24 h after infection with *T. brucei* bloodstream forms. Parasitaemia appeared in the bloodstream on the fourth day. No differences in survival rate were observed between the treated and negative control untreated groups during the 7 days of the experiment (Figure 6.17.A). In all three curcuminoid-treated groups, average parasitaemia was lower than in the untreated control, particularly on day 4 PI. In contrast, there was a very clear difference between the positive control group, which was treated with 7 mg/kg diminazene, and the negative control group, in that no parasitaemia was observed in this group. The experiment was repeated with one treated group (AS-HK014) in addition to the positive and negative control groups. In this experiment, the number of AS-HK014 doses was increased to 4 administrations, the first one after 6 h from incubation and 24, 48 and 72 hours after the first injection. Very similar results were obtained from this repeat and no differences were observed in survival rates between treated and control groups (Figure 6.17.B).

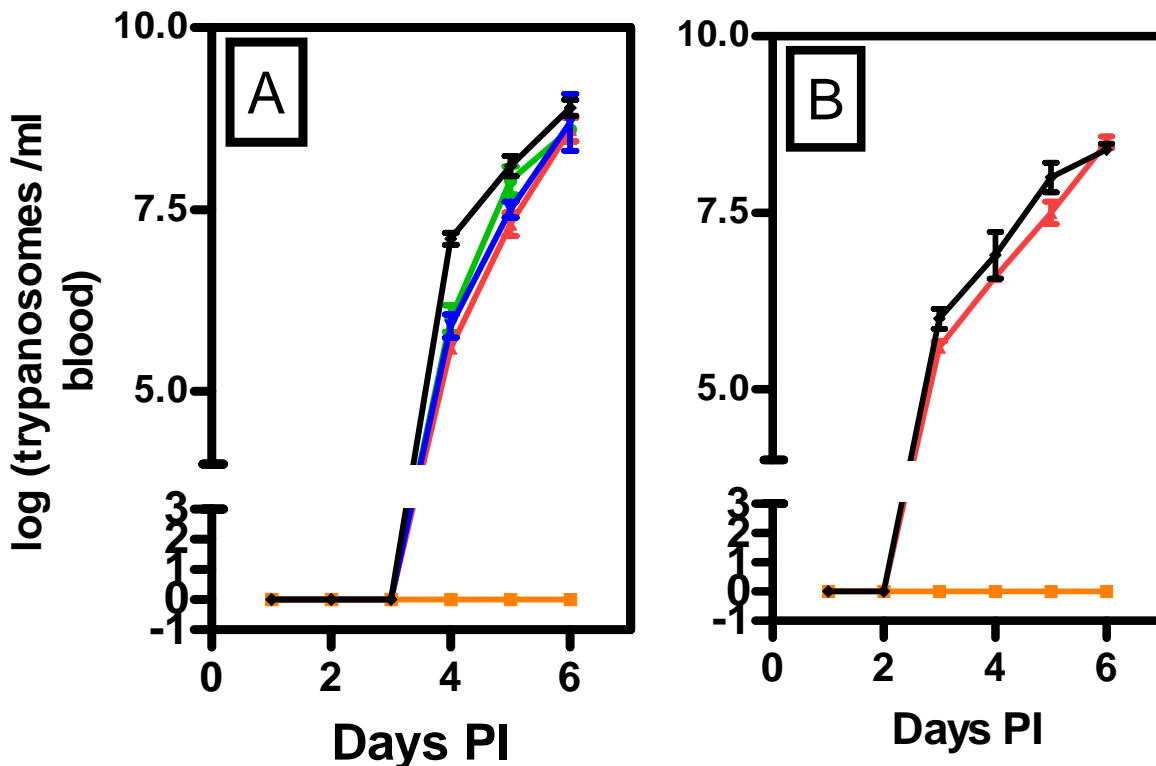


Figure 6.17 Changes in parasitaemia levels of mice infected with bloodstream forms of *T. brucei* when treated with some curcumin compounds.

AS-HK009 (●), AS-HK014 (▲) and AS-HK027 (▼). Drugs were given at 50 mg/kg in 2 doses at 6 and 24 h after infection (A) or 4 doses at 6, 24, 48 and 72 h after infection (B). Drug free (◆) and diminazene 7 mg/kg (■) were used as negative and positive controls, respectively. Mice were euthanized on day 6 PI. Data shown are the average and SE of five mice of each group.

6.8 Discussion

The possible mechanism of action of curcumin analogues was investigated in this study. The analogues studied, AS-HK009, AS-HK014 and AS-HK027, were selected from a small library of curcumin compounds. The choice of these compounds was based on the results from Alamar Blue assays, showing that they have a strong antiparasitic activity against *T. brucei* BSF. The ability of these compounds to cause cell lysis was first investigated. Monitoring cell lysis either by direct cell count, spectrophotometric or the propidium iodide assay, it was very clear that compound AS-HK014 caused the most rapid lysis of the cells. This compound caused cell lysis in less than an hour at a concentration below 1 μM .

Since the cytotoxicity effects of curcumin on cancer cells is due to the pro-oxidant activity of this compound through the generation of ROS (Bhaumik *et al*, 1999), we investigated whether curcuminoids increase the level of ROS in *T. brucei* BSF. None of the tested curcumin analogues caused an elevation in the ROS levels, and in fact seemed to display anti-oxidant properties, as all the curcuminoids reduced the level of ROS in the incubation medium. Therefore their mode of action could not be due to an inhibition of the enzymes that protect the cell from damage by ROS. However, a recent study on *Leishmania donovani* promastigote treated by curcumin at 25 μM resulted in an increase of ROS production within two hours (Das *et al*, 2008). This result appears to conflict with our findings in *T. brucei*. However, curcumin has been found to act as antioxidant or as pro-oxidant agent in different cell types (Ahsan *et al*, 1999). These two reactions are depending on the drug concentration in the different species of parasites (Das *et al*, 2008). For instance, in *Plasmodium* 1 μM curcumin was enough to cause ROS generation, whereas concentrations below 1 μM lead to decrease in ROS concentration (Le Bras *et al*, 2005).

Several observations lead to the conclusion that most of the curcuminoids, as exemplified by AS-HK014, tested display trypanocidal activity rather than a trypanostatic effect. Using several investigative techniques, it was consistently shown that these compounds rapidly kill the trypanosomes instead of merely preventing further growth. In addition, low concentrations of these compounds, with a delayed onset of parasite killing, did not cause any apparent change in the proportion of cells in G1, S or G2/M phase of the cell cycle, which would have been indicative of cell cycle arrest. Instead, the curcuminoids seem to rapidly cause a permeabilisation of the plasma membrane, as visualised with propidium iodide, leaving the nuclear DNA intact and unfragmented. This effect on plasma

membranes seems to precede any effect on the mitochondrial membranes: the Ψ_m remained unaffected up to the point of cell death. For example, incubation for 16 hours with AS-HK014 or 0.3 μM AS-HK096 lead to two distinct populations being observed in flow cytometry with TMRE: cells with a normal Ψ_m identical to the untreated control, and those with a completely depolarised mitochondrial membrane. Considering the timing and dosage, the latter event is most probably the result of plasma membrane breach.

The question of the mechanism of the trypanocidal mode of action of curcumin and its analogues thus appears to narrow down to how these compounds compromise the plasma membrane of the trypanosome. Some authors (Das *et al.*, 2008; Cao *et al.*, 2006) have described lipid peroxidation in response to curcumin treatment, following increases in ROS. However, as we find that curcumin actually protects *T. b. brucei* against ROS that it itself generates, this does not seem a likely mechanism. This conclusion is more in line with the emerging consensus in the literature that curcuminoids usually protect against lipid peroxidation (reviewed by Goel *et al.*, 2008) and we will next focus on parameters such as plasma membrane potential, calcium influx and intracellular pH.

The extraordinary efficacy of some curcuminoids, killing trypanosomes both very potently and rapidly *in vitro*, shows that these compounds must be taken up efficiently by the parasite. We have tried to assess this using the only radiolabel available: [^3H]-curcumin from Moravek – no labelled compound is available for any of the analogues and the cost of custom synthesis is prohibitive. The rate of 1 μM curcumin uptake, at 0.088 ± 0.06 $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$ was found to be slow compared to other substrates (e.g. hypoxanthine, glucose, adenosine, uracil; (de Koning & Jarvis, 1998) but uptake rates are concentration dependent and the low specific activity of the label did not allow us to obtain K_m or V_{max} values. Moreover, curcumin itself displays only moderate trypanocidal activity and it is well possible that the more potent activity of some curcuminoids is the result of much faster influx. Even so, this rate is comparable to the V_{max} of the highly successful trypanocides pentamidine and diminazene aceturate on the TbAT1/P2 transporter (0.068 ± 0.007 and 0.049 ± 0.010 $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$, respectively; (De Koning, 2001b; De Koning *et al.*, 2004).

Curcumin is clearly not principally taken up by passive diffusion, as its accumulation is saturable, being inhibited both by unlabelled curcumin and by AS-HK014. This in itself is a significant finding as we are unaware of any studies assessing curcumin uptake in any cell type and the compound is sufficiently lipophilic ($\text{cLogP} = 2.56$; ChemDraw Pro 10.0,

CambridgeSoft) to have a significant diffusion rate across biomembranes. However, there is no correlation between cLogP and trypanocidal activity (Figure 6.18).

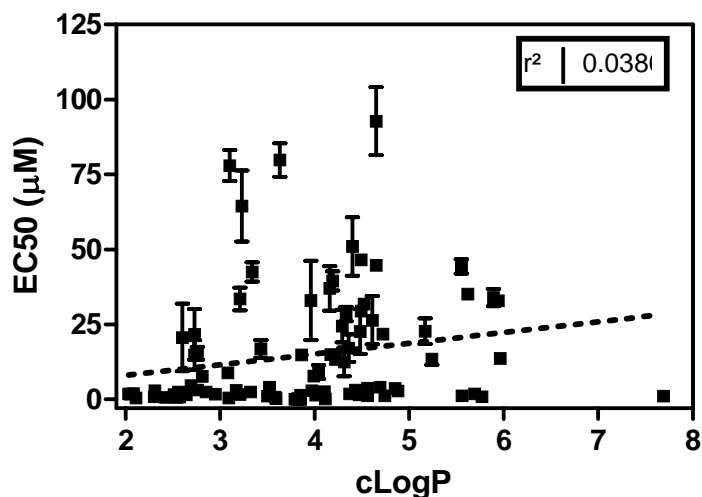


Figure 6.18 The correlation between cLogP and trypanocidal activity of curcumin analogues.

Curcumin displays considerable structural similarity with pentamidine, being a symmetrical molecule with two substituted benzene rings connected by a 7-atom linker chain. It was thus reasonable to test whether curcumin inhibits the previously reported pentamidine transporters HAPT and LAPT (De Koning, 2001b). However, neither curcumin nor AS-HK014 inhibited these transporters at relevant concentrations and the route of entry for these compounds remains unresolved for the moment. While the TbAT1/P2 adenosine transporter is also known to transport pentamidine, its substrate selectivity is now very well understood (De Koning *et al.*, 2005) and, as pentamidine recognition depends on its amidine domains, which are not present in curcuminoids, this transporter is also not expected to contribute to curcumin uptake.

With regard to the *in vivo* study, it was shown that compounds AS-HK009, AS-HK014, and AS-HK027 have no significant effect on trypanosomes in an infected mouse model (ICR strain); at drugs level up to 50 mg/kg body weight there was no effect of the compound on parasitaemia. Given that the motivation for this study is to identify compounds that can be used *in vivo* this finding is worrying. This finding maybe referred

to the bioavailability of curcumin which is the most major problem for this compound (Anand *et al*, 2007). The bioavailability of curcumin is due to the rapid metabolism and poor absorption of curcumin (Maheshwari *et al*, 2006; Anand *et al*, 2007). Holder *et al* (1978) found that 75% of curcumin was excreted in the faeces when it was intraperitoneally administered in rats. It is also possible that curcumin compounds get bound to serum protein such as albumin before they could unleash their therapeutic effect. But this is a weak possibility because all the previous studies found negligible amounts of curcumin in blood plasma after oral administration in rats (Wahlstrom & Blennow, 1978; Ravindranath & Chandrasekhara, 1980; Ravindranath & Chandrasekhara, 1981) or after oral or intraperitoneal administrations in mice (Pan *et al*, 1999).

Though important findings were made in this study, however the full mechanism of action of the curcumin on trypanosomes remains unknown. Comprehensive investigations taking into consideration the findings in this study are therefore required to fully decipher the mode of action of curcumin on *T. b. brucei*.

Chapter seven

7 curcumin Structure-activity relationship in *T. brucei*

7.1 Introduction

The urgent need for new drugs against African trypanosomiasis is clear, as all drugs that are used now against this disease are expensive and moderately to extremely toxic. Furthermore, the parasites became resistant to some of these drugs.

Curcumin, a yellow dye extracted from *curcuma longa*, exhibits a broad range of the biological activities. It has anti-oxidant (Rajakrishnan *et al*, 1999), anti-inflammatory (Srimal & Dhawan, 1973; Mukhopadhyay *et al*, 1982), anti-cancer (Sharma *et al*, 2001; Shukla *et al*, 2003) and anti-protozoal properties (Rasmussen *et al*, 2000; Koide *et al*, 2002; Nose *et al*, 1998; Perez-Arriaga *et al*, 2006; Cui *et al*, 2007a; Reddy *et al*, 2005).

Based on these properties of curcumin, 158 compounds that are analogues of curcumin have been synthesized to improve the potency of curcumin as antitrypanosomal agents. All these compounds contain a 7-carbon spacer between aromatic rings except one compound (AS-HK129) which contains a 5-carbon spacer. Significantly, many of these analogues were more active than curcumin (see chapter 5). Some of these compounds such as AS-HK025, AS-HK073 and AS-HK088, were previously synthesized and tested against *Plasmodium falciparum* with EC₅₀ values of 2.3, 7.9 and 8.4 μ M, respectively (Mishra *et al*, 2008). All these analogues were tested in triplicate against *T. brucei* 427 to determine the EC₅₀ values. To date, no systematic attempts have been made to correlate curcuminoid structure to antiprotozoal activity. However, the extensive data set obtained in our study allows us to attempt just that. The most active compounds were used as pharmacophore to design new lead compounds. In this chapter, a comprehensive study will be presented with respect to the structure-activity relationship (SAR) of curcumin and its 157 analogues with regards to their antitrypanosomal activities.

7.2 Results and discussion:

To establish an extensive structure-activity relationship (SAR) for curcumin analogues as anti-trypanosomal agents, the analogues were divided into ten groups based on their structural characteristics. To see whether the methoxy substitutions on the aromatic ring exert any effect on the biological activity of curcuminoids, the demethylated analogues AS-HK033, AS-HK032 and AS-HK019, and the methylated analogues AS-HK073, AS-HK075, AS-HK074 and AS-HK020 were evaluated for antitrypanosomal activity. It was found that the mono-*O*-demethylated curcuminoid AS-HK033 exhibited approximately 3-fold higher activity than the parent compound, curcumin (AS-HK001). Further demethylation to the di-*O*-demethylated analogue AS-HK032, however, resulted in about 2-fold decrease in activity, though it still was more active than compound AS-HK001. Compound AS-HK019, the demethylated analogue of the parent curcuminoid AS-HK002, was 7-fold more active than the parent with an EC₅₀ value of $0.65 \pm 0.02 \mu\text{M}$. Rather surprisingly, it has been found that compound AS-HK073, the *O*-methyl analogue of compound AS-HK001, was almost 6-fold more active than its parent compound AS-HK001, whereas the mono- and di-*O*-methylated analogues AS-HK075 and AS-HK074 were almost 3-fold more active than their parent compound AS-HK002. However, the *O*-methylated analogue AS-HK020 was slightly less active than the parent curcuminoid AS-HK003. The contribution from the methoxy and hydroxyl substitutions is thus not straightforward. The hydroxyl groups can function as both H-bond acceptor and donor, whereas the methoxy groups can only accept such bonds. Both substitutions would increase electron density on the rings increasing the binding contribution from any Π -stacking, but the methoxy substitution would do so more effectively. The data in Table 7.1 is consistent with an interpretation that a hydroxyl substitution is favoured on position R3, and that activity is mostly determined through binding of 1 ring, as substitutions on the second ring appear to be less critical (compare AS-HK001, AS-HK033 and AS-HK019).

To test for the effect of an additional polar group on the aromatic ring of curcuminoid, the nitro analogue AS-HK041 was synthesized and evaluated for antitrypanosomal activity and it turned out that the presence of a nitro substituent resulted in a significant (6-fold) decrease in activity, positively as a result of steric effects, but this was not further explored. The nitro group would also remove electron density from the ring reducing Π -stacking energy.

In order to see the effect of higher alkyl ether analogues on the biological activity of curcuminoid analogues, the *n*-propyl ether AS-HK079, allyl ethers AS-HK044 and AS-HK045, 3,3-dimethylallyl ethers AS-HK077, AS-HK076 and AS-HK078, and 2-hydroxyethyl ethers AS-HK024, AS-HK023 and AS-HK081 were evaluated for antitrypanosomal activity. The assay results have demonstrated that the activity of these alkyl ether analogues was 1 to 4-fold more active than their respective parent curcuminoids, except for compound AS-HK081, which displayed 6.5-fold higher activity than the parent compound AS-HK002.

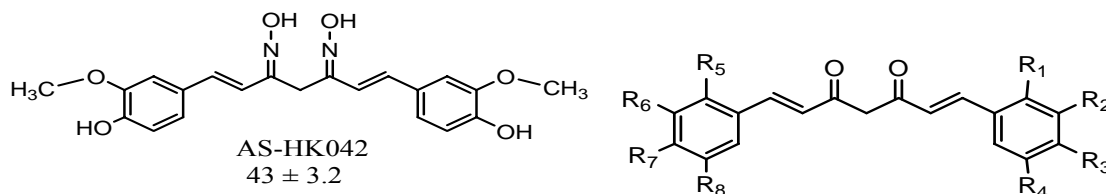
In addition to the ether derivatives, the ester analogues, i.e. the acetates AS-HK025, AS-HK026 and AS-HK027, and the benzoates (AS-HK028, AS-HK029 and AS-HK063-AS-HK067) were subjected to biological evaluation. The results demonstrated that the acetate analogues were about 1.5 to 3-fold more active than their parent curcuminoids (see Table 1). However, it is worth noting that their EC₅₀ values were still in excess of 1 μM. For the benzoate analogues, some displayed 2 to 4-fold higher activity (compounds AS-HK066 and AS-HK065) while others displayed almost identical activity (compounds AS-HK028 and AS-HK029) to those of the parent curcuminoids. Moreover, replacing the two hydroxyl (OH) groups in compound AS-HK001 with two cyanomethyl (OCH₂CN) groups in compound AS-HK080 also improved the antitrypanosomal activity by > 3-fold, but was not improved relatively to the tetra-methoxy analogue AS-HK073.

Introduction of two groups of ethyl acetate to compound AS-HK001 at positions 3 and 7 (AS-HK101) increases the activity by 21-fold, whereas introduction of two groups of O-biotin (AS-HK100) or of isobutyric acid (AS-HK102) did not cause any change in the activity of compound AS-HK001. However, introducing one group of isobutyric acid to the original compounds AS-HK001 and AS-HK002 (AS-HK103 and AS-HK104) or two isobutyric acid groups to the original compounds AS-HK002 and AS-HK003 (AS-HK105 and AS-HK106) increases the activity between 2- to 4-fold. Thus, the introduction of an ester directly at positions 3 and/or 7 has a little or no effect on activity, whereas linking an including acetyl, acetyl ester through the carbon to the oxygen group at position three and seven was clearly increased trypanocidal potency (AS-HK101). It is certainly possible that the spatial orientation of the ester group, as a major H-bond donor/acceptor group is responsible for this, and should be further explored.

The introduction of one or two groups of bromopentane ((C₅H₁₀Br) AS-HK108-AS-HK113) or of azylpentoxy ((C₅H₁₀N₃) AS-HK116) reduces the antitrypanosomal activity by 2- and 5-fold, respectively. In contrast, introduction of one or two pentyl pyridinium

(C₁₀H₁₅N) groups on either of the two benzene rings increases the activity dramatically by ~125-fold and resulting in EC₅₀ values between 0.02 μM and 0.07 μM (AS-HK119-AS-HK124). Indeed, the increase is so dramatic that it is legitimate to ask whether these compounds function as curcuminoids or as pyridinium compounds on the trypanosomes. In a separate study (Alsalabi, Roderko and De Koning, unpublished), a series of long-chain alkyl pyridinium ions was shown to have mid-nanomolar activity against the same *T. brucei* strains as employed here. Possibly the combination of both motifs to generate a very potent trypanocide has a priority for future synthesis.

Modification of the two keto groups in the linker chain of AS-HK001 with two hydroxyl imine (=N-OH), giving AS-HK042, or with two methoxy imines (=N-OCH₃), giving AS-HK012, reduces the activity by 17- and 6-fold, respectively. Given that the hydroxyl imines are good hydrogen bond donors and acceptors through the hydroxyl group and even the imine nitrogen should be able to function as an H-bond acceptor, the loss of activity on the modification of the keto groups is likely due to steric effects, interfering directly with either binding to a specific target or with uptake by the parasite.



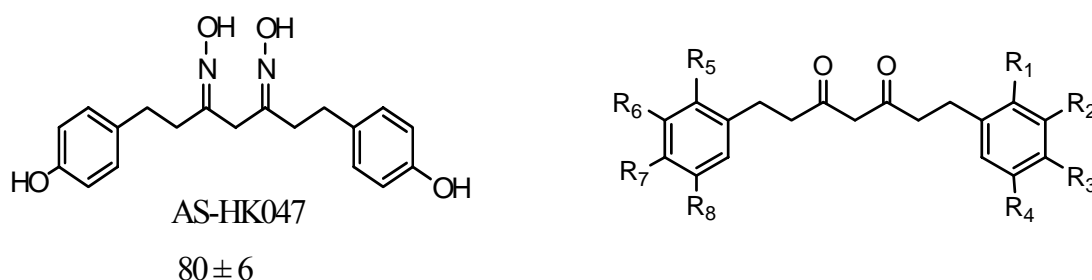
AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μM)
001	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	2.5 ± 0.4
002	H	O-CH ₃	OH	H	H	H	OH	H	4.6 ± 0.8
003	H	H	OH	H	H	H	OH	H	7.7 ± 1.1
033	H	OH	OH	H	H	O-CH ₃	OH	H	0.87 ± 0.06
032	H	OH	OH	H	H	OH	OH	H	1.8 ± 0.1
019	H	OH	OH	H	H	H	OH	H	0.65 ± 0.02
073	H	O-CH ₃	O-CH ₃	H	H	O-CH ₃	O-CH ₃	H	0.4 ± 0.07
075	H	O-CH ₃	O-CH ₃	H	H	H	OH	H	1.7 ± 0.7
074	H	O-CH ₃	O-CH ₃	H	H	H	O-CH ₃	H	1.6 ± 0.4
020	H	H	O-CH ₃	H	H	H	OH	H	8.9 ± 1.4
041	H	O-CH ₃	OH	NO ₂	H	O-CH ₃	OH	H	15 ± 2
044	H	O-CH ₃	O-C ₃ H ₅	H	H	O-CH ₃	OH	H	2.4 ± 0.1
045	H	O-CH ₃	O-C ₃ H ₅	H	H	O-CH ₃	O-C ₃ H ₅	H	1.6 ± 0.1
079	H	O-CH ₃	O-C ₃ H ₇	H	H	O-CH ₃	O-C ₃ H ₇	H	1.3 ± 0.4
076	H	O-CH ₃	O-C ₅ H ₉	H	H	O-CH ₃	O-C ₅ H ₉	H	1.2 ± 0.2
077	H	O-CH ₃	OH	H	H	O-CH ₃	O-C ₅ H ₉	H	1.4 ± 0.2
078	H	O-CH ₃	O-C ₅ H ₉	H	H	H	O-C ₅ H ₉	H	1.9 ± 0.3
023	H	O-CH ₃	O-C ₂ H ₄ OH	H	H	O-CH ₃	O-C ₂ H ₄ OH	H	1.8 ± 0.8
024	H	O-CH ₃	OH	H	H	O-CH ₃	O-C ₂ H ₄ OH	H	2.9 ± 1.1
081	H	O-CH ₃	OH	H	H	H	O-C ₂ H ₄ OH	H	0.70 ± 0.3
025	H	O-CH ₃	OC(O)CH ₃	H	H	O-CH ₃	OC(O)CH ₃	H	1.6 ± 0.5
026	H	O-CH ₃	OC(O)CH ₃	H	H	H	OC(O)CH ₃	H	1.5 ± 0.3
027	H	H	OC(O)CH ₃	H	H	H	OC(O)CH ₃	H	3.2 ± 0.4
028	H	O-CH ₃	OH	H	H	O-CH ₃	OC(O)-C ₆ H ₅	H	3.1 ± 0.7
029	H	O-CH ₃	OH	H	H	H	OC(O)-C ₆ H ₅	H	3.7 ± 0.4
065	H	H	OH	H	H	O-CH ₃	OC(O)-C ₆ H ₅	H	1.2 ± 0.3
066	H	H	OH	H	H	H	OC(O)-C ₆ H ₅	H	4.2 ± 1.7
080	H	O-CH ₃	O-CH ₂ CN	H	H	O-CH ₃	O-CH ₂ CN	H	0.75 ± 0.5
100	H	O-CH ₃	O-biotin	H	H	O-CH ₃	O-biotin	H	2.1 ± 0.3
101	H	O-CH ₃	OCH ₂ C(O)OEt	H	H	O-CH ₃	OCH ₂ C(O)OEt	H	0.12 ± 0.02
102	H	O-CH ₃	OC(O)C(CH ₂)CH ₃	H	H	O-CH ₃	OC(O)-C(CH ₂)CH ₃	H	2.6 ± 0.5
103	H	O-CH ₃	OC(O)C(CH ₂)CH ₃	H	H	O-CH ₃	OH	H	1.1 ± 0.2
104	H	O-CH ₃	OC(O)C(CH ₂)CH ₃	H	H	H	OH	H	1.0 ± 0.02
105	H	O-CH ₃	OC(O)C(CH ₂)CH ₃	H	H	H	OC(O)C(CH ₂)CH ₃	H	2.0 ± 0.3
106	H	H	OC(O)C(CH ₂)CH ₃	H	H	H	OC(O)C(CH ₂)CH ₃	H	2.3 ± 0.2
108	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	4.0 ± 1.4
109	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	H	O-CH ₃	OH	H	3.1 ± 1.1
111	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	H	H	OH	H	4.6 ± 1.4
112	H	O-CH ₃	OH	H	H	H	O-C ₅ H ₁₀ Br	H	5.4 ± 1.7
113	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	H	H	O-C ₅ H ₁₀ Br	H	6.0 ± 1.2
116	H	O-CH ₃	O-(CH ₂) ₅ -N ₃	H	H	O-CH ₃	O-(CH ₂) ₅ -N ₃	H	13 ± 2.9
119	H	O-CH ₃	O(CH ₂) ₅ -N-pyridine	H	H	O-CH ₃	O(CH ₂) ₅ -N-pyridine	H	0.07 ± 0.02
120	H	O-CH ₃	O(CH ₂) ₅ -N-pyridine	H	H	O-CH ₃	OH	H	0.02 ± 0.01
122	H	O-CH ₃	O(CH ₂) ₅ -N-pyridine	H	H	H	O(CH ₂) ₅ -N-pyridine	H	0.06 ± 0.02
123	H	O-CH ₃	OH	H	H	H	O(CH ₂) ₅ -N-pyridine	H	0.04 ± 0.02
124	H	O-CH ₃	O(CH ₂) ₅ -N-pyridine	H	H	H	OH	H	0.03 ± 0.01

AS-HK123 is an isomerise to compound AS-HK120.

Scheme 7.1 Changes in chemical groups and their effect on the efficacy of original curcumin compounds.

The reduced analogues of curcuminoids were next tested against *T. b. brucei* s427. For the tetrahydro analogues AS-HK007- AS-HK009, AS-HK085 and AS-HK087, the first two analogues (i.e. AS-HK007 and AS-HK008) were approximately 8- and 5-fold less active

than their respective parent compounds. However, the number and nature of the oxygen functions on the aromatic rings strongly determined the antitrypanosomal activity of the tetrahydro analogues. Thus, the analogue AS-HK009 was 3-fold more active than its parent curcuminoid AS-HK003 and was almost 9-fold more active than compound AS-HK008, the 3''-*O*-methoxy analogue. However, replacing the two keto groups in compounds AS-HK009 with two hydroxyl imine (=N-OH) (given AS-HK047) reduces the activity by 10-fold less than the original compound AS-HK003 and 32-fold less than compound AS-HK009, consistent with a steric interference with target binding as observed for the unsaturated curcuminoids above. Combined, those observations support the hypothesis that 1- the unsaturated and saturated curcuminoids likely act on the same primary target and 2- the keto groups of the linker are directly involved in binding to the intracellular target. The reduced linkers will allow more conformational flexibility, explaining the increased influence of the hydroxyl substitutions on the rings.



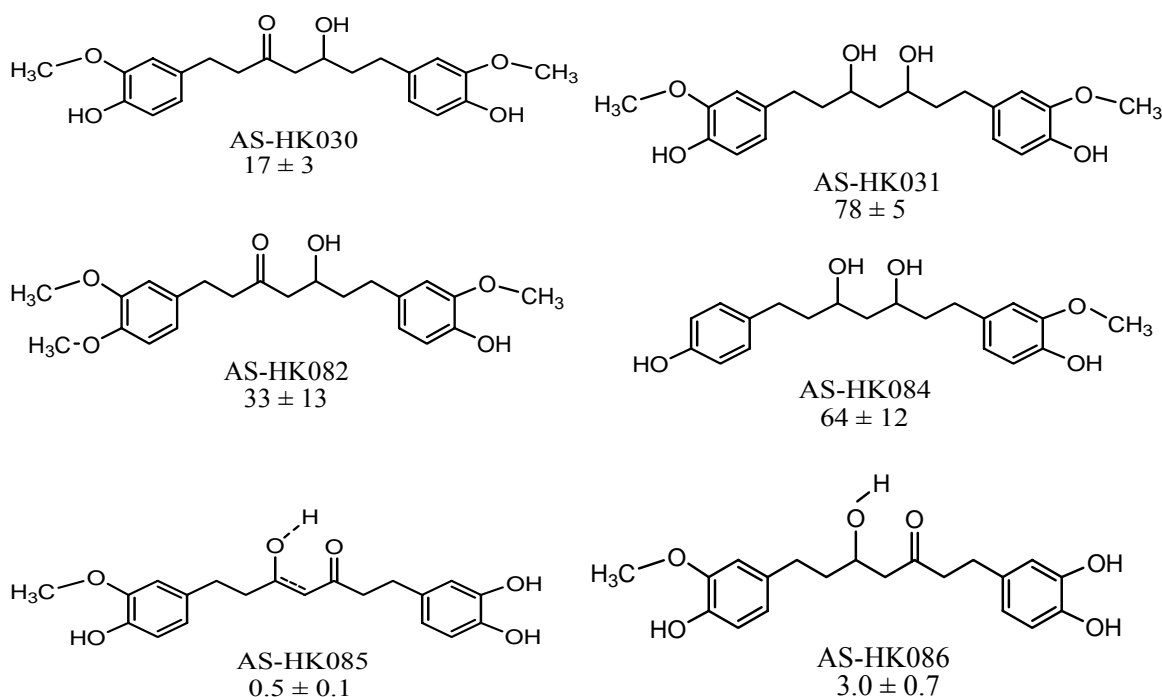
AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μM)
007	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	21 ± 11
008	H	O-CH ₃	OH	H	H	H	OH	H	22 ± 8
009	H	H	OH	H	H	H	OH	H	2.5 ± 0.4
083*	H	O-CH ₃	OH	H	H	H	OH	H	15 ± 5
087	H	OH	OH	H	H	OH	OH	H	1.9 ± 0.6

* Same structure with compound AS-HK008.

Scheme 7.2 Efficacy of reduced curcumin compounds on their activity.

The hexahydro analogues AS-HK030, AS-HK031 and AS-HK082 exhibited very low activity, being 7-, 27- and 73-fold less active than the conjugated compound AS-HK024, respectively. However, removing one methoxy group from compound AS-HK031 to prepare AS-HK084 does not significantly change activity. The compound AS-HK086 was 3.5-fold less active than the corresponding conjugated compound AS-HK033. Compound AS-HK085 was the most active analogue in this series, apparently owing to the double

bond introduced between carbons 3 and 4 of the linker, resulting in a 6-fold higher activity compared to AS-HK086.

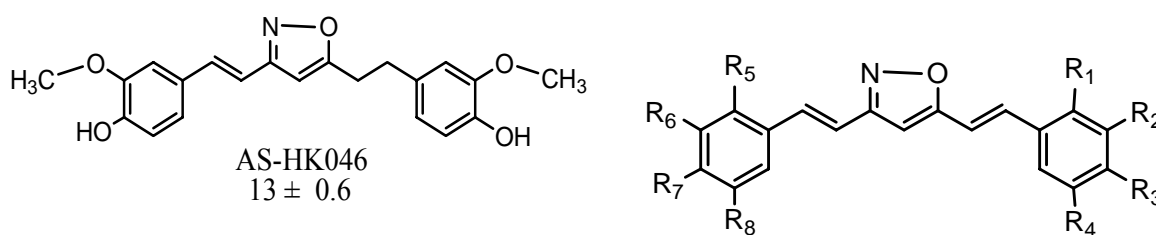


Scheme 7.3 Efficacy of reduced curcumin compounds on their activity.

Oxazol and isoxazol analogues:

Replacing the diketo group in compounds AS-HK001, AS-HK002 and AS-HK003 with isoxazolyl ring reduces the activity from 2- to 10-fold in compounds AS-HK004, AS-HK005 and AS-HK006, respectively. The activity remains low when additional acetyl (AS-HK043) and pentyl (AS-HK022) groups were added to these compounds, as described above for the equivalent derivatives of AS-HK001-003. In addition, reducing one bond from AS-HK004, resulting in AS-HK046, leads to a further decrease in activity. Interestingly, replacing the diketo feature in AS-HK001 with an isoxazolyl ring (AS-HK004) reduces the activity and introducing an isobutyric acid group to AS-HK001 does not change the activity, the activity is increased by 2-fold when both groups are together introduced in compound AS-HK107. Similarly, introducing one group of bromopentane (C₅H₁₀Br) to AS-HK004 (AS-HK115) does not make any significant changes in the activity. Introduction of one or two pentyl pyridinium (C₁₀H₁₅N) groups in AS-HK001 while also replacing the diketo group with an isoxazolyl ring resulted of obtaining two of

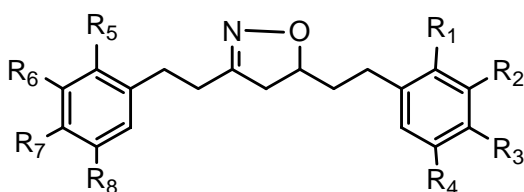
the best active compound of curcumin analogues against *T. brucei* (compound AS-HK126 and AS-HK125) with EC_{50} values of 0.01 μ M and 0.03, respectively. The profound increase in activity with this pyridinium substitution was already described above (AS-HK119-124) and the fact that the same substitution on isoxazole analogues leads to a very similar or even higher activity strength hence the argument that in this case the pyridinium moieties maybe the active element. While this has been explored further, the investigation of the pyridinium ions is not part of this thesis.



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μ M)
004	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	9.2 ± 2.3
005	H	O-CH ₃	OH	H	H	H	OH	H	37 ± 7.4
006	H	H	OH	H	H	H	OH	H	25 ± 5.5
043	H	O-CH ₃	O-C ₂ H ₅	H	H	O-CH ₃	O-C ₂ H ₅	H	7.9 ± 0.0
022	H	O-CH ₃	O-C ₅ H ₁₁	H	H	H	OH	H	14 ± 1.1
021	H	O-CH ₃	O-C ₅ H ₁₁	H	H	H	O-C ₅ H ₁₁	H	NS
107	H	O-CH ₃	C ₄ H ₈ O ₂	H	H	O-CH ₃	C ₄ H ₈ O ₂	H	1.4 ± 0.1
115	H	O-CH ₃	O-C ₅ H ₁₀ Br	H	H	O-CH ₃	OH	H	2.1 ± 0.02
125	H	O-CH ₃	OC ₁₀ H ₂₀ BrN	H	H	O-CH ₃	OC ₁₀ H ₂₀ BrN	H	0.03 ± 0.004
126	H	O-CH ₃	OC ₁₀ H ₂₀ BrN	H	H	O-CH ₃	OH	H	0.01 ± 0.003

Scheme 7.4 Effect of changes in chemical groups on the activity of oxazole curcumin analogues. NS= not solvent.

Converting the diketo group in the reduced compounds AS-HK007, AS-HK008 and AS-HK009 into isoxazol group reduces the activity up to 7-fold in compounds AS-HK088, AS-HK089 and AS-HK090, respectively. Furthermore, the addition of two acetyl groups to compound AS-HK088, giving AS-HK091, does not improve the activity of this compound. Thus the oxazole and isoxazole analogues consistently loose some of their trypanocidal activity compared to the di-keto potent compounds- with the exception of the alkylpyridinium series. This could be due to the conformational restrictions of the central ring structure.



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μM)
088	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	17 ± 4.6
089	H	O-CH ₃	OH	H	H	H	OH	H	23 ± 7.5
090	H	H	OH	H	H	H	OH	H	26 ± 8.1
091	H	O-CH ₃	O-C ₂ H ₅	H	H	O-CH ₃	O-C ₂ H ₅	H	13 ± 4.8

Scheme 7.5 The effect of introduction and reduction of different chemical groups on the activity of the reduced oxazole curcumin analogues.

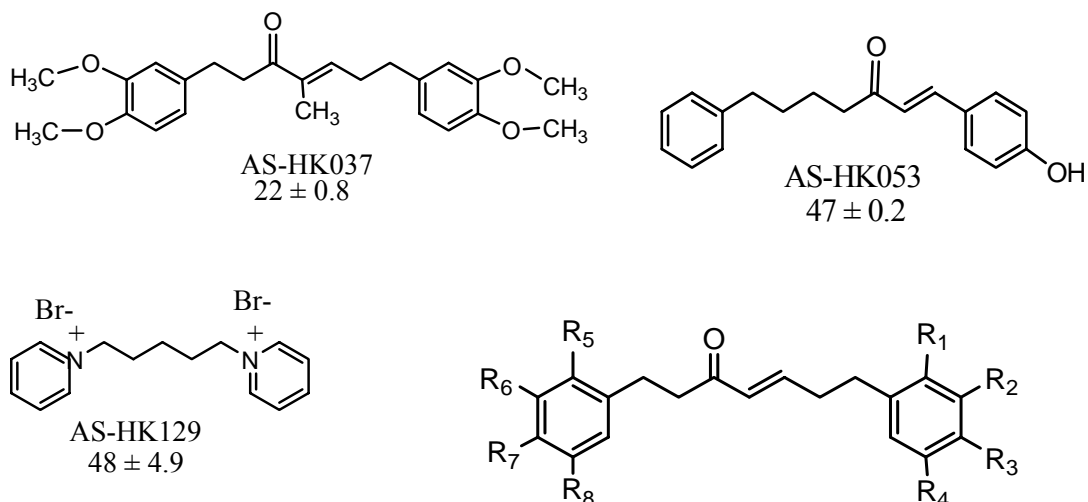
Other linker modifications:

We next explored mono-oxygenated analogues of the heptyl linker chain. We first synthesized the mono keto analogue AS-HK014, which was obtained by dehydration of compound AS-HK030. We discovered that the enone AS-HK014 exhibited very high antitrypanosomal activity of $0.053 \pm 0.007 \mu\text{M}$, which was 50-fold more active than the parent compound AS-HK001, or 340-fold more active than its immediate precursor, the hexahydro analogue AS-HK030. The activity of AS-HK014 was superior to the activity of the standard veterinary trypanocide diminazene aceturate, which displayed an activity of $0.10 \pm 0.01 \mu\text{M}$ against s427.

In order to study more about structure-activity relationship and to find any possible additional potent analogues by using the mono keto analogue AS-HK014 as the lead structure, we further explored different analogues (Scheme 7.6). It was found that the mono-demethylated analogue AS-HK034, the completely demethylated compound AS-HK035, the methylated analogues AS-HK018, AS-HK036, AS-HK048, and AS-HK051 were all more active than their respective parent curcuminoids AS-HK001-AS-HK003, except for compound AS-HK035 whose activity was about the same as that of its parent compound AS-HK001. The unsubstituted analogue AS-HK054 also displayed similar activity. However, none of them showed higher activity than the enone AS-HK014. However, adding methyl (CH₃) at C₄ of the linker chain to compound AS-HK036 to produce AS-HK037 drops the activity from $1.9 \mu\text{M}$ to $22 \mu\text{M}$, and further side chain introductions in the linker were not attempted.

We then investigated the alkyl ether and the acetate analogues of the enone AS-HK014 (i.e. compounds AS-HK094-AS-HK098) and it was found that all the analogues exhibited high activity. The monoacetate AS-HK097 and the diacetate AS-HK096 displayed the highest activity, at 0.089 ± 0.03 and 0.052 ± 0.01 μM , or 28 and 48-fold more active than the natural parent curcuminoid AS-HK001, respectively. Clearly, compound AS-HK096 has a similar activity as the enone compound AS-HK014, so replacement of the two hydroxyl groups in AS-HK014 by two acetyl groups in compound AS-HK096 does not change its activity, although ether substitutions did. Introduction of one cyanomethyl (OCH_2CN) group (compound AS-HK092) reduced the activity of AS-HK014 to 1.4 ± 0.4 μM , respectively, only 2-fold more active than the parent compound AS-HK001. Surprisingly, the introduction of a second cyanomethyl to make the symmetrical AS-HK093 restored almost all the trypanocidal activity 0.087 ± 0.03 μM .

Introducing an pentyl bromide ($\text{OC}_5\text{H}_{10}\text{Br}$) ether at R_3 in compound AS-HK118 or at R_3 and R_7 in compound AS-HK117 reduced the activity of AS-HK014 by 10- or 22-fold. However, the presence of one or two *O*-pentyl pyridinium groups in compounds AS-HK128 and AS-HK127, while increasing the activity relative to AS-HK001 by 2- and 7-fold, respectively, but actually reduced the activity of AS-HK014, on contrast to identical substitutions on the di-keto scaffold (schemes 7.4 and 7.6). Only one compound (AS-HK129) contains a 5-carbon spacer; this compound exhibited low antitrypanosomal activity with an EC_{50} value of 48 μM (scheme 7.6).

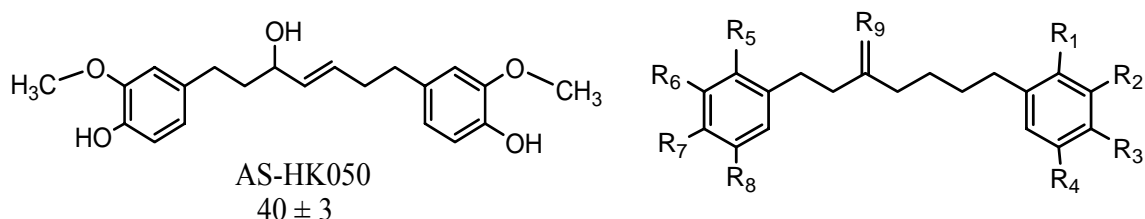


AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μM)
014	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	0.053 ± 0.007
057	H	H	H	H	H	H	H	H	2.8 ± 0.8
051	H	H	OH	H	H	H	OH	H	2.6 ± 0.5
035	H	OH	OH	H	H	OH	OH	H	2.5 ± 0.5
048	H	H	OH	H	H	O-CH ₃	OH	H	3.0 ± 0.3
034	H	O-CH ₃	OH	H	H	OH	OH	H	0.75 ± 0.1
018	H	O-CH ₃	O-CH ₃	H	H	O-CH ₃	OH	H	0.14 ± 0.05
036	H	O-CH ₃	O-CH ₃	H	H	O-CH ₃	O-CH ₃	H	1.9 ± 0.2
094	H	O-CH ₃	O-C ₅ H ₁₁	H	H	O-CH ₃	O-C ₅ H ₁₁	H	1.1 ± 0.3
095	H	O-CH ₃	OH	H	H	O-CH ₃	O-C ₅ H ₁₁	H	0.86 ± 0.3
096	H	O-CH ₃	O-C(O)-CH ₃	H	H	O-CH ₃	O-C(O)-CH ₃	H	0.052 ± 0.01
097	H	O-CH ₃	O-C(O)-CH ₃	H	H	O-CH ₃	OH	H	0.089 ± 0.03
098	H	O-CH ₃	O-C ₂ H ₄ OH	H	H	O-CH ₃	OH	H	0.27 ± 0.06
092	H	O-CH ₃	OH	H	H	O-CH ₃	O-CH ₂ CN	H	1.4 ± 0.4
093	H	O-CH ₃	O-CH ₂ CN	H	H	O-CH ₃	O-CH ₂ CN	H	0.087 ± 0.03
117	H	O-CH ₃	OC ₅ H ₁₀ Br	H	H	O-CH ₃	OC ₅ H ₁₀ Br	H	1.2 ± 0.1
118	H	O-CH ₃	OC ₅ H ₁₀ Br	H	H	O-CH ₃	OH	H	0.5 ± 0.1
127	H	O-CH ₃	OC ₁₀ H ₁₅ N	H	H	O-CH ₃	OC ₁₀ H ₁₅ N	H	0.35 ± 0.01
128	H	O-CH ₃	OC ₁₀ H ₁₅ N	H	H	O-CH ₃	OH	H	1.2 ± 0.2

Scheme 7.6 The effect of introduction and reduction of different chemical groups on the activity of the mono keto curcumin analogues.

In order to see the contribution of the olefinic group in the enone AS-HK014 to the antitrypanosomal activity, the corresponding dihydro analogue AS-HK038 was prepared and it was found that removal of the conjugated olefinic function resulted in almost complete loss of activity. The activity of AS-HK038 was 640-fold less active than that of AS-HK014. A similar result was also observed for the saturated keto analogues AS-HK049 (Scheme 7.7). To assess whether the keto function was also essential for activity, the keto group in compound AS-HK014 was replaced with hydroxyl group (-OH) to prepare the enol AS-HK050. Another changes were performed on compound AS-HK038 by replacing the keto group with hydroxyl imine (=N-OH) or with methoxy imine (=N-O-CH₃) to prepare AS-HK010 and AS-HK013, respectively. All these changes resulted in an almost

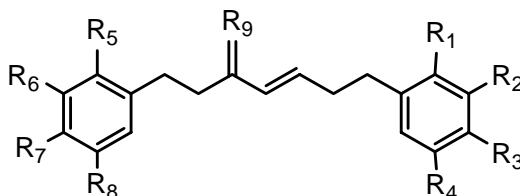
complete loss of activity. Furthermore, addition or reduction of the substitutes such as OH and O-CH₃ on one or both rings of these compounds generally did not cause any marked increase in the antitrypanosomal activity (AS-HK011, AS-HK52, AS-HK058 and AS-HK61). We conclude that an essential structural feature for a curcuminoid to exhibit strong antitrypanosomal activity is the presence of an α,β -keto system at the heptyl chain.



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	EC ₅₀ (μM)
058	H	H	H	H	H	H	H	H	O	44 ± 2.4
052	H	H	H	H	H	H	OH	H	O	23 ± 4.3
038	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	O	32 ± 0.4
049	H	O-CH ₃	OH	H	H	H	OH	H	O	45 ± 1.0
061	H	H	H	H	H	H	H	H	N-OH	33 ± 1.5
010	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	N-OH	51 ± 9.8
011	H	H	OH	H	H	H	OH	H	N-OH	93 ± 11
013	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	N-O-CH ₃	34 ± 3.8

Scheme 7.7 The effect of introduction and reduction of different chemical groups on the activity of the reduced mono keto curcumin analogues.

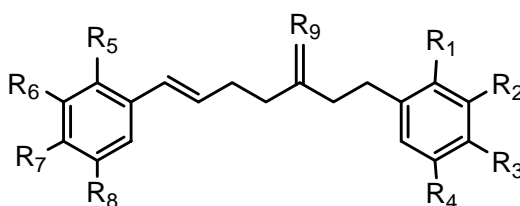
To further investigate about the importance of the substitution on the aryl rings, all the AS-HK014 substitution were removed (AS-HK057) resulting in a 53-fold decrease in activity. In another investigation about the importance of the keto function in the activity, the keto group in compound AS-HK014 was also replaced with hydroxylimine (=N-OH) group to prepare AS-HK039 or with methoxy imine (N-O-CH₃) to prepare AS-HK040. The results were similar to those obtained in the last group and the activity was lost (566-fold less active than compound AS-HK014).



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	EC ₅₀ (μ M)
057	H	H	H	H	H	H	H	H	O	29 \pm 0.8
039	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	NOH	29 \pm 2.4
040	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	NOCH ₃	30 \pm 1.8

Scheme 7.8 The effect of replacing the keto group by hydroxylimine and methoxy imine groups on the activity of the mono keto curcumin analogues.

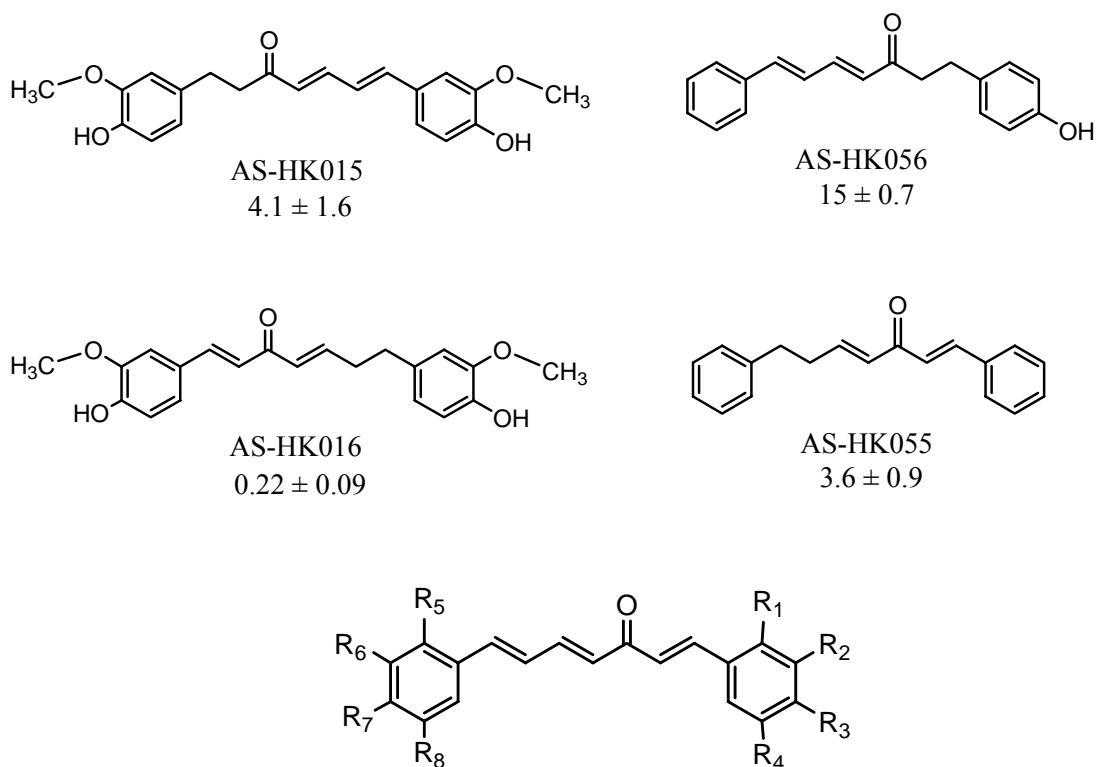
Similarly, removing all the substitutes on the both aryl rings, and changing the double bond position from C₄-C₅ to C₁-C₂ (AS-HK059) resulted in a decrease in activity by 245-fold compared to AS-HK014. Reinstating on of the aryl substitutions (hydroxyl at position R₃; AS-HK053) resulted in even lower activity. Similarly, replacing the keto group in AS-HK059 with hydroxylimine (=N-OH) group or with methoxy imine (N-O-CH₃) to prepare AS-HK060 and AS-HK062, respectively, also resulted in a further decrease in activity (>640-fold less active than AS-HK014).



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	EC ₅₀ (μ M)
059	H	H	H	H	H	H	H	H	O	14 \pm 1.9
053	H	H	OH	H	H	H	H	H	O	47 \pm 0.2
060	H	H	H	H	H	H	H	H	NOH	35 \pm 0.8
062	H	H	H	H	H	H	H	H	NOCH ₃	34 \pm 2.9

Scheme 7.9 The effect of changing the bond position with replacing the keto group by hydroxylimine and methoxy imine groups on the activity of the mono keto curcumin analogues.

Within the framework of the most active compound, AS-HK014, we investigated whether additional olefinic function would give rise to an increase in biological activity. The dienones AS-HK015, AS-HK016, AS-HK055 and AS-HK056 were synthesized and assessed for antitrypanosomal activity (Scheme 7.10). The introduction of a further conjugated double bond, either at position 1,2 (AS-HK055) or 6,7 (AS-HK056) of AS-HK014 linker, reduced this compound trypanocidal activity by 1.5- and 6-fold, respectively. This appears to indicate that the conjugation as such is not the sole determinant in activity, but that flexibility of the linker must be retained as much as feasible within the requirement of a single double bond conjugated with the keto group. Consistent with this view, the trienone AS-HK017 displayed lower activity than the corresponding dienone AS-HK016 and similar to AS-HK015 ($P > 0.05$), leading to the conclusion that the double bond C6-C7 is particularly unfavourable. None of the thirty trienone compounds (Scheme 7.10) displayed superior activity to AS-HK014, but a few had submicromolar EC_{50} values. On the other hand, the trienone AS-HK017 and AS-HK130-AS-HK158 were also tested for antitrypanocidal activity. In this group, 19 out of 30 exhibited EC_{50} values higher than the original compound AS-HK001, with EC_{50} values between 0.19 μM and 2.5 μM . However, none of them showed higher activity than the compound AS-HK014.



AS-HK	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	EC ₅₀ (μM)
151	H	H	H	H	H	H	H	H	2.9 ± 0.4
152	H	H	OH	H	H	H	H	H	1.7 ± 0.2
154	H	OH	H	H	H	H	H	H	0.68 ± 0.07
153	H	H	O-CH ₃	H	H	H	H	H	5.7 ± 0.1.0
017	H	O-CH ₃	OH	H	H	O-CH ₃	OH	H	1.1 ± 0.3
130	O-CH ₃	H	O-CH ₃	H	O-CH ₃	H	H	O-CH ₃	7.1 ± 0.08
132	H	OH	O-CH ₃	H	O-CH ₃	H	H	O-CH ₃	0.72 ± 0.04
135	O-CH ₃	H	H	O-CH ₃	O-CH ₃	H	H	O-CH ₃	2.1 ± 0.02
136	H	H	O-CH ₃	H	O-CH ₃	H	H	O-CH ₃	3.5 ± 0.4
137	H	OH	H	H	O-CH ₃	H	H	O-CH ₃	0.33 ± 0.04
139	H	H	H	H	O-CH ₃	H	H	O-CH ₃	2.0 ± 0.06
141	H	OH	O-CH ₃	H	H	OH	H	H	0.28 ± 0.02
142	H	H	O-CH ₃	H	H	H	O-CH ₃	H	4.9 ± 0.22
143	H	OH	H	H	H	H	O-CH ₃	H	0.58 ± 0.03
145	OH	O-CH ₃	H	H	H	O-CH ₃	O-CH ₃	H	0.19 ± 0.01
146	H	OH	O-CH ₃	H	H	O-CH ₃	O-CH ₃	H	0.38 ± 0.06
147	H	H	OH	H	H	O-CH ₃	O-CH ₃	H	0.65 ± 0.02
148	H	H	OH	H	H	H	O-CH ₃	H	1.7 ± 0.22
149	H	OH	O-CH ₃	H	O-CH ₃	H	O-CH ₃	H	1.0 ± 0.09
150	O-CH ₃	H	O-CH ₃	H	O-CH ₃	H	O-CH ₃	H	7.0 ± 0.4
157	OH	O-CH ₃	H	H	H	H	H	H	1.8 ± 0.2
158	O-CH ₃	H	H	O-CH ₃	H	H	H	H	4.1 ± 0.4
133	O-CH ₃	H	O-CH ₃	H	H	H	Cl	H	18 ± 1.7
134	H	H	Cl	H	H	H	Cl	H	8.0 ± 0.7
138	Cl	H	H	H	O-CH ₃	H	H	O-CH ₃	1.9 ± 0.04
140	H	OH	O-CH ₃	H	H	H	Cl	H	2.5 ± 0.2
144	H	OH	H	H	H	H	Cl	H	0.97 ± 0.2
156	Cl	H	H	H	H	H	H	H	4.1 ± 0.05
131	H	NO ₂	H	H	O-CH ₃	H	H	O-CH ₃	7.1 ± 0.05
155	H	NO ₂	H	H	H	H	H	H	1.1 ± 0.1

Scheme 7.10 The effect of introduction and reduction of different chemical groups on the activity of the dienone and trienone curcumin analogues.

In conclusion, the structure activity analysis of 158 curcumin analogues for trypanocidal activity indicated the following outcomes:

- Converting the diketo groups (C=O) into isoxazol group reduces the activity of curcumin.
- The activity increases when a pentyl pyridinium (OC₁₀H₁₅N) group was added. For example, the O-pentyl pyridinium compound AS-HK126 gave the best activity of all the analogues with an EC₅₀ value of 0.01 μM.
- O-linked substitution on the aryl rings replacing the hydroxyl groups of curcumin, present a complex pattern. In general, alkyl ethers, acetates, benzoate esters, biotin, isobutyric acid and similar substitution do not lead to substantially increased activities compared to curcumin.
- Reduction of one of the diketo groups to a hydroxyl (OH) group or reduction of both groups, to a diol, leads to a reduced activity, whereas conversion only one of these groups to a conjugated enol increases the activity.
- Introduction of further double bonds to the linker leads to a substantial loss of activity.
- The further reduction of the enol structure either at the double bond or the keto group, or replacement of the keto by hydroxylimine or methoxy imine similarly destroys the anti-trypanosomal activity.
- The activity of the compounds improves when groups such as OH, MeO, O-C₂H₅, O-C₂H₄OH, O-C₃H₇, C₆H₅-COO and O-CH₂COOC₂H₅ are substituted together with OH and MeO at R₂, R₃, R₆ and R₇. The activity also improved when two groups of O-biotin (C₁₀H₁₅N₂O₃S) or ethyl acetate (O-CH₂COOC₂H₅) were added.

Chapter eight

8 General discussion

The increasing burden of parasitic diseases, including African trypanosomiasis and leishmaniasis, is a major concern in the world and management and control of the disease is fraught with numerous problems. Toxicity of antiparasitic agents is an important problem. For instance, melarsoprol, used in the late-stage of sleeping sickness, is reported to cause a fatal reactive encephalopathy in 10% of patients (Barrett, 2000; Fairlamb, 2003). Pentavalent antimonials (Pentostam and Glucantime), which are antileishmanial drugs, also have severe side effects such as malaise, vomiting and anorexia (Chang *et al*, 1985; Kreier & Baker, 1987). The cost and the availability of the drugs are also crucial factors in the control of the diseases. For instance, DMFO, a drug used to treat the late stage of *T. b. gambiense* infections costs about US\$ 250 per patient whilst US\$ 9, 000 is spent for the hospitalisation and treatment of leishmaniasis with a regimen of Amphotericin B (Marty & Rosenthal, 2002; Rosenthal & Marty, 2003).

A major and difficult issue associated with antimicrobial agents is the problem of drug resistance. For example, resistance to melarsoprol (Bacchi, 1993; Brun *et al*, 2001; Legros *et al*, 1999; Balasegaram *et al*, 2006; Delespaux & De Koning, 2007), diminazene (Geerts *et al*, 2001; Anene *et al*, 2006) and pentavalent antimonials (Sundar, 2001b; Hadighi *et al*, 2006) has been reported. The emergence and spread of parasites resistant to the commonly used anti-trypanosomiasis and anti-leishmaniasis drugs means that current treatments are losing their efficacy in certain regions, rendering the diseases nearly untreatable. Thus, the limitations of registered drugs used against these two diseases and the problems associated with these drugs, necessitate the urgent development of new therapies to combat kinetoplastid diseases.

A basic guide in the selection of novel anti-trypanosoma/leishmania drugs should be their affordability and availability. In the light of this, detailed study of natural compounds is important. Our decision to investigate analogues of choline and curcumin as potential antitrypanosomal and antileishmanial compounds is therefore appropriate. Curcumin is a natural yellow pigment, which is extracted from *curcuma longa* rhizomes. The compound is not only used as natural food additive and spice but has also been used traditionally to treat a large number of infectious and non-infectious diseases.

Using standard drug assessment methods it was clearly demonstrated in this study that choline and curcumin analogues have anti-parasitic activities against trypanosomes and leishmania. Of the 98 curcuminoid compounds screened in this study, most were found to

possess activity against these species. Likewise, five out of the seven choline compounds tested in this study showed strong activity against bloodstream forms of *Trypanosoma brucei brucei* (Tb427WT, TbAT1-KO and TbAT1-KO-B48). Interestingly, four of the latter compounds inhibited the promastigotes of *Leishmania major* and *L. mexicana*, and amastigotes of *L. mexicana*, with EC₅₀ values below that of pentamidine, one of the recommended first-line drugs for the treatment of leishmaniasis.

The toxicity test on human HEK cell line with choline and curcumin compounds performed in this study did not highlight any *in vitro* toxic effects. The same outcome was found when some selected curcumin compounds were incubated with rat hepatocytes for up to 2 h. It is worth noting that in a study conducted elsewhere it was concluded that curcumin has no toxicity at doses up to 10 g per day in humans (Aggarwal *et al*, 2003). Coupled with the finding that the compounds were non-toxic to HEK cells, the findings in this study are highly encouraging since these compounds could be developed further to become lead compounds for further evaluation as anti-leishmanial and/or anti-trypanosomal drugs.

A finding in this study worth in-depth exploration is the observation that the nature of the chemical side chain on the choline analogue influenced the efficacy of the compounds. For instance, the bisthiazolium compound T4 was observed to be 13 times more active against the *T. brucei* bloodstream forms, than its close analogue T3, although the sole difference was the presence of a methoxy (T4) or hydroxyl (T3) group in the alkyl side chain on the thiazolium ring (structures see Figure 3.1, page 60). However, the corresponding monothiazolium, T1 (equivalent to T3 containing only one ring structure), also displayed a 4.4-fold higher trypanocidal activity than T3. This leads to the testable hypothesis that a ‘methoxy-T1’ would have the best activity of this series. These finding seems to support similar trends observed in tests against *Plasmodium* species (Hamze *et al*, 2005) and are also in line with activities against *Leishmania* species (Table 3.1, page 64). These observations highlight the urgent need for a systematic structure/activity analysis with a much larger sample size.

The presence of mediated transport of choline by *T. brucei* was also investigated using the classic uptake technique. The outcome indicates that, unlike in *Plasmodium*, these organisms do not express choline transporters. It was also concluded that the HAPT and LAPT transporters were not involved in the transport of choline or choline analogues into the parasites. These findings suggest that the mode of action of these compounds could not be attributed to an inhibition of a choline transporter. This conclusion was consistent with

the observation that the test compounds did not change the lipid composition of *T. b. brucei* (see below).

To investigate the possible mode of action of these compounds, we employed various scientific methods. Whereas some of the curcumin analogues rapidly killed trypanosomes, the actions of the choline compounds were mostly trypanostatic in nature. None of the choline compound tested caused parasite death as a consequence of plasma membrane disruption or by interfering with choline transport or phospholipid metabolism. The analysis of phospholipid composition after incubation with high concentrations of choline lipids did not identify any changes in overall content of the main phospholipids, including PC, PE and sphingolipids. Thus it is highly unlikely that the choline analogues act directly as inhibitors or substrates of enzymes of the phospholipid biosynthesis pathways. However, several of the analogues were identified in the mass spectra, showing their entry into the cells. One possibility would be that the compounds induced apoptosis in trypanosomes, as reported for some prostaglandins (Figarella *et al.*, 2005; Figarella *et al.*, 2006). Indeed, some of the analogues, particularly G25 and MS1 seemed to induce a rapid depolarisation of the mitochondrial membrane and others (T1 and M38) appear to cause DNA degradation – phenomena often associated with apoptosis (Figarella *et al.* 2005). However, none of the compounds clearly induced both phenomena, nor did any of them induce the formation of Reactive Oxygen Species (ROS) which was reportedly the mechanism by which prostaglandins induced apoptosis in trypanosomes (Figarella *et al.* 2006). It must therefore be concluded that the mechanism of action is not identical for the entire group of compounds, but that none of them induce an apoptosis-like effect on trypanosomes. Future studies will focus on the mitochondrion as a site of action for choline analogues.

Similarly, the mechanism of action of curcumin against trypanosomes and leishmania could not be completely deciphered. However, the diverse pharmacological activities of curcumin against various cell types have been reported in literature. The compound was reported to attack multiple cellular targets in various cell types including the inhibition of numerous enzymes such as protein kinase C, cytochrome P450 and telomerase among others. For example, Cui *et al.*, (2006) reported that incubation of three human cancer cell lines (Bel7402, HL60 and SGC7901) with 1 μ M curcumin for 120 h leads to inhibition of telomerase followed by induction of apoptosis. Curcumin was reported to prevent activation of protein kinase C in mouse fibroblast cells by phorbol esters, pointing to a possible effect on cellular regulation (Liu *et al.*, 1993), in mammalian plasma membrane

preparations (Mahmmoud, 2007) and in neutrophils (Jancinova *et al*, 2009). The cytochrome P450 (CYP) was also inhibited by curcumin in rat (Oetari *et al*, 1996) and human (Volak *et al*, 2008) liver cells. Moreover, the inhibitory effect of both curcumin and curcumin analogues on the five major human drug-metabolizing CYPs has been also reported by Appiah-Opong *et al*, (2007) and Appiah-Opong *et al*, (2008), respectively.

To explore the mechanisms of the chemopreventive effects of curcumin, we have tested whether curcumin can induce apoptosis in trypanosomes. Apoptosis is a mode of cell death and characterized by plasma membrane blebbing, cell shrinkage, chromatin compaction and collapse of the cell into small intact fragments (Pal *et al*, 2001). Yoshino *et al* (2004) and Su *et al* (2006) also reported that curcumin-mediated apoptosis is closely related to increase in the production of intracellular reactive oxygen species (ROS) in HL60 and human colon cancer Colo 205 cells, respectively. Our findings resulted that none of any of these signs was observed on the treated trypanosomes with curcuminoids. Therefore, the mechanism of curcumin's action by this way is still improbable.

The pro-oxidant activity of curcumin by generation of ROS has been reported in different types of cells. For instance, Bhaumik *et al* (1999), Cui *et al* (2007) and Das *et al* (2008) reported that curcumin was found to induce hyperproduction of ROS followed by loss of mitochondrial membrane potential in AK-5 tumor cells (a rat histiocytoma), *Plasmodium falciparum* and *Leishmania donovani* promastigotes, respectively. On the other hand, this compound has been widely demonstrated to have potent antioxidant activities and inhibit ROS in human hepatoma G2 cells (Chan *et al*, 2005) and diabetics by inhibiting Ca²⁺ influx and protein kinase C (Balasubramanyam *et al*, 2003). However, curcumin has been found to act both as pro-oxidant and anti-oxidant agent in the DNA cleavage (Ahsan *et al*, 1999), human RBCs (Banerjee *et al*, 2008) and *Plasmodium* (Le Bras *et al*, 2005). According to these studies it was suggested the pro- or anti-oxidant properties of curcumin analogues will dominate depending on the compounds concentration and other conditions. The results we report have suggested that on *T. brucei* curcumin analogues only act as anti-oxidant, at concentrations ranging from 20 µM to 0.01 µM.

If curcumin analogues are to be used routinely as antiparasitic drugs then the potential for early onset of parasite resistance to the drug must be anticipated. Since curcumin itself is currently being used extensively in food preparation, it is anticipated that a mono-therapy with curcumin could usher an early onset of parasite resistance as a consequence of drug pressure, although this is not necessarily true for curcumin analogues. However, a way to circumvent this is to combine this compound with another antiparasitic drug in what could

be referred to as curcumin combination therapy (CCT) similar to the artemisinin combination therapy currently being used against malaria. The possibility of synergy with other antiparasitic drugs should be urgently investigated.

One of the aims in this study is to improve the pharmacological profile of curcumin against kinetoplastid parasites (e.g. improve their activity, selectivity). To achieve this aim, the structure activity relationship (SAR) of these compounds was evaluated against *T. brucei* bloodstream forms. This strategy aims to achieve an iterative improvement of the biological activity (i.e. trypanocidal activity, therapeutic index) by a continuous feedback of screening results to the synthesis strategy. The partnership with Apichart Suksamrarn and Chatchawan Changtam of Ramkhamhaeng University, Bangkok, has provided exciting results and insights. Among other observations, it was clear that activity was improved in the following cases. The first case was when the diketo linker was reduced to a conjugated enol. The second was when a pentyl pyridinium ($\text{OC}_{10}\text{H}_{15}\text{N}$) group, or two groups of O-biotin ($\text{C}_{10}\text{H}_{15}\text{N}_2\text{O}_3\text{S}$) or ethyl acetate ($\text{O-CH}_2\text{COOC}_2\text{H}_5$) were added. Finally, activity was increased when groups such as OH, MeO, O- C_2H_5 , O- $\text{C}_2\text{H}_4\text{OH}$, O- C_3H_7 , $\text{C}_6\text{H}_5\text{-COO}$ and O- $\text{CH}_2\text{COOC}_2\text{H}_5$ are substituted together with OH and MeO at R_2 , R_3 , R_6 and R_7 .

From the observations made in this study, it can be concluded that certain choline and curcumin analogues possess promising antiparasitic activities against trypanosomes and leishmania. There is now a need to develop these compounds to become pre-clinical drugs candidates taking into consideration some of the important information provided in this thesis. Of particular importance would be the improvement of bioavailability and pharmacokinetics of these classes of compounds.

Appendices

1- CBSS BUFFER (CARTER'S BALANCED SALT SOLUTION)

Components	Quantity per litre
MES	6.4 g
NaCl	5.7 g
KCl	342.9 mg
CaCl ₂	44.1 mg
MgSO ₄	17.2 mg
NaH ₂ PO ₄	904.8 mg
MgCl ₂	60.9 mg
Glucose	2.7 g

Adjust to final pH 6.0 and store at 4°C.

2- ASSAY BUFFER

Components	Quantity per litre
Glucose	2.53 g
HEPES	8.0 g
MOPS	5.0 g
NaHCO ₃	2.0 g
KCl	347.5 mg
MgCl ₂ ·6H ₂ O	62.5 mg
NaCl	5.7 g
NaH ₂ PO ₄ ·2H ₂ O	913.5 mg
CaCl ₂ ·2H ₂ O	40.7 mg
MgSO ₄ ·7H ₂ O	19.9 mg

Adjust to final pH 7.3 and store at 4°C. Note- for uptake assays using yeast, assay buffer without the addition of glucose is used for the final wash and re-suspension (all other quantities remain the same).

3- PSG (PHOSPHATE-BUFFERED SALINE PLUS GLUCOSE)

Components	Quantity per litre
Na ₂ HPO ₄ (anhydrous)	13.48 g
NaH ₂ PO ₄ ·2H ₂ O	0.78 g
NaCl	4.25 g

Make to one litre with dH₂O (PS buffer). Dissolve 10 g of glucose in approximately 200 ml of dH₂O (Glucose solution). Add six volumes of PS to four volume of glucose solution (PSG buffer). Adjust to pH 8.0 exactly and store at 4°C.

4- OIL MIXTURE

Components	Quantities
Mineral oil (Sigma)	50 ml
di-n-butyl phthalate	350 ml

5- 2% SDS

Components	Quantities
Sodium dodecyl sulphate	10 g
dH ₂ O	500 ml

6- Preparation of perfusion solutions:**6.1- Stock solutions:****6.1.1- Hank's Buffer (10x):**

Components	Quantity per litre
NaCl	80 g
KCl	4 g
MgSO ₄ .7H ₂ O	2 g
Na ₂ HPO ₄ .2H ₂ O	0.6 g
KH ₂ PO ₄	0.6 g

store at 4°C.

6.1.2- Krebs-Henseleit Buffer (2x):

Components	Quantities
Distal H ₂ O	785 ml
16.09% NaCl	200 ml
1.10% KCl	150 ml
0.22 M KH ₂ PO ₄	25 ml
2.74% MgSO ₄ .7H ₂ O	50 ml
0.12 M CaCl ₂ .6H ₂ O	100 ml

All these stocks should be stored at 4°C. The previous solutions were transferred to a 2000 ml brown bottle and bubbled with 95% O₂ and 5% CO₂ for 10 minutes. In the mean time, 9.71 g of NaHCO₂ was dissolved in 1000 ml distilled water and also bubbled as the salt solution. The second solution was added to the first solution in order to get Krebs-Henseleit Buffer stock, which was stored at 4° C.

6.2- Perfusion solutions:

The solutions that are used for perfusion the rat liver and preparation of cell suspension were filtered and stored at 4° C.

6.2.1- Hank I (500 ml):

Components	Quantities
NaHCO ₂	1.05 g
HEPES	1.05 g
bovine serum albumin (BSA)	3.33 g
fraction V, Cat. No. 9418, Sigma	114 mg
distilled water	450 ml
Hank's Buffer (10x)	50 ml

6.2.2- Hank II (500 ml):

Components	Quantities
NaHCO ₂	1.05 g
HEPES	1.5 g
CaCl ₂ .2H ₂ O	147 mg
distilled water	450 ml
Hank's Buffer (10x)	50 ml

6.2.3- Krebs-Albumin Buffer (500 ml):

Components	Quantities
HEPES	1.5 g
bovine serum albumin (BSA)	5 g
distilled water	250 ml
Krebs-Henseleit Buffer (2x)	250 ml

6.2.4- Krebs-HEPES Buffer (500 ml):

Components	Quantities
HEPES	1.5 g
distilled water	250 ml
Krebs-Henseleit Buffer (2x)	250 ml

The pH of these four perfusion solutions was adjusted to 7.4 with 5 N NaOH.

7- Total cell protein measurement (Lowry Assay):

Solutions used:

0.5 M NaOH, 1% w/v CuSO₄, 2% w/v Na-K-tartrate, 2% w/v Na₂CO₃ and Folin's (ciocaltau) reagent.

All the previous solutions should be stored at 4 °C. The protein standard (200 µg/ml bovine serum albumin in 0.5 M NaOH) was stored at -20 °C.

1- On the day of the assay two solutions were made up:

- solution A:

Components	Quantities
1% CuSO ₄	1 ml
2% Na-K-tartrate	1 ml
2% Na ₂ CO ₃	98 ml

- solution B:

Components	Quantities
Folin's reagent	5 ml
H ₂ O	15 ml

2- The standard curve was set up in plastic test-tubes with lids as follows:

BSA (µl)	0	125	250	500	750	1000
0.5 M NaOH (µl)	1000	875	750	500	250	0
Protein conc. (µg/ml)	0	25	50	100	150	200

8- Determination of Reduced Glutathione by Fluorimetry:

Solution I:

Components	Quantity per litre
Na ₂ HPO ₄	35.6 g
EDTA	1.86 g

Solution II:

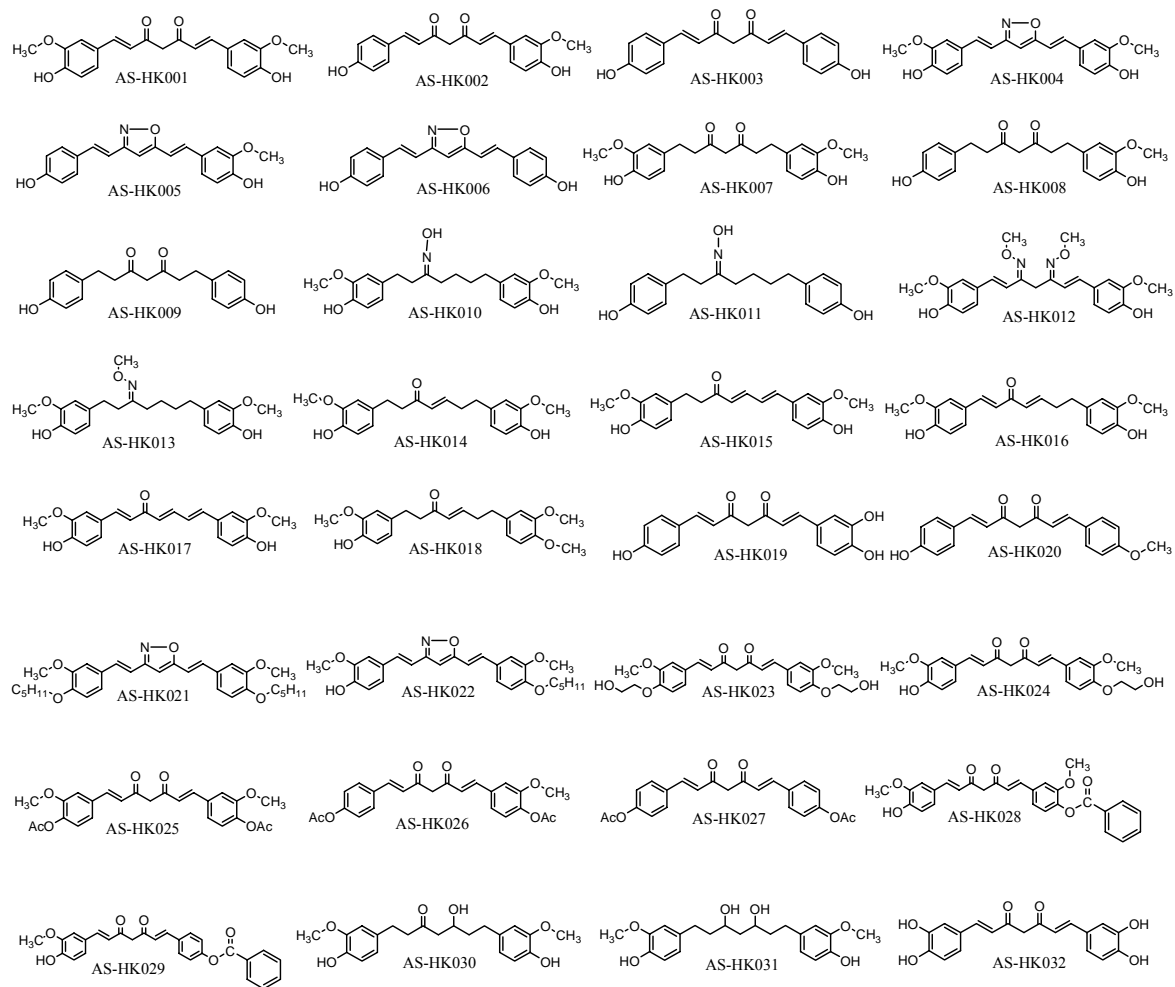
Components	Quantity per 200 ml
NaH ₂ PO ₄ .H ₂ O	6.13 g
EDTA	0.372 g

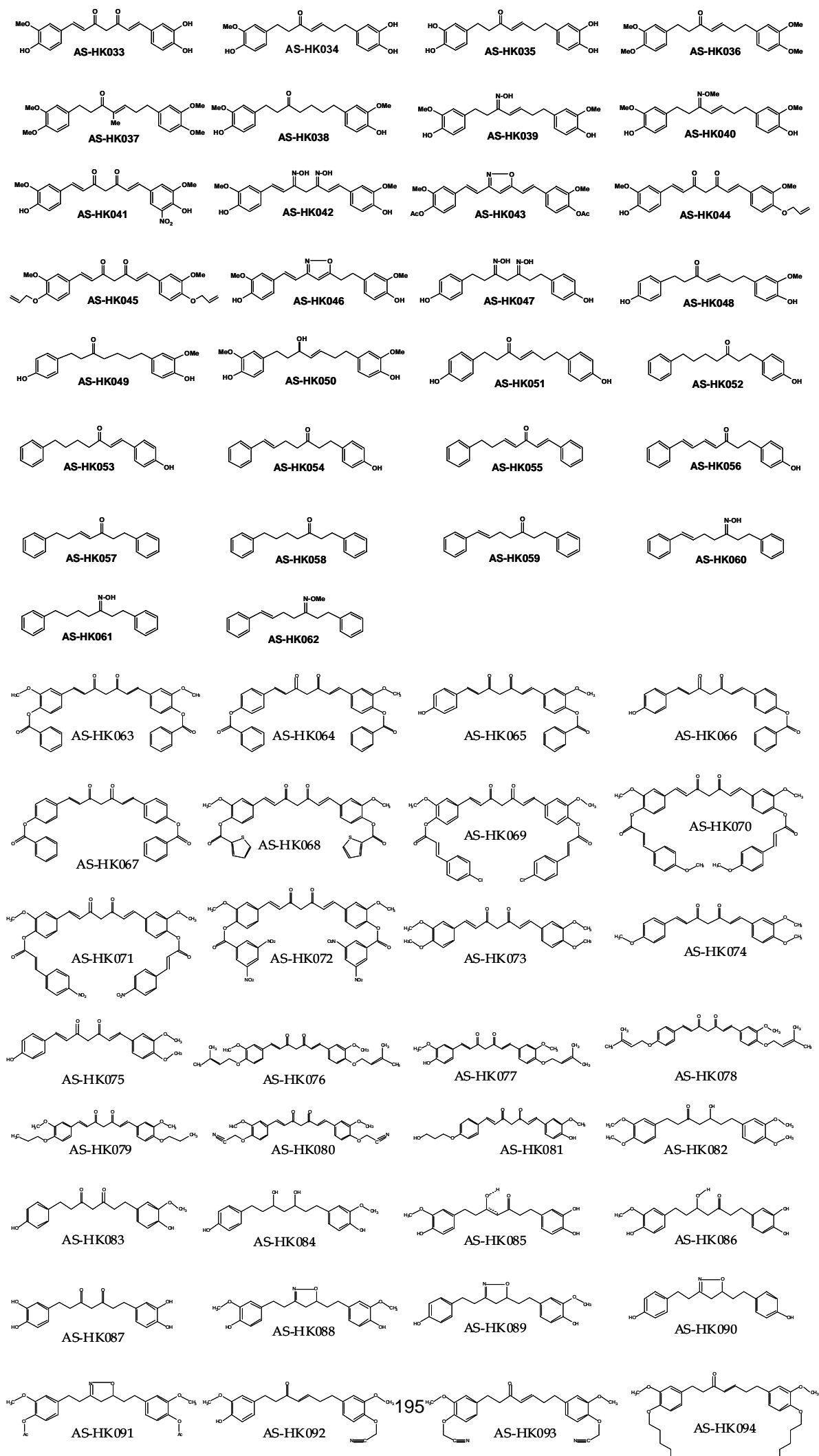
The pH of solution I was adjusted to 8.0 by using the solution II, and the buffer was stored at room temperature.

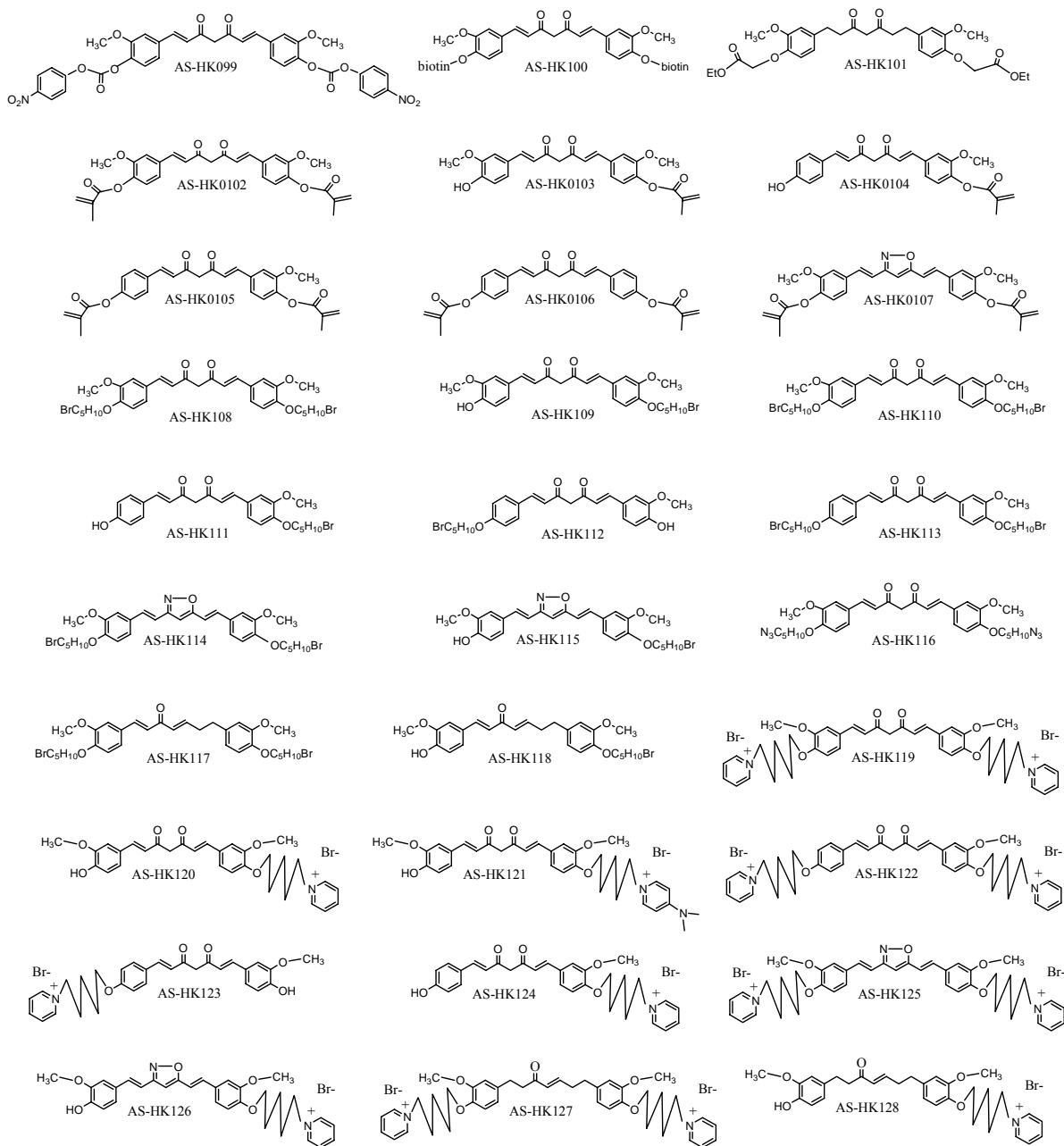
The standard was 1 mM GSH (3 mg/ml) in 10 % TCA, and stored at -20 °C.

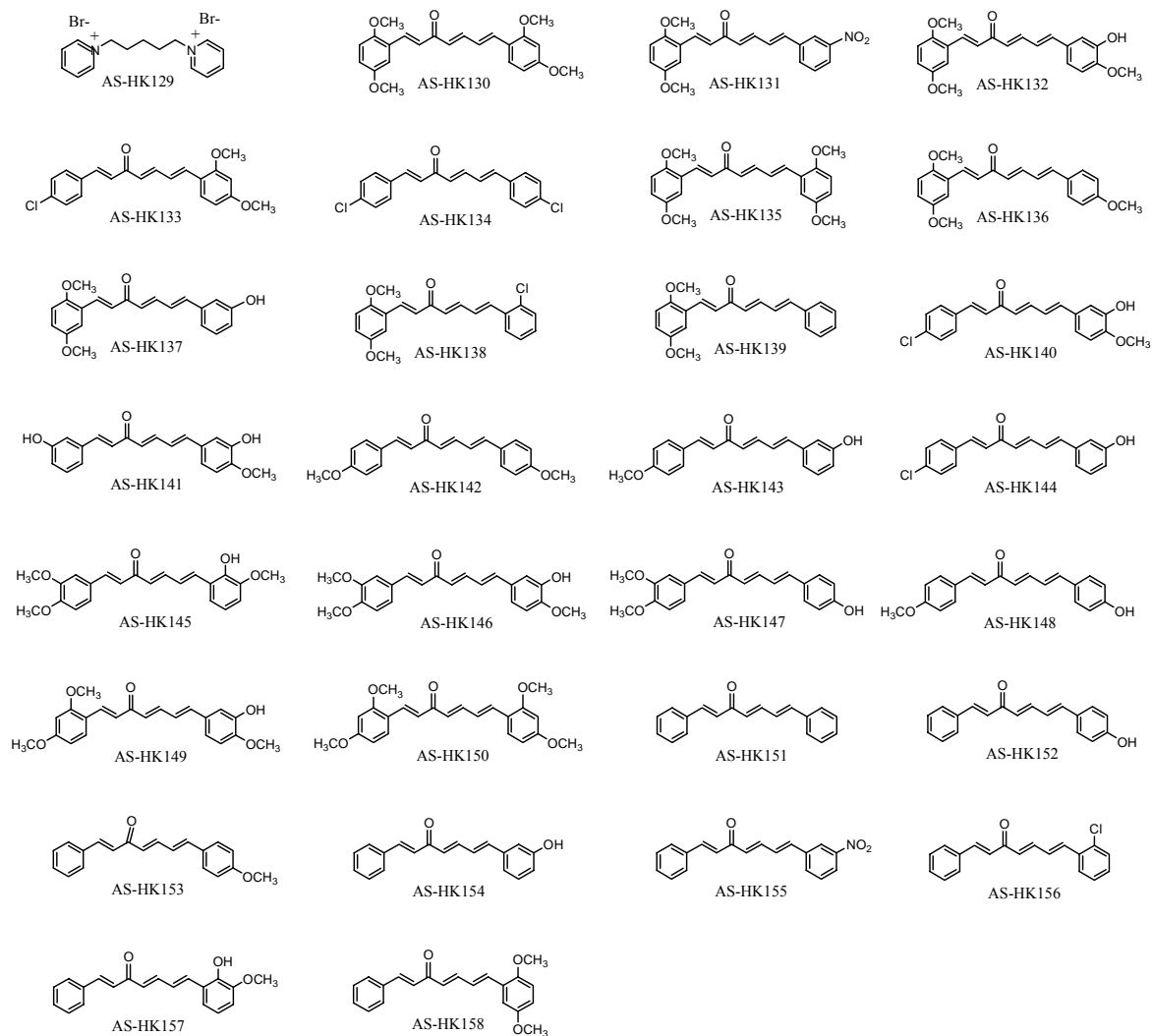
1mM GSH (μl)	0	10	25	50	75	100
TCA (μl)	1000	990	975	950	925	900
GSH conc. (μM)	0	10	25	50	75	100

APPENDIX II: STRUCTURES OF CURCUMIN ANALOGUES





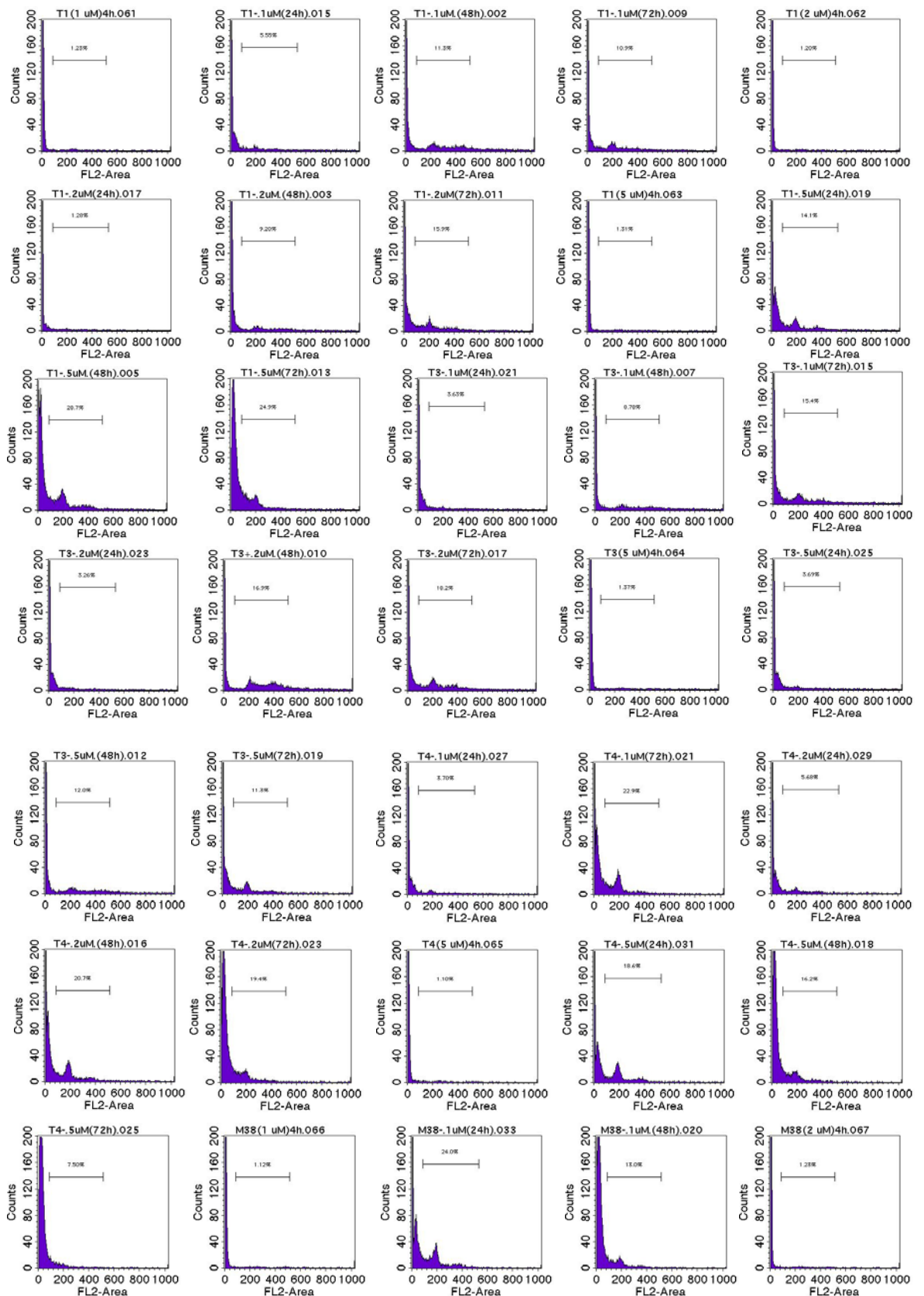


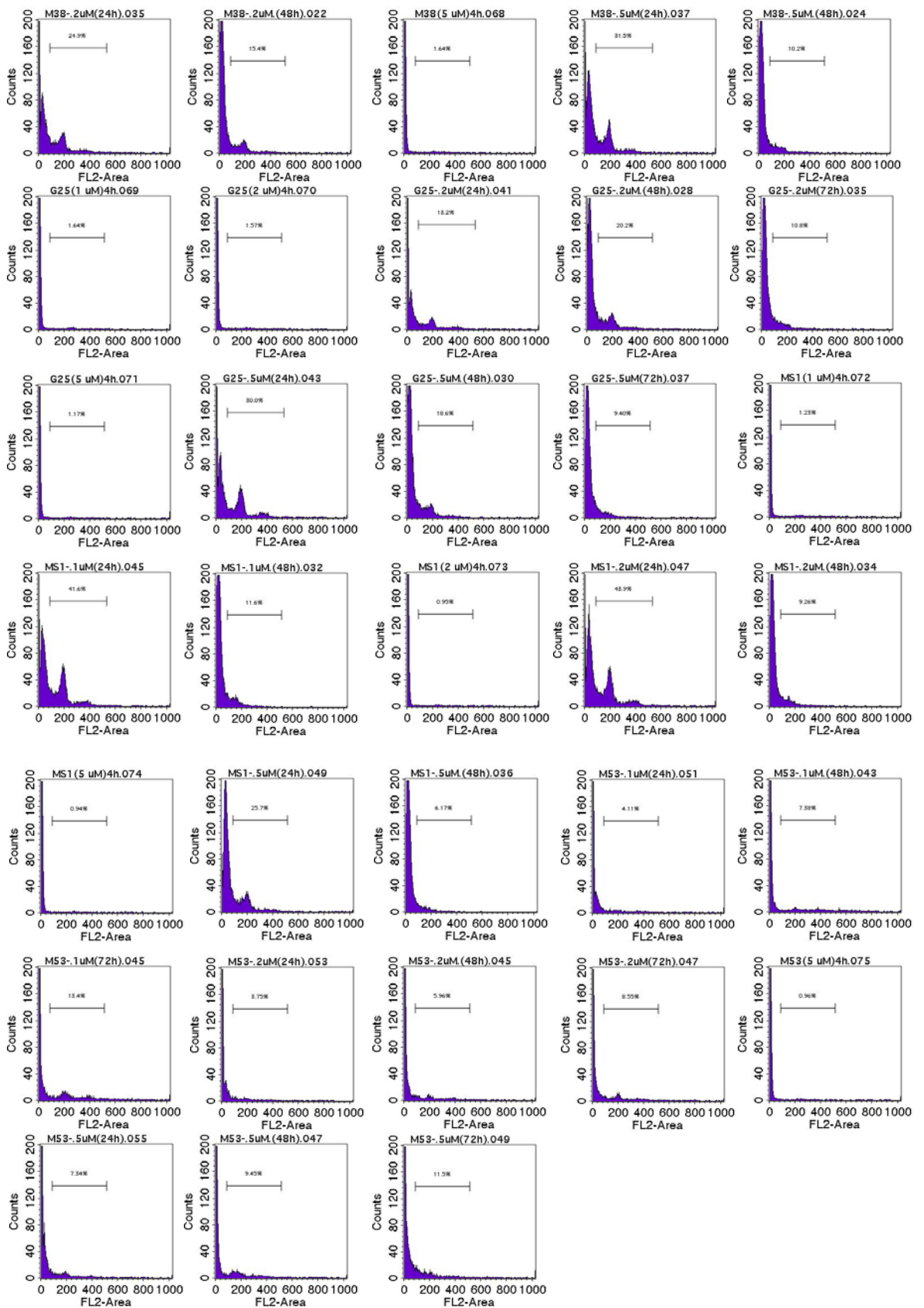


APPENDIX III: SUPPLEMENTARY DATA

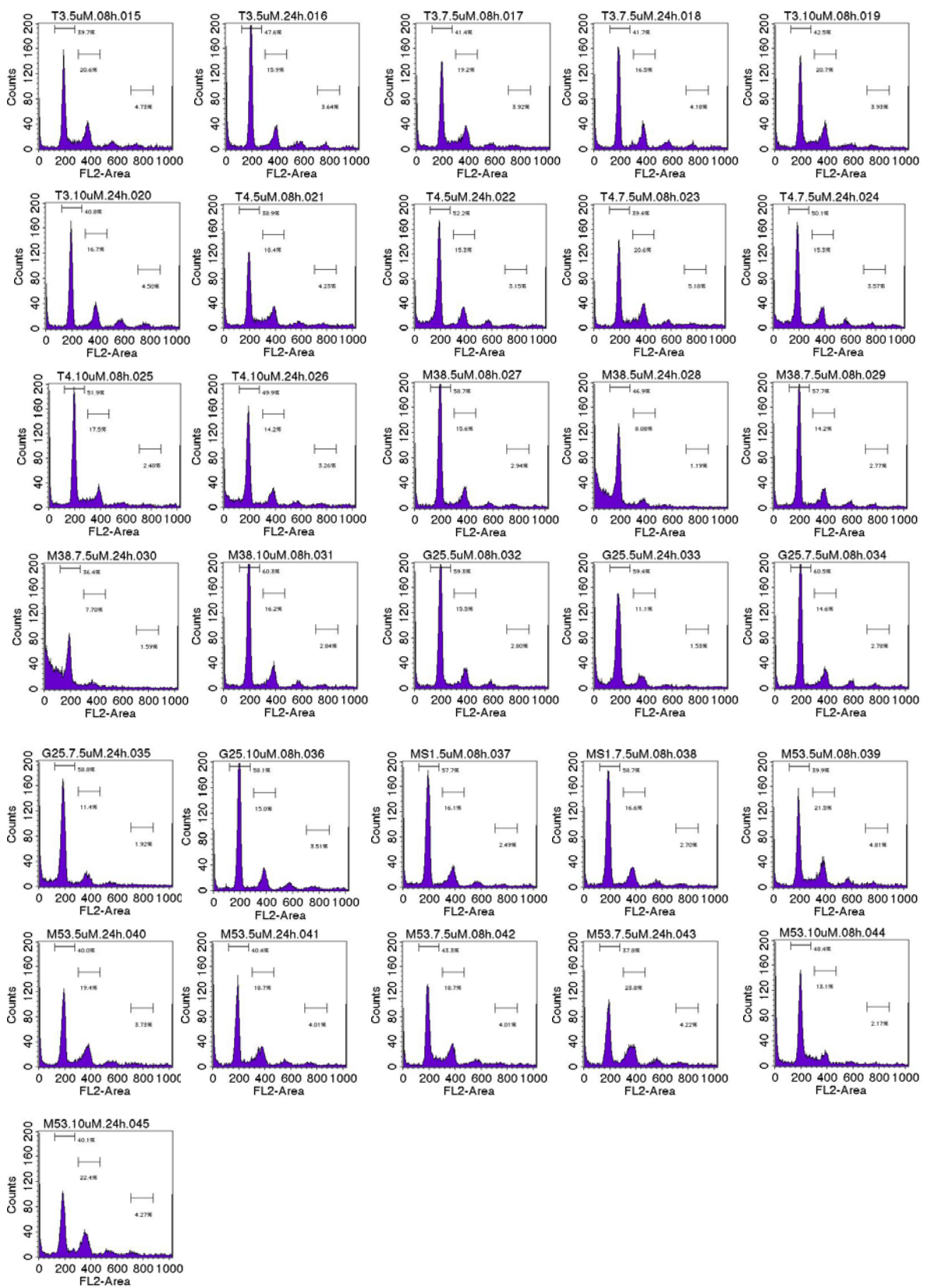
1- Choline compounds:

1.1 Cellular permeability

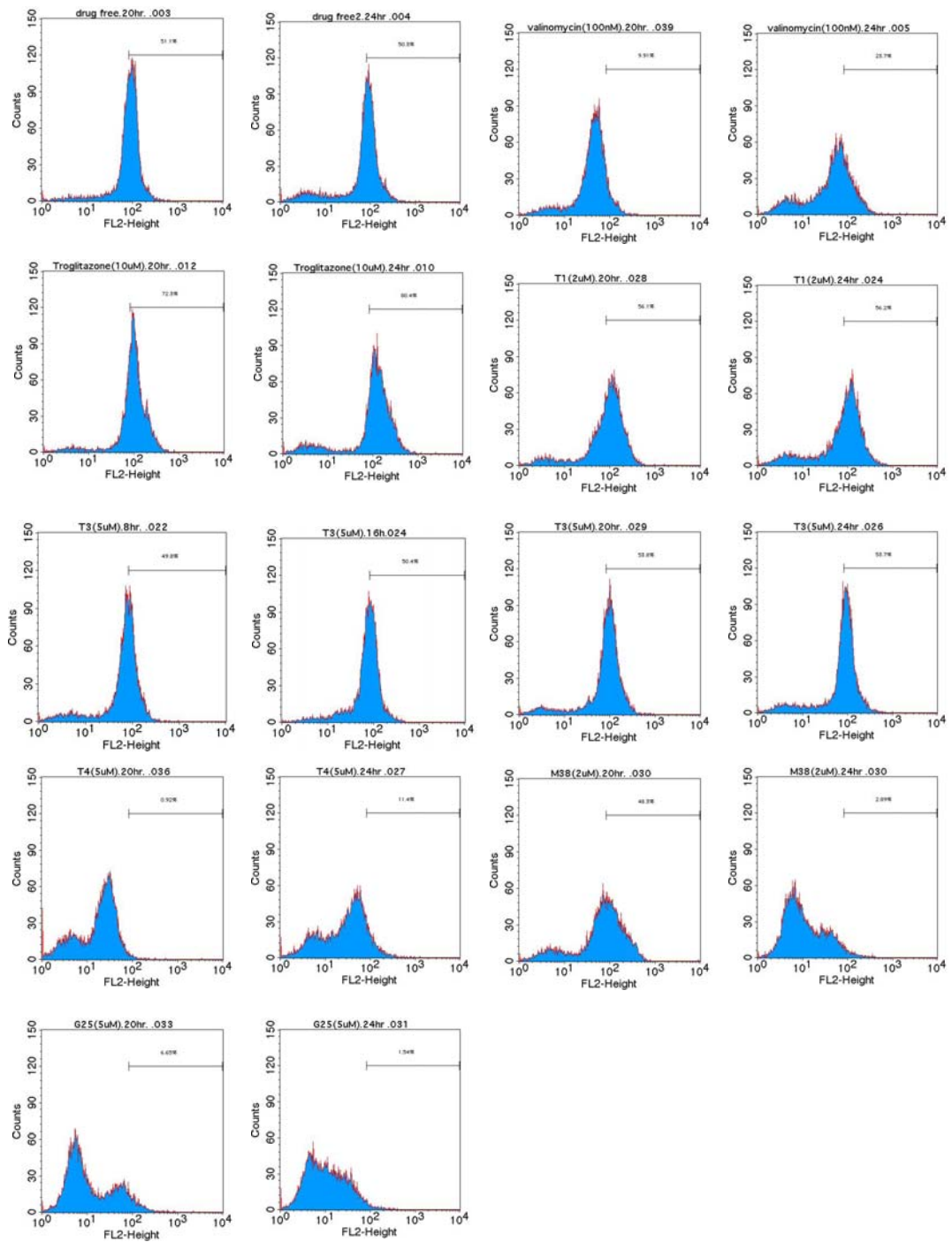




1.2 DNA content

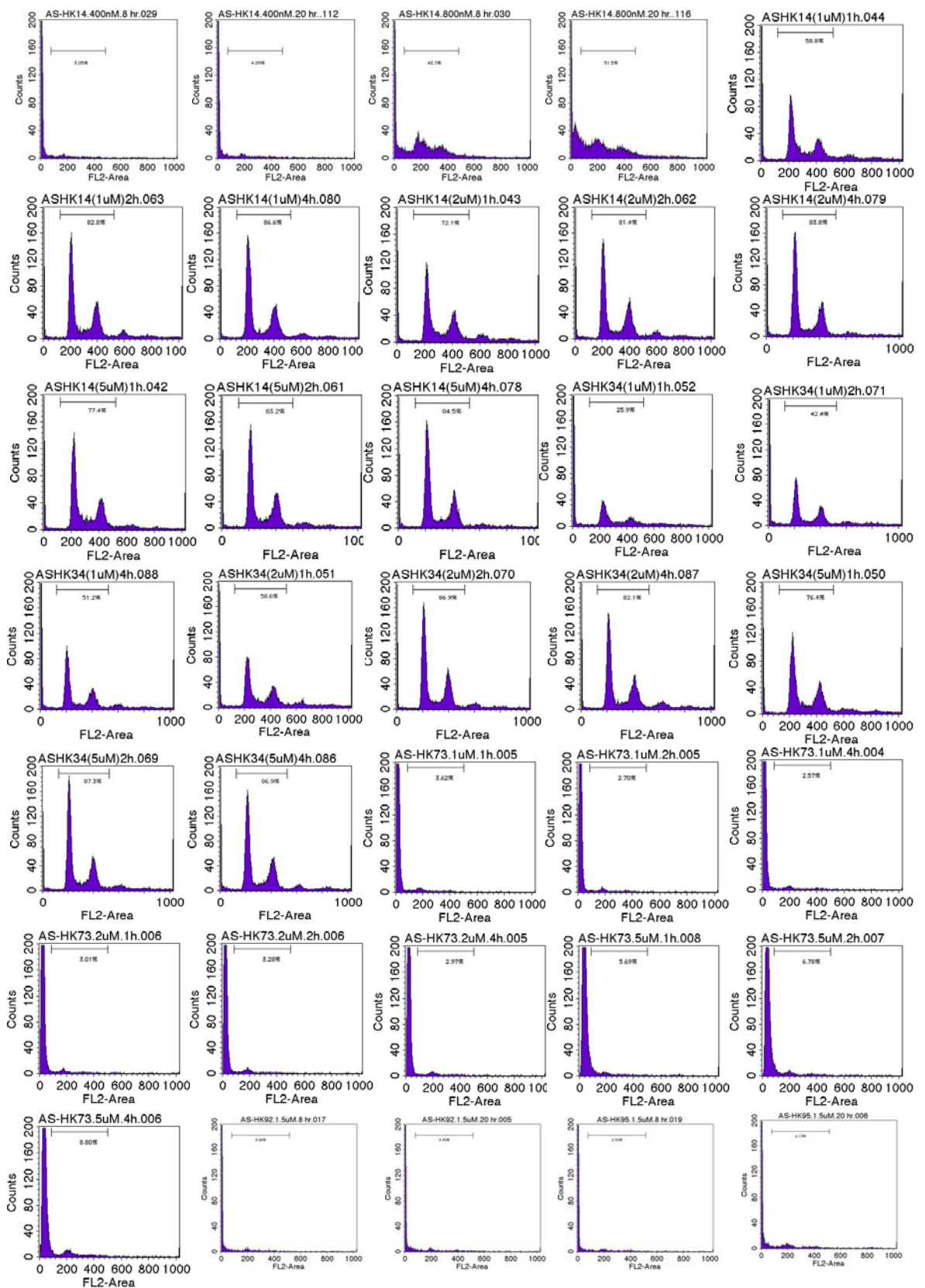


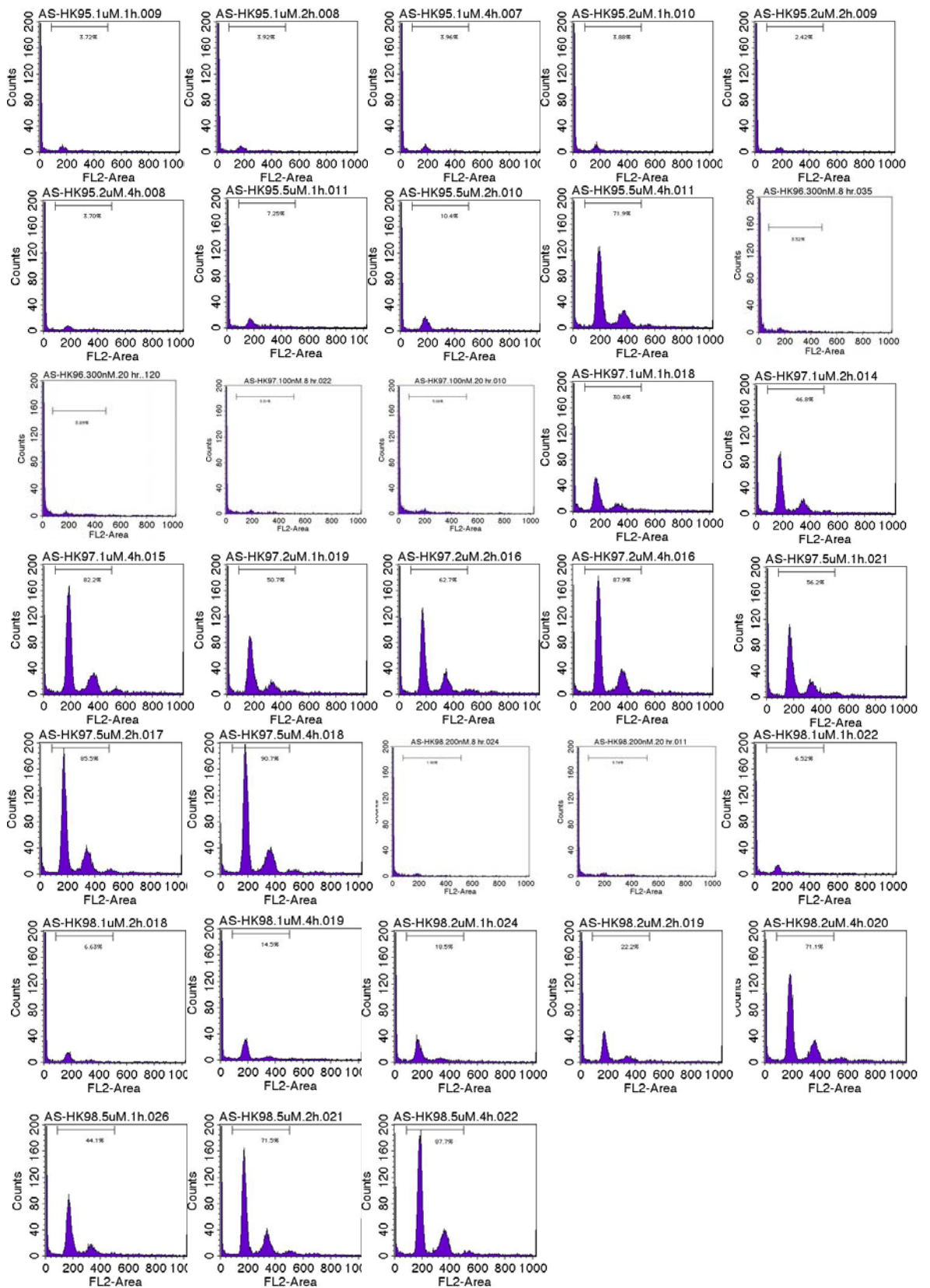
1.3 Mitochondrial membrane potential:



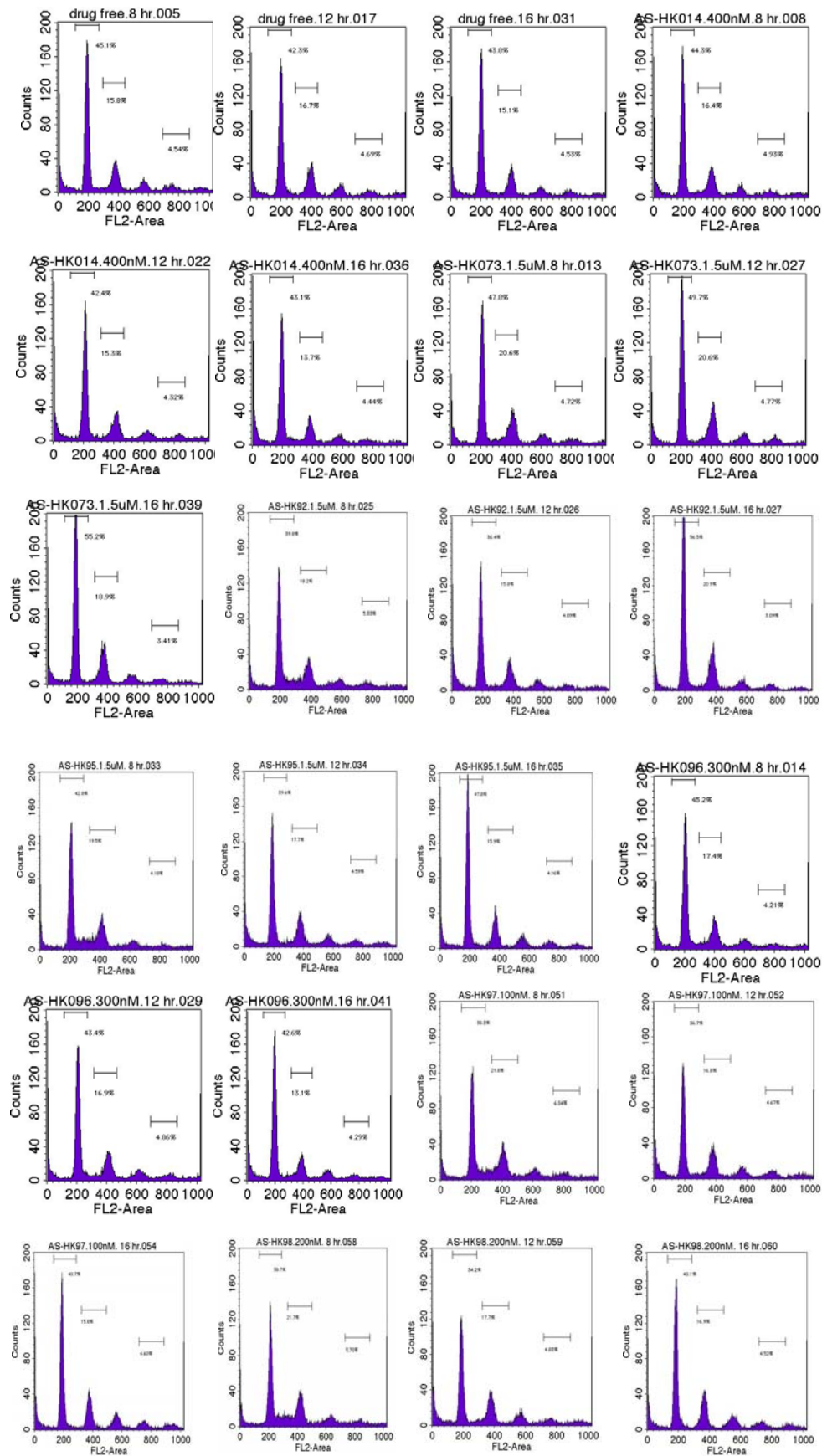
2 Curcumin compounds

2.1 Cellular permeability:

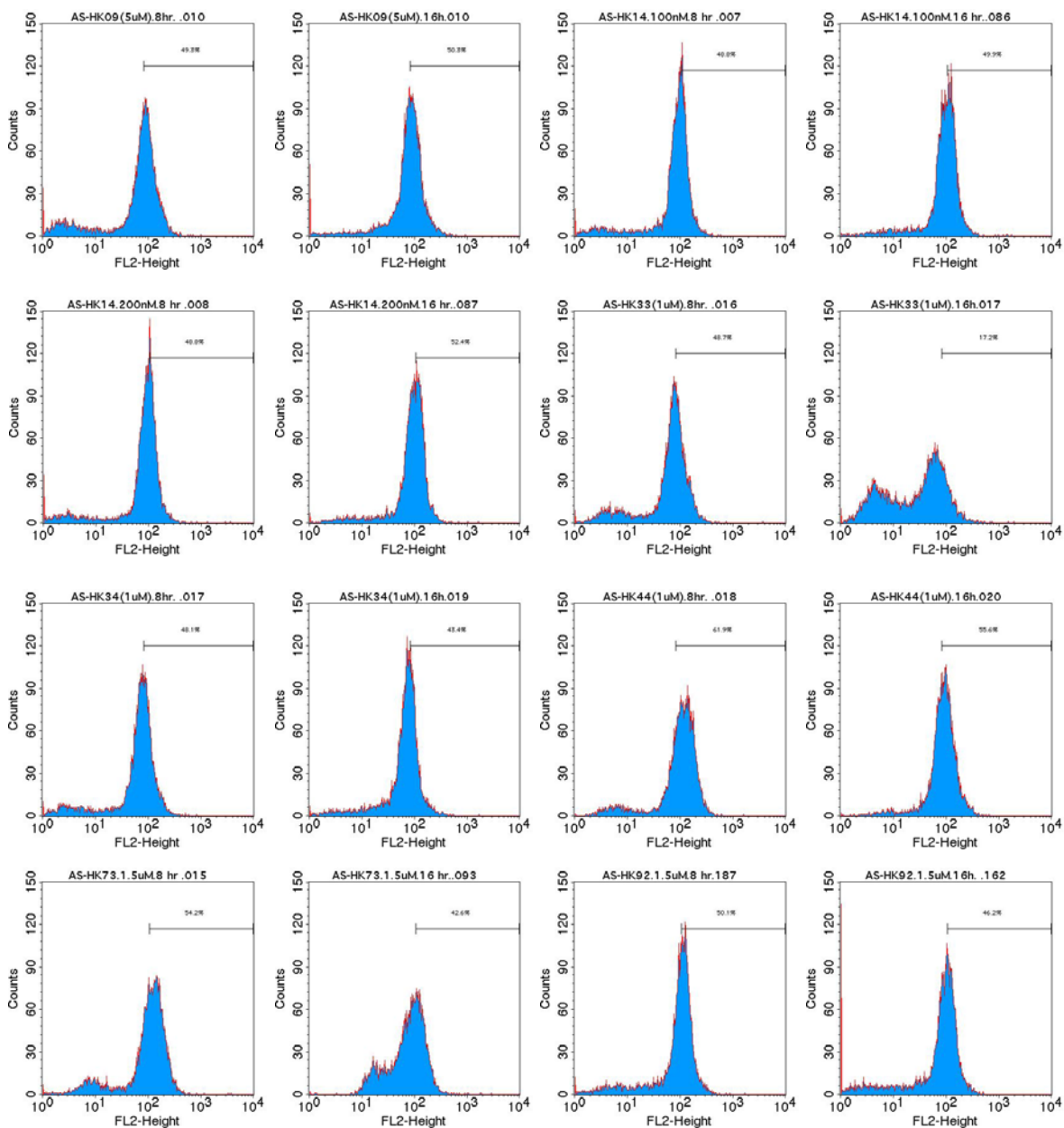




2.2 DNA content



2.3 Mitochondrial membrane potential



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