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STUDIES ON THE CHEMOTHERAPY OF LEISHMANIA DONOVANI INFECTIONS USING COMPOUNDS THAT AFFECT TRYPANOTHIONE METABOLISM

by

CHRYSANTHI C. MATARA

Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy in the Faculty
of Veterinary Medicine

Department of Veterinary Parasitology
University of Glasgow
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DEDICATION

Στους γονείς μου και στη θεία μου Δήμητρα

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DECLARATION

I declare that this thesis has been composed by myself from the results of research conducted under the supervision of Dr. F.W. Jennings and Professor G.H. Coombs and has not been used in any previous application for a degree. All sources of information have been acknowledged by means of a reference.

Signed

C.C. MATARA

Date 15th October 1991

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Figure 40. The effect of 8-hydroxyquinoline compounds against the promastigotes of L. donovani

SUMMARY

The hypothesis that compounds that inhibit the synthesis or interfere with the activity of trypanothione are likely to act synergistically against trypanosomatids was tested using Leishmania donovani as the target organism. Studies involved determining the efficacy of drugs and drug combinations against L. donovani growing in mice and as promastigotes and amastigotes in vitro.

The compounds tested included the antitrypanosomal drugs DL-α-difluoromethylornithine (DFMO, Eflornithine^R), diminazene diaceturate (Berenil^R), pentamidine (Lomidine^R) (pentamidine is also recognised as antileishmanial drug), melarsonyl potassium (Trimelarsan^R, Mel W^R), other experimental compounds reported to have activity against trypanosomes (various nitroimidazoles and guanylhydrazones, DL-buthionine-(S,R)-sulfoximine), and also the established antileishmanial drug, sodium stibogluconate (Pentostam^R). Most of these compounds were reported recently to be highly successful in the combination chemotherapy of experimental murine trypanosomiasis with central nervous system involvement.

Many of the compounds were shown to have antileishmanial activity but none of the combinations appeared to be synergistic under the conditions used. However, at high concentrations, some were more effective than the recognised antileishmanial drug sodium stibogluconate.

The antileishmanial activity of DFMO was studied in more detail using different regimens to assess the potential of the drug for treating visceral leishmaniasis and prevent relapse. DFMO showed good activity but did not give a total clearance of parasites from all tissues.

The potential of some promising drug combinations was also tested for clearing the parasites from tissues other than the liver, such as the spleen and the bone marrow. These combinations reduced significantly the number of parasites but without giving a total clearance.

An experimental compound, octyl degrol oil, which allowed a slow release of the drugs, was also investigated and the results showed

a beneficial effect when it was used with sodium stibogluconate.

A number of experimental compounds with potential antitrypanosomal activity were available in limited quantities. These included DNA intercalators, platinum and rhodium drug complexes and 8-hydroxyquinolines and were tested as potential antileishmanials. The results from this study suggested that the DNA intercalators and the 8-hydroxyquinolines have antileishmanial activity and further studies evaluating these compounds would be worthwhile.

1. INTRODUCTION

1.1. THE PARASITE

1.1.1. EVOLUTION, CLASSIFICATION, GEOGRAPHICAL DISTRIBUTION, TRANSMISSION AND VECTORS

1.1.1.1. The systematic position of the genus Leishmania

It is now generally accepted that living organisms are separated into five kingdoms: Monera, Protista, Plantae, Fungi and Animalia. Protozoa were regarded as a phylum within the kingdom Animalia, in classifications accepted until 1960's. After that time it became increasingly apparent that a new classification was necessary to take into account the enormous number of species of protozoa being reported for the first time and our increasing understanding of the biology and structure of unicellular organisms.

Protozoa are now best considered as a subkingdom within the Protista and seven distinct phyla have been recognised (Levine *et al*., 1980): Sarcomastigophora, Labyrinthomorpha, Apicomplexa, Microspora, Ascetospora, Myxozoa and Ciliophora.

Leishmanias are included in the Sarcomastigophora and a recent classification is outlined in Table 1.

The genus *Leishmania* may be defined as follows: parasitic protozoa of the order Kinetoplastida, family Trypanosomatidae: heteroxenous, with promastigotes and paramastigotes in the alimentary tract of an insect host and rounded amastigotes living and dividing in the macrophage cells of a vertebrate host.

As far as is known, the only invertebrate hosts are the blood-sucking phlebotomine sand-flies (Diptera: Nematocera: Pshychodidae: Phlebotominae) of the genera *Lutzomyia*, *Phlebotomus* and *Psychodopygus*. The males feed on fruit juices only, and therefore play no direct role in transmission of leishmaniasis. The probable evolutionary history of *Leishmania* from parasites of insects and eventually to one of mammals, implies that infected sand-flies are primary hosts, though for convenience they are termed vectors.

Table 1

The revised scheme for the classification of *Leishmania spp.*.
(From Levine *et al.*, 1980; Lainson and Shaw, 1987).

Kingdom PROTISTA Haeckel, 1866
Sub-Kingdom PROTOZOA Goldfuss, 1817
Phylum SARCOMASTIGOPHORA Honigberg and Balamuth, 1963
Sub-phylum MASTIGOPHORA Deising, 1866
Class ZOOMASTIGOPHOREA Calkins, 1909
Order KINETOPLASTIDA Honigberg, 1963, *emend*. Vickerman, 1976
Sub-order TRYPANOSOMATINA Kent, 1880
Family TRYPANOSOMATIDAE Doflein, 1901, *emend*. Grobben, 1905
Genus *Leishmania* Ross, 1903

The vertebrate hosts are a variety of mammals. The parasites are particularly common in canids (visceralising species) and rodents (mainly species causing cutaneous disease). Important hosts also occur in edentates, marsupials, procyonids, primitive ungulates and primates. Rare infections are recorded in New World chiropterans.

Until recently, it was considered that there are no sexual processes and multiplication in both the invertebrate and the vertebrate host is by binary fission. Recent unpublished findings, however, now suggest that sexual processes do occur.

Transmission among the mammalian reservoir hosts is predominantly by the bite of the infected insect. The mechanism of transmission has been the subject of much debate for many years. The understanding of the mechanisms of and factors influencing transmission remain to be fully investigated.

Distribution is through most tropical and sub-tropical America, Africa, India and parts of eastern Asia (but unknown in Australasia), in central Asia, the Mediterranean basin and some neighbouring European countries.

1.1.1.2. Evolution of the genus Leishmania

It is now generally accepted by most protozoologists, that the eukaryote Protista evolved from a flagellate stock (Butschli, 1882) and that the promastigote of the Trypanosomatidae probably approximates most closely to the ancestral form. However, opinions are divided regarding the origin of the trypanosomatids. Basically there are two schools of thought:

1. an origin from monogenic parasites of the invertebrates, with subsequent adaptation to vertebrates;

2. an origin in vertebrates, with secondary transmission by blood-sucking invertebrates.

There is unlikely ever to be any conclusive evidence on one side or the other, however, and the best one can suggest is that the modern trypanosomatids might have stemmed from both sources, with the weight of present-day opinion favouring an evolution from monogenic invertebrate flagellates (Lainson and Shaw, 1987).

All the known leishmanias develop as promastigotes, or morphological modifications of the form, in species of phlebotomine sand-flies. Speculations on the evolution of the existing species of Leishmania are only possible after a comparative study of a large number of these organisms. However, although we are at last beginning to appreciate the multiplicity of leishmanial parasites, our knowledge of the biology and biochemistry of most of them is still very poor. This is particularly the case with the developmental stages in the sand-fly which, if we are right in regarding these insects as the primitive and primary hosts, are most likely to provide us with evolutionary clues.

1.1.1.3. Classification of subgenera and species of the genus Leishmania

It is very obvious that the genus Leishmania is comprised of quite a considerable number of species. It is becoming increasingly apparent that these species are themselves made up of lower categories which might variously be regarded as subspecies, serological ("serodemes"), enzyme ("zymodemes"), or genetic ("schizodemes") variants; a situation that reflects the continuing process of speciation.

The taxonomic division of Leishmania into species is complicated due to morphological similarities of the organisms and the relatively few easily recognisable characters by which one species can be distinguished from another. Early classifications were based on the pathology of the diseases and the parasite's geographical distribution.

The more important characters used today for classification of the leishmanias are listed in the Table 2.

It has been particularly difficult to establish an adequate classification of Leishmania in the New World. There, the speciation of the genus has developed greatly, mainly due to the availability of an abundant variety of possible vectors and the multiplicity of reservoir hosts of the parasites. In addition, the ecological conditions have changed constantly as a result of deforestation and

Table 2

Characters[a] used in the classification and identification of the subgenera and/or species of the genus *Leishmania*.

A. Morphology

B. Behaviour of the parasite in:

   1. The natural sand-fly host
   2. The vertebrate host
   3. Specified *in vitro* culture media

C. Host response

D. Biochemistry

E. Geographical distribution

F. Clinical features in human infections

[a] Modified from W. Peters (1982).

settlement by humans (Riggione and Hernandez, 1981).

Diverse classifications have been proposed for the leishmanias. Recently Lainson and Shaw (1987) proposed a new classification which is as follows:

1. The subgenus *Leishmania* Saf'janova 1982

Definition: With the characters of the genus: life cycle in the insect host limited to the midgut and foregut of the alimentary tract: type-species *Leishmania* (*Leishmania*) *donovani* (Laveran and Mesnil, 1903) Ross, 1903. Old and New World.

The *Leishmania* (*L.*) *donovani* complex:

L. (*L.*) *donovani* (Laveran and Mesnil, 1903) Ross, 1903. (Old World).

L. (*L.*) *infantum* Nicolle, 1908 (Old World).

L. (*L.*) *chagasi* Cunha and Chagas, 1937 (New World).

Other possible species:

L. (*L.*) *archibaldi* Castellani and Chalmers, 1919. Sudan: in rodents, the genet, the serval-cat, and man.

Leishmania (*L.*) sp. Kenya: in man, and occasional dogs.

Leishmania (*L.*) sp. Eastern Pyrenees: in man.

Leishmania (*L.*) sp. Italy: in foxes and dogs.

Leishmania (*L.*) sp. Iraq: in canids and man.

Leishmania (*L.*) sp. China; Inner Mongolia: in man.

Other species, outside the *donovani* complex:

L. (*L.*) *tropica* (Wright, 1903) Luhe, 1906.

L. (*L.*) *aethiopica* Bray, Ashford and Bray, 1973.

L. (*L.*) *gerbilli* Wang, Qu and Guan, 1964.

L. (*L.*) *major* Yakimoff and Schokhor, 1914 *emend*. Bray, Ashford and Bray, 1973.

Leishmania (*L.*) sp. Namibia: in man and the sand-fly (*Phlebotomus rossi*).

Leishmania (*L.*) sp. Namibia: in the hyrax (*Procavia capensis*).

Leishmania (*L.*) sp. Ethiopia: in the rodent (*Arvicanthis* sp.).

The *Leishmania* (*L.*) *mexicana* complex (New World)

L. (*L.*) *mexicana* Biagi, 1953 *emend*. Garnham, 1962.

L. (L.) enriettii Muniz and Madina, 1948.

L. (L.) amazonensis Lainson and Shaw, 1972.

L. (L.) aristidesi Lainson and Shaw, 1979 emend. Lainson and Shaw, 1986

L. (L.) venezuelensis Bonfante-Garrido, 1980.

Leishmania (L.) sp. Dominican Republic: in man

Leishmania (L.) sp. Belize, Central America: in man.

Possible additional members of the L. (L.) mexicana complex:

L. (L.) pifanoi Medina and Romero, 1959 emend. Medina and Romero, 1962.

L. (L.) garnhami Scorza et al., 1979.

Leishmania (L.) sp. Trinidad: in the sand-fly (Lutzomyia flaviscutellata), rodents and marsupials.

Leishmania (L.) sp. Vale do Ribeiro, Sao Paulo State, Brazil: in man.

Leishmania (L.) sp. Caratinga, Minas Gerais State, Brazil: in the rodent Proechimys dimidiatus.

Leishmania (L.) sp. Caratinga, Minas Gerais State, Brazil: in man.

The Leishmania (L.) hertigi complex (New World):

L. (L.) hertigi Herrer, 1971.

L. (L.) deanei Lainson and Shaw, 1977.

2. The subgenus Viannia n. subgen.

Definition: With the characters of the genus: Life cycle in the insect host including a prolific and prolonged phase of development as rounded or stumpy paramastigotes and promastigotes, attached to the wall of the hindgut (pylorus and/or ileum) by flagellar hemidesmosomes, with later migration of flagellates to the midgut and foregut. Type-species Leishmania (Viannia) braziliensis Vianna, 1911, emend. Matta, 1916.

Distribution: the American tropics and sub-tropics.

The Leishmania (V.) braziliensis complex:

L. (V.) braziliensis Vianna, 1911 emend. Matta, 1916.

L. (V.) guyanensis Floch, 1954.

L. (*V*.) *panamensis* Lainson and Shaw, 1972.

L. (*V*.) *peruviana* Velez, 1913.

Unnamed species of the *L*. (*V*.) *braziliensis* complex:

Leishmania (*V*.) sp. Belize, Central America: in man.

Leishmania (*V*.) sp. Para State, Brazil (south of the Amazon River): in the sloth (*Choloepus didactylus*).

Leishmania (*V*.) sp. Itaituba, Para State, Brazil: in the common opossum (*Didelphis marsupialis*).

Unnamed species of the subgenus *Viannia*:

Leishmania (*L*.) sp. Para State, Brazil: in the sand-fly (*Lutzomyia tuberculata*).

Leishmania (*V*.) sp. Para State, Brazil: in an unidentified sand-fly (*Psychodopygus* sp.).

Leishmania (*V*.) sp. Para State, Brazil: in the sand-fly (*Lutzomyia ubiquitalis*).

Leishmania (*V*.) sp. Para State, Brazil: in the nine banded armadillo (*Dasypus novemcinctus*).

Leishmania (*V*.) sp. Para State, Brazil: in man.

Leishmania (*V*.) sp. Para State, Brazil: in man.

Questionable leishmanial parasites:

Leishmania herreri Zeledon, Ponce and Murillo, 1979. In the sand-flies (*Lu*. *ylephiletor*; *Lu*. *shannoni*; *Lu*. *trapidoi*) and the sloths (*Choloepus hoffmani*; *Bradypus griseus*).

Leishmania sp. (Barbosa *et al*., 1976). From man, Mato Grosso State, Brazil.

In contrast to the mammalian *Leishmania* species, the taxonomic status of lizard leishmanias is uncertain, mainly because the presence of intracellular amastigotes within the lizard host has never been well established. Some authorities maintain that the *Leishmania* species of lizards form a sufficiently distinct group to be ranked as *Sauroleishmania* at generic level (Ranque, 1973; Lainson and Shaw, 1987) while others (Wallbanks *et al*., 1985) suggested that such species might be classified within the genus

Trypanosoma. This suggestion followed from work showing that promastigotes of Trypanosoma platydactyli and Leishmania tarentolae had similar isoenzyme profiles. Recent studies (Simpson and Holz, 1988) of DNA and lipid composition, however, do not support the suggestion of Wallbanks et al., but give conflicting evidence that the lizard leishmanias are more closely related to mammalian leishmanias than to trypanosomes.

1.1.2. MORPHOLOGY, ULTRASTRUCTURE AND LIFE CYCLES

1.1.2.1. Morphology

The basic organelles of the leishmanial parasites are essentially similar and morphological differences at light-microscope level largely consist of differences in size and disposition of the nucleus and the kinetoplast.

Electron micrographs of the amastigote and promastigote stages have confirmed size differences seen at light-microscope level and have also disclosed differences in the number of subpellicular tubules (peripheral fibrils) and the distance between these structures (Gardener et al., 1977; Veress et al., 1980, see section 1.1.2.2. below).

By ordinary transmitted-light microscopy, amastigotes of Leishmania are best studied in impression-smears and sections after staining with the Romanowsky stains (Giemsa's or Leishman's stain). The organisms appear as round or oval bodies 2 to 5 µm in length which contain a nucleus and a kinetoplast and lie within vacuoles in the cytoplasm of the host cell. However, in impression smears, large numbers of extracellular forms following the rupture of infected cells are commonly seen. Leishmania donovani is the smallest (2-3 µm) (Simpson, 1968) of all leishmanias, whereas the relatively large size of the amastigotes of L. deanei in Brazilian porcupines (Lainson and Shaw, 1977) sets it apart from all other known leishmanias.

The promastigotes of all Leishmania species are fairly similar in size and morphology, possessing a long anterior flagellum and

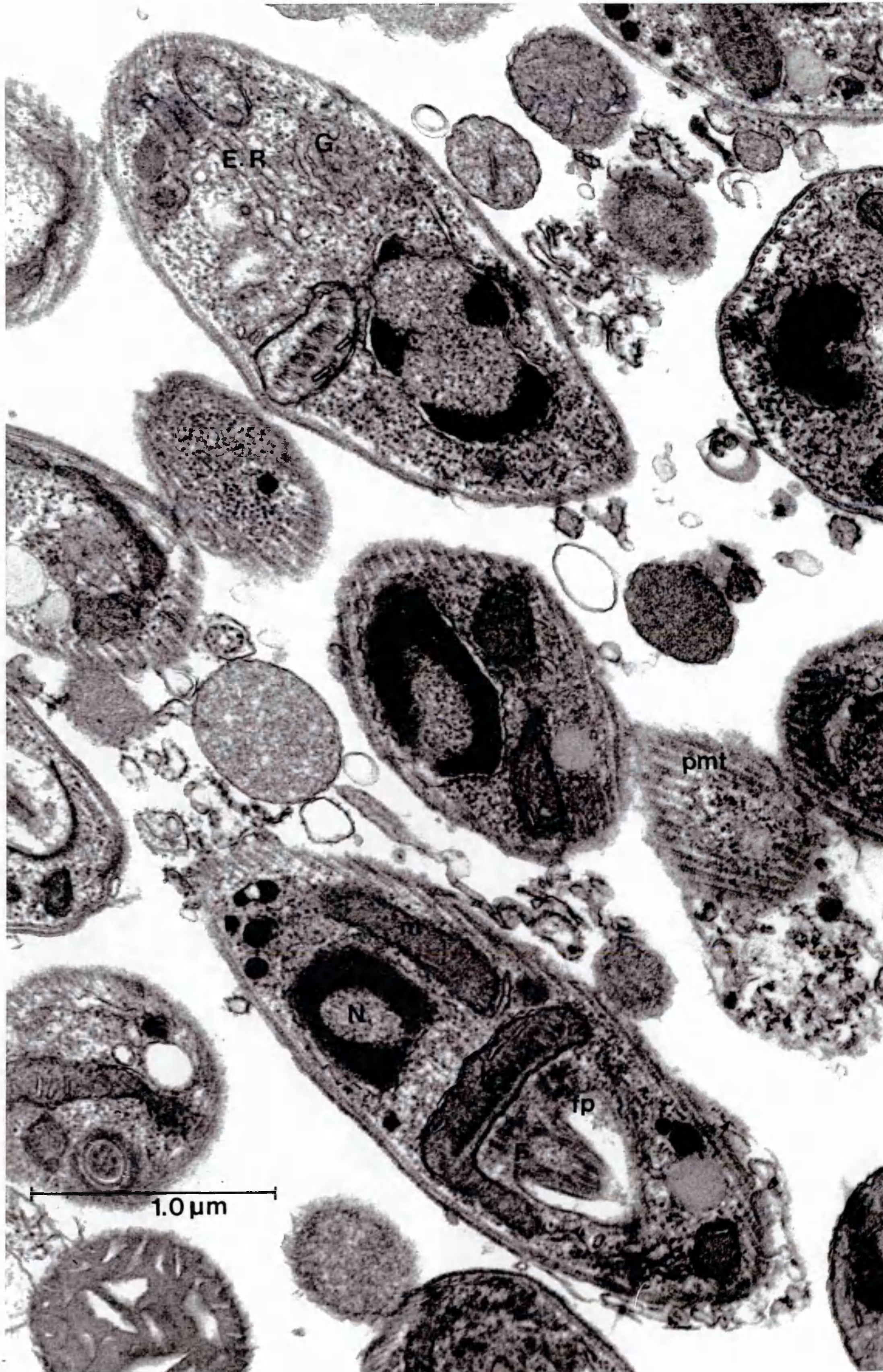


Fig. 1. A transmission electron micrograph of longitudinal section of L. donovani amastigotes. Note kinetoplast (K), mitochondrion (m), nucleus (N), subpellicular microtubules (pmt), Golgi apparatus (G), endoplasmic reticulum (E.R) and flagellum (F) in flagellar pocket (fp). x 44,000.
(Micrograph courtesy of L. Tetley).

having the approximate dimensions of 2 x 20 µm. However, in some species of Leishmania, distinct forms (nectomonad promastigotes, haptomonad promastigotes, paramastigotes) do occur in the sand-fly midgut and these are thought to correspond to different parts of the developmental cycle (Killick-Kendrick et al., 1974).

A wide variety of promastigotes is seen in in vitro cultures (usually nectomonad promastigotes, haptomonad promastigotes and rosettes of promastigotes). However, in cultures it is not possible to relate such forms to a particular sequence of development as in the sand-fly.

Transformation of amastigotes to promastigotes in vitro has been described in detail for L. mexicana by Hart et al. (1981c). The process of differentiation was reported to involve three morphologically distinct intermediates, including a division stage.

1.1.2.2. Ultrastructure

Both of the stages are typical of all trypanosomatids, in having many sub-cellular structures in common with most eukaryotic cells (See Figure 1). They do, however, have some unique features.

The plasma membrane of all stages of Leishmania is a typical, trilaminar unit membrane 2-4 nm in width, beneath which lies an array of subpellicular microtubules. The subpellicular microtubules impart a high degree of cell rigidity often making it difficult to disrupt leishmanial cells properly for cell fractionation and probably serve as a cytoskeleton (Vickerman and Preston, 1976). They have been used as potential means of separating Leishmania species. Various workers have counted these tubules. L. donovani had the smallest microtubule count of the species investigated. Veress et al., (1980) found 91-93 in L. donovani from human cases with either mucocutaneous or visceral infections in Sudan. The practical value of these techniques, however, compared with other specific methods of characterisation is limited.

Cytochemical and lectin-binding studies reveal that the unit membranes of amastigotes and promastigotes have polysaccharide components (Dwyer et al., 1974).

Uptake of macromolecules is by pinocytosis, but no detailed study of this process accompanied by cytochemical analysis appears to have been made.

A contractile or pulsatile vacuole lies next to the plasma membrane of the reservoir of promastigotes, but little is known of its true function in pathogenic flagellates, nor if its role is other than osmoregulatory (Molyneux and Killick-Kendrick, 1987).

At the anterior end of the parasite the plasma membrane is invaginated to form the flagellar pocket, from which the flagellum emerges. In this region, the network of subpellicular microtubules is absent. The characteristic axonemal structure of the flagellum is observed in Leishmania promastigotes; nine pairs of peripheral axonemal doublets encircle a central pair. This arrangement is observed distal to the basal plate.

The flagellar apparatus facilitates locomotion and attachment to the substratum and may also play a part in feeding mechanisms (Vickerman and Preston, 1976).

In the amastigote, the flagellum is not "free" or outside the body of the parasite and thus has no locomotory function.

The flagella of all paramastigotes and promastigotes are modified within the sand-fly for attachment to cuticular surfaces of the hind and foregut with the formation of hemidesmosomes. This modification of the flagella for attachment is a phenomenon common to all associations between trypanosomatids and invertebrates, with the attachment by hemidesmosomes to cuticle apparently being present in all trypanosomatids in insects.

There are three features of Leishmania structure that have been found in no other eukaryotic organism other than trypanosomatids. These are: the exceptional organisation of the mitochondrion and its DNA, the kinetoplast DNA (kDNA), and the presence of glycosomes and megasomes (Alexander and Vickerman, 1975; Vickerman and Preston, 1976; Opperdoes and Borst, 1977; Coombs et al., 1986).

The single highly branched mitochondrion, approximating to 10% of the total cell volume in the amastigote (Coombs et al., 1986) contains larger quantities of mitochondrial DNA than that found in

other cells (Vickerman and Preston, 1976). The DNA present in the mitochondrion is arranged in a slightly concave disc (0.83 μm in depth), called the kinetoplast, which lies just posterior to the basal body of the flagellum and within a constant spatial distance throughout the life cycle.

By rotary shadowing or darkfield electron microscopy, this fibrous kDNA disc of kinetoplastid flagellates, has been shown to be made up of several thousand mini-circles and a small number (50) of maxi-circles, organised in a characteristic network structure (Barker *et al*., 1982). The contour length of mini-circles of kDNA, which is group rather than species specific (as is the number of mini-circles), is 0.29 μm. During division the kinetoplast divides before the nucleus (Vickerman, 1974).

The process of transformation of leishmanias from amastigotes to promastigotes has been reported to be associated with an increase in the number of mitochondrial profiles per section of flagellate (Vickerman, 1974). In both forms of the parasite, the mitochondrion contains plate-like cristae and respiration is cyanide-sensitive (Vickerman, 1974; Hart *et al*., 1981c).

Ultrastructural studies of *Leishmania* reveal the presence within the cytoplasm of membrane-associated organelles such as the endoplasmic reticulum and Golgi apparatus, characteristic of other eukaryotes. Golgi products are packaged as lysosomes and play a role in intracellular digestion. Lysosomes of *Leishmania* have been little studied and assumptions to date have been made by comparison with what is known of their role in other kinetoplastids. This relates with digestion of ingested macromolecules after fusion with Golgi-derived primary lysosomes and also to the exocytosis of multivesiculate bodies into the flagellar reservoir. Vickerman and Preston (1976) also suggested that primary lysosomes may control organelle turnover, after fusing with organelles or structures within the parasite to form autolysosomes, in a material recycling process.

Alexander and Vickerman (1975) first reported the presence of megasomes in *L. m. mexicana* amastigotes.

Megasomes are lysosome-like organelles variable in shape but often large, occupying as much as 15% of the amastigote cell volume (Coombs *et al*., 1986). They contain several putative lysosomal enzymes including arylsulphatase and a cysteine proteinase (Pupkis *et al*., 1986). It has been suggested that they may play a role in the survival of *L. mexicana* amastigotes within the host (Coombs and Pupkis, 1986; Coombs and Baxter, 1984).

Glycosomes are specialised membrane-bound organelles which contain a number of enzymes associated with several important biochemical pathways such as glycolysis (Opperdoes, 1984; Mottram and Coombs, 1985) and *de novo* purine and pyrimidine biosynthesis (Hassan *et al*., 1985; Opperdoes, 1987).

They appear to be unique to trypanosomatids. They were first identified in *Trypanosoma brucei* (Opperdoes and Borst, 1977) and reported since then in *T. cruzi*, *Crithidia fasciculata* and *C. luciliae* (Taylor *et al*., 1980) and *L. m. mexicana* (Coombs *et al*., 1982).

There appears to be large variation between trypanosomatids in the abundance of these organelles and this is probably an indication of their importance to different species and stages. Bloodstream forms of *T. brucei* have an average of 240 (Opperdoes *et al*., 1984), *L. tropica* promastigotes are reported to contain 50-100 (Opperdoes, 1987) whereas the amastigotes of *L. m. mexicana* were found to have only around 10 (Coombs *et al*., 1986).

Although symbionts have not been observed in *Leishmania* species, Molyneux (1974) reported the presence of virus-like particles (VLPs) throughout the cytoplasm of *L. hertigi* cultured promastigotes, initially isolated from the tropical porcupine (*Coendou rothschildi*) in Panama and Brazil. It is not known if *L. hertigi* acquired the VLPs from a sand-fly vector or from the mammalian host. All stocks examined had VLPs organised in paracrystalline arrays or associated with induced tubules, in the cytoplasm of promastigotes (Molyneux and Ashford, 1983). The number of VLPs was drastically reduced upon transformation of promastigotes to amastigotes. VLPs were not transmissible to other *Leishmania* nor

to cells or lines susceptible to viruses (Molyneux and Killick-Kendrick, 1987).

1.1.2.3. Life cycles

The behaviour and life cycles of different species of Leishmania in the invertebrate host are not uniform. Defined sequences of morphological types occur within the sand-fly vector and life cycles of different species differ in the initial sites of establishment. In the vertebrate host, the parasite's life cycle involves existence in the reticulo-endothelial (RES) system. In this location, the parasites cause damage to a greater or a lesser extent.

The disease is initiated in the vertebrate host by the bite of an infected female phlebotomine sand-fly in search of a blood meal. The elongated, flagellated, extracellular promastigote forms of the parasite that occur in the sand-fly's foregut are known as metacyclics and are particularly adapted for surviving in mammals. They are inoculated into the mammalian skin during the blood meal whereupon they are rapidly phagocytosed by mononuclear phagocytes.

Having been taken into mononuclear phagocytes, the promastigotes transform into the amastigote form.

Lysosomes fuse with the phagosomes containing the organisms but the amastigotes resist phagolysosomal enzymes by unknown mechanisms. Leishmania amastigotes are remarkable in that, they are obligate intramacrophage-phagolysosome microorganisms.

The amastigote form divides by binary fission eventually causing the infected cell to rupture and release amastigotes which can be phagocytosed and so infect further macrophages. Multiplication of the amastigotes within the phagolysosomes of the macrophages of the skin, mucosa and viscera, gives rise to cutaneous, mucosal and visceral leishmaniasis, respectively. In the case of mucocutaneous leishmaniasis, primary infections occur at the level of the skin and later spread to the soft parts of the nasal septum, palate and pharynx (Mauel, 1979).

Amastigotes taken up by a feeding sand-fly transform back to promastigotes within the insect's midgut, after at least one initial

division. They then multiply, undergo developmental changes and depending on the species of *Leishmania*, colonise the pylorus and ileum (*L. braziliensis* complex) and/or the abdominal midgut (mammalian leishmanias other than the *braziliensis* complex) and finally migrate to stomodaeal valve, oesophagus, pharynx and proboscis where they are found as metacyclics.

A variety of morphological changes occur within the promastigotes as they divide and establish themselves within the sand-fly. They have been described in detail by Molyneux and Killick-Kendrick (1987). Most of the experimental work on the developmental changes that occur in promastigotes, however, has been carried out using promastigotes grown *in vitro*. The biochemical differences between the developmental stages of promastigotes and amastigotes are discussed in section 1.1.3..

The change from infective promastigote to the intracellular amastigote is the most important developmental change that occurs in terms of the clinical disease and is associated with large changes in morphology and several metabolic switches. At the light-microscope level, this process was found to be completed within 12-24 hours (Alexander, 1975; Ardehali and Khoubyar, 1978).

The factors that initiate the promastigotes to amastigotes transformation have not been yet fully elucidated. Temperature appears to play a very important role, the change in temperature from the sand-fly (27°C) to the definitive host (37°C) is considerable. It has been reported that a rise in temperature of a *L. braziliensis panamensis* promastigote culture from 26°C to 35°C resulted in amastigote-like forms (Darling and Blum 1987; Smejkal *et al*., 1988). A similar effect had been shown previously by various other workers with several other species of *Leishmania* (Hunter *et al*., 1984; Lawrence and Robert-Gero, 1985). This temperature change also induces the synthesis of several heat shock proteins which are thought to play a part in the transformation of the parasite (Hunter *et al*., 1984; Lawrence and Robert-Gero, 1985; van der Ploeg *et al*., 1985; Shapira *et al*., 1988; Toye and Remold, 1989).

Although amastigote type forms can be produced axenically from promastigotes in culture (see above) there has been great difficulty in attempting to culture these extracellular forms. Probably the most successful has been the growth of *L. m. pifanoi* (Pan, 1984) and *L. braziliensis* (Eperon and McMahon-Pratt, 1989) in cell free media although it is unknown how closely related these are to the intracellular amastigotes (Evans, 1987).

The *in vitro* culture of *Leishmania* species is also temperature-dependent. *L. donovani* amastigotes grow best in macrophages at temperatures ranging between 35°C and 37°C (Berman and Neva, 1981) while *L. m. mexicana* and *L. tropica*, at 34-35°C (Biegel *et al*., 1983). It has been suggested that this temperature sensitivity may account for the site specificity of the different cutaneous and visceral forms of leishmaniasis, presumably a cutaneous site would be several degrees cooler than a visceral site (Biegel *et al*., 1983).

1.1.3. PHYSIOLOGY AND BIOCHEMISTRY

Biochemical and physiological studies of leishmanial parasites began in the late 1940s. There were various reasons for these studies.

(1). Leishmanial parasites represent an interesting model system of cell development as they readily transform *in vitro*.

(2). They are involved in a complicated form of intracellular parasitism and an understanding of the biochemistry behind the survival mechanism could lead to the development of ways of interfering with it.

(3). Biochemical differences between parasite and host could represent potential targets for further drug development.

Since that time, the relationship between promastigote metabolism and amastigote function within a host cell has continued to be a concern among investigators. The knowledge of promastigote metabolism has increased considerably and techniques to investigate amastigote biochemistry both *in vitro* and within host cells have begun to appear. Some differences between multiplicative

promastigotes and metacyclic promastigotes have been reported (Mallinson and Coombs, 1989), but as yet the specific characteristics of metacyclic forms have not been extensively studied. The question of promastigote-amastigote correlations, however, remains an overriding consideration.

As there is a general lack of information on the amastigote stage, most of the work discussed here, except where stated otherwise, refers to promastigotes in general.

1.1.3.1. Energy metabolism

The energy metabolism of _Leishmania_ has been quite extensively studied and has been reviewed by Mukkada (1985) and Glew _et_ _al_. (1988). _Leishmania_ species studied to date take up glycerol, glucose, and a number of related monosaccharides (von Brand _et_ _al_., 1967). The work of Zeledon (1960a, b, c, d) has also shown the uptake of numerous amino acids by promastigotes. Enzymes of the glycolytic and tricarboxylic acid (TCA) cycle are present in both the amastigote and promastigote and their respiration is cyanide sensitive. Promastigotes contain enzymes that allow them to utilise carbohydrates or amino acids as energy substrates. With some species, amino acids appear to be used mainly during the earlier stages of promastigote growth _in_ _vitro_ whereas glucose tends to be utilised more during the stationary phase of growth (Marr and Berens, 1977; Cazzulo _et_ _al_., 1985). Mukkada (1985) suggested that _Leishmania_ promastigotes utilise amino acids rather than carbohydrates as their respiratory substrate. However, it has been suggested that these findings reflect the length of time that the promastigotes have been maintained in continuous culture _in_ _vitro_. Hart and Coombs (1982) reported that low sub-passage promastigotes of _L_. _m_. _mexicana_ preferentially utilised glucose rather than amino acids whilst the reverse was true for high sub-passage promastigotes.

Meade _et_ _al_. (1984) studied some of the enzymes involved in the carbohydrate and amino acid metabolism of the amastigotes and promastigotes in _L_. _donovani_ and showed that there are quantitative

rather than qualitative differences between the stages. However, the amastigotes of L. m. mexicana do appear to differ considerably from promastigotes. The amastigotes, unlike promastigotes, utilise fatty acids in preference to glucose for their energy metabolism. There are also higher levels of enzymes responsible for the β-oxidation of fatty acids in the amastigote than in the promastigote (Hart et al., 1981b; Hart and Coombs, 1982). This may reflect differences in the respective environments of the different stages: the sand-fly gut may be rich in amino acids and/or carbohydrates whereas the lysosomes may have a plentiful supply of fatty acids.

1.1.3.2. Surface Membrane Biochemistry

The interest in leishmanial parasite membranes, especially their external surfaces, stems from the potential role that these organelles play in allowing the parasite to evade macrophage digestive systems. Furthermore, knowledge of the nature and sources of leishmanial antigens and their processing by host macrophages is essential to a complete understanding of the various immune responses to infection. Finally, the development of techniques for the isolation of amastigotes stimulated an interest in the kinetics of various molecular exchanges which must occur between parasite and host cell through membranes maintained by both participants.

Pioneering studies on leishmanial membrane structure and chemistry are reported in the papers of Dwyer and his associates on L. donovani (Dwyer, 1980; Dwyer and Gottlieb, 1983, 1984, 1985; Gottlieb and Dwyer, 1981a,b). L. donovani membranes contain about 40 peptides which can be separated as distinct bands by electrophoresis (SDS-PAGE) and at least 24 glycoprotein/ glycopeptides. The extent to which these macromolecular components are involved in determining the clinical infection has yet to be determined, but Dwyer (1981) has reported several of them to be external, antigenic, and responsible for antigenic crossreactivity between L. donovani and L. tropica, L. braziliensis and Trypanosoma cruzi.

Three phosphomonoesterase activities have been shown to be present on the external surface of promastigotes (Dwyer and Gottlieb, 1985). There is a non-specific acid phosphatase as well as distinct 5'- and 3'-nucleotidases (Gottlieb and Dwyer, 1981a, b). These enzymes were clearly external with their active sites directed into the extracellular environment (Gottlieb and Dwyer, 1981a). Although exact functions of these enzymes are unknown, attractive hypotheses concern their potential role in purine uptake and an alteration of the phagolysosomal contents to enhance survival of the parasite. Equivalent 3'-nucleotidases are not found in mammalian cells and so may prove useful in diagnosis or as a target for chemotherapy.

An ATPase which is thought to be responsible for proton pumping, an adenylate cyclase which may be involved in the regulation of cyclic AMP levels and three phospholipases which have been implicated in the pathology of trypanosomatid infections, are also associated with surface membrane (Dwyer and Gottlieb, 1985).

A limited amount of information on the transport of exogenous materials across the parasite surface membrane reveals the active uptake of several nutrients, notably the amino acids methionine and proline (Simon and Mukkada, 1984; Law and Mukkada, 1979), hexose sugars (Zilberstein and Dwyer, 1985), alpha-aminoisobutyric acid (Lepley and Mukkada, 1983), purines and nucleosides (Hansen et al., 1982; Aronow et al., 1987).

The major cell surface glycoconjugate of leishmanial promastigotes is lipophosphoglycan (LPG) (King et al., 1987). Amastigotes were also reported (Schnur et al., 1972) to express LPG, however, it is not clear which of the two forms of the parasite produces it more efficiently. LPG has a highly immunogenic nature and as an antigen is useful for serotyping Leishmania strains (Greenblatt et al., 1983) and was found to protect mice against cutaneous leishmaniasis (Handman and Mitchell, 1985), suggesting the possibility of a molecular defined Leishmania vaccine (see section 1.3.2.). Turco (1988) reviewed the current information about the activities and functions that have been demonstrated or suggested

for LPG. Several are believed to involve attachment and entry of the parasite into the macrophage, while the others are thought to be crucial in enabling the parasite to survive within the phagolysosomal system (see section 1.1.4.).

The finding of a proteinase (gp63) on the surface of promastigotes and amastigotes has stimulated much interest in its biological function. Its role in parasite attachment and survival are discussed elsewhere.

1.1.3.3. Purine and Pyrimidine metabolism

Purine and pyrimidine nucleotides can be synthesised _de novo_ in mammalian cells from simple precursors (glycine, formate, CO_2, glutamine and aspartate). Alternatively, bases and nucleosides can be "salvaged" from exogenous sources or they may be the products of DNA and RNA breakdown within the cell. These can be converted to the appropriate nucleotides which are then used in the synthesis of DNA and RNA.

There have been extensive reviews recently on the purine and pyrimidine metabolism of parasitic protozoa (Hassan and Coombs, 1987) and the leishmanias in particular (Marr and Berens, 1985). Like most parasitic protozoa, the leishmanias appear to be unable to synthesise the purine ring _de novo_ and so rely on exogenous sources for DNA and RNA synthesis. However, they do appear to be able to synthesise _de novo_ at least part of their pyrimidine requirements.

Leishmania purine metabolism has been investigated extensively and several differences from the mammalian system have been identified and offer opportunities for chemotherapeutic attack (see section 1.3.). Work on _L. m. mexicana_ amastigotes and promastigotes has shown only quantitative differences between the stages in the enzymes involved in these pathways. However, this is not the case with _L. donovani_ for which differences have been reported. Notably, promastigotes contain adenine aminohydrolase whereas amastigotes contain adenosine deaminase.

1.1.3.4. Polyamines

The occurrence, metabolism and function of polyamines in trypanosomatids have captured the interest of a number of current investigators. These low molecular weight aliphatic compounds, include simple diamines such as putrescine and the common polyamines spermidine and spermine. Spermidine, spermine and their precursor putrescine, are ubiquitous components of mammalian cells. They appear to play important roles in cellular phenomena such as replication and differentiation. Studies with specific inhibitors of polyamine biosynthesis and with mutants unable to synthesise polyamines, have demonstrated that both differentiation and a normal rate of growth require polyamines. Polyamine content, especially of putrescine and spermidine, varies with the type of cell and its division state and its synthesis is closely controlled during the cell cycle and, in higher eukaryotes, is hormonally regulated (Tabor and Tabor, 1976; Janne _et al_., 1978; Bacchi _et al_., 1979).

The metabolic pathways of polyamine synthesis are given by Bacchi (1981). The main routes of polyamine biosynthesis in animals and microorganisms originate from ornithine and methionine. Ornithine is decarboxylated by ornithine decarboxylase (ODC) to yield putrescine. Methionine is phosphorylated and combined with adenosine to form S-adenosyl-L-methionine (SAM). Decarboxylated SAM, generated through SAM decarboxylase, provides aminopropyl groups for spermidine and spermine synthesis. The latter are formed by the addition of one or two aminopropyl groups to putrescine catalysed by spermidine and spermine synthetase, respectively.

Polyamines have been detected in several species of trypanosomatids and they are formed by this same route (see above). Initial studies on polyamine metabolism in _T_. _b_. _brucei_ bloodstream forms revealed that putrescine and spermidine but not spermine were present (Bacchi _et al_., 1977) and that there was sufficient ornithine decarboxylase (ODC) activity to account for _de novo_ synthesis of polyamines. S-adenosyl-L-methionine decarboxylase (SAM decarboxylase) was present as indicated by blockade by methylglyoxal bis (guanylhydrazone) (MGBG) of

[^{14}C]methionine incorporation into spermidine in intact cells and of CO_2 production from [^{14}C]SAM in cell extracts (Bacchi *et al*., 1979; Bitonti *et al*., 1986a) and by MGBG inhibition of cell growth (Chang *et al*., 1978).

Putrescine and spermidine have been detected in both amastigote and promastigote stage of *Leishmania* spp., however, reports regarding the presence of spermine are conflicting. Krassner and Morrow (1977) initially detected putrescine, spermidine and spermine in *L. donovani* amastigotes and recently transformed promastigotes; spermine was not found in established promastigote cultures. Schnur *et al*. (1979) found all three polyamines in serially cultured promastigotes of *L. tropica*, and *L. aethiopica*. Bachrach *et al*., (1979) identified these polyamines in promastigotes of several *Leishmania* spp.. Coombs and Sanderson (1985) observed high intracellular and extracellular putrescine levels in *L. mexicana* late log phase promastigotes and it was suggested that these may be responsible for the insensitivity of promastigotes of this species to α-difluoromethylornithine (DFMO) (Coombs *et al*., 1983). Polyamine levels have been found to be higher in promastigotes than amastigotes and the relative levels of each polyamine do vary with species and stage of *Leishmania*. During promastigote growth in culture, polyamine levels are highest in exponential phase.

The polyamine biosynthesis inhibitors have some potential as therapeutic agents against diseases, including cancer, that involve deranged cellular proliferation. However, their most promising use is for the treatment of diseases caused by parasitic protozoa. These organisms appear to be particularly sensitive to the interruption of polyamine synthesis, and the biochemical reasons for this sensitivity are under active investigation. Bacchi (1981) reviewed possible polyamine functions unique to trypanosomatids, and thus of potential interest as chemotherapeutic targets. One promising polyamine function is the activation of α-glycerophosphate dehydrogenase, a key respiratory enzyme, by spermidine or spermine in bloodstream *Trypanosoma brucei* (Bacchi *et al*., 1979). The pathway involved has not been demonstrated in *Leishmania*. It also has been

demonstrated that spermidine synthesis can be blocked in two ways, namely through inhibition of ornithine-putrescine conversion by DFMO (Bacchi et al., 1980) or through inhibition of the methionine-spermidine pathway by methylglyoxal bis guanylhydrazone (MGBG) (Chang et al., 1978).

1.1.3.5. Proteinases

These degradative enzymes hydrolyse proteins into peptides and amino acids for nutritional purposes and are implicated in a number of cellular processes including post-translational modification of proteins, rapid turnover of cellular proteins which allows rapid adaptation to environmental changes and in the pathogenicity of various microorganisms (North, 1982).

Interest has been greatly stimulated recently by the findings that the major surface antigen of Leishmania, known as gp63, is a proteinase (Etges et al., 1986). Two models for the binding of infective promastigotes to macrophages propose that this enzyme is a key surface molecule of the parasite during the initial phases of infection (Bordier, 1987). The molecular weight of this proteinase has been variously reported as varying between 63 kDa (Etges et al., 1986) and 68 kDa (Fong and Chang, 1981). Similar enzymes appear to be present in all species (Bouvier et al., 1987) and the proteinase has been shown to be apparently present in promastigotes and amastigotes of L. donovani, L. major and L. m. mexicana (Lockwood et al., 1987). It has been recently shown that the proteinase is a zinc metalloproteinase (Bouvier et al., 1987). Jahnig and Etges (1988) determined the secondary structure of this surface proteinase and showed that it appears to be a novel kind of membrane-anchored proteinase.

The role of this proteinase has not been yet elucidated. It has been proposed that it plays a role in protecting the parasite from microbicidal enzymes in the sand-fly gut (Bordier, 1987). However the findings that it is more abundant on the surface of the virulent than avirulent promastigotes of L. m. amazonensis (Chaudhuri and Chang, 1987) and that it appears to be more abundant on the surface

of the more infective stationary phase promastigotes than log phase promastigotes of *L. braziliensis* (Kweider *et al*., 1987) argues that it may play a role in protecting the parasite in the macrophage.

It has been also recently shown that the enzyme incorporated into liposomes prevents breakdown of protein inside the vesicle by macrophages (Chaudhuri *et al*., 1989). This strongly supports the theory that the enzyme aids survival by protecting the parasites from microbicidal attack.

A species and stage specific cysteine proteinase was found localised in megasomes of amastigotes of *L. mexicana* and not in any other stage or either *L. donovani* or *L. major* (Pupkis *et al*., 1986). More recently it has been shown that *L. m. mexicana* contains multiple cysteine proteinases that are of three types (Robertson and Coombs, 1990). The finding that cysteine proteinase inhibitors have antileishmanial activity against amastigotes of *L. m. mexicana in vitro* (Coombs and Baxter, 1984) shows the importance of these proteinases.

A large degree of developmental regulation of proteinases occurs in different species of *Leishmania* (Lockwood *et al*., 1987; North *et al*., 1988) and it has been suggested that the synthesis of these proteinases may be a prerequisite for the successful transformation to the amastigote and survival in macrophages. The appearance of a proteinase in stationary phase promastigotes of *L. donovani* that is not present in the log phase organisms or the amastigotes is particularly interesting but as yet unexplained.

Other aspects on the biochemistry of *Leishmania* are dealt with in section 1.3.3..

1.1.4. LEISHMANIA AND THE MACROPHAGE

The interaction of *Leishmania* with its host cell, the macrophage, is one of the most interesting aspects of leishmaniasis. Macrophages are involved in several areas of the host's immune response to invading micro-organisms. These cells have a wide array of antimicrobial defences which include phagocytosis and the

concomitant production of oxygen free radicals with subsequent exposure to the degradative enzymes of the lysosomal system. Thus, the cell types that are largely responsible for the killing of invading microorganisms are also the host cells for leishmaniasis. The parasite itself displays a number of highly elaborate strategies that enable evasion of the host's defence system during all stages of the immune response. Basically, the evasion mechanisms can be attributed to three principles:

(1). evasion from cytotoxic serum components,

(2). strategies for survival within macrophages, and

(3). modulation of the T-cell immune response.

1.1.4.1. Entry of Leishmania into the macrophage

Immediately after transmission by the vector and before entry into macrophages, extracellular promastigotes are exposed to and escape from the cytotoxic environment of the serum. This serum resistance of infective promastigotes is not caused by their failure to bind and activate complement (Bogdan et al., 1990), but rather appears to be linked to the expression of developmentally regulated surface antigens (da Silva et al., 1989). It is also possible that the saliva of the insect vector somehow contributes to the relative serum resistance of metacyclics, since salivary gland lysates enhance their infectivity (Titus and Ribeiro, 1988). Preliminary experiments revealed species-specific differences between the serum resistance of metacyclics from various Leishmania isolates that do not, however, strictly correlate with the severity of the disease (Franke et al., 1985). In contrast, amastigotes of L. donovani and L. mexicana, were shown to resist lysis by human serum despite efficient complement component type 3 (C3) fixation and binding, whereas amastigotes of L. major were readily killed (Mosser et al., 1985). It is assumed that the high serum sensitivity of amastigotes of L. major may contribute to the limitation of the disease to the skin (oriental sore) as opposed to the visceralization seen in L. donovani infections.

The two major surface glycoconjugates of Leishmania, gp63

(Bordier, 1987) and the lipophosphoglycan (LPG) (Turco, 1988), mediate the uptake of different developmental stages of promastigotes (log phase and metacyclics), (on which the expression of the LPG is developmentally modified), by macrophages. They function either directly or indirectly (via bound host proteins) as ligands for various macrophage receptors among which the complement receptors CR1 and CR3 appear to be the most important (Bordier, 1987; da Silva et al., 1989). "Serum-independent binding of Leishmania promastigotes to the CR3 receptor is mediated by a sequence containing arginine-glycine-aspartic acid (RGD) in gp63 or iC3b which are deposited on LPG or gp63 due to activation of locally produced complement components" (Tait and Sacks, 1988). Synthetic peptides based on this sequence competitively inhibited the serum independent binding to CR3 of gp63 coated beads, C3b-coated erythrocytes and whole parasites (Russel, 1987). The binding of promastigotes was also reported to be inhibited by anti-fibronectin (Fn) antibodies (Quaissi, 1988) although it is now unclear whether this is because attachment is mediated via Fn, iC3b, or gp63, since all share cross-reactive RGD sequences and all these ligands are thought to be expressed or deposited on the surface of infecting promastigotes.

Serum-dependent binding is mediated primarily by C3b (which accounts for 80% of the C3 deposited on macrophage) deposition on LPG and binding to CR1 receptor (Puentes et al., 1988). Secondary interactions might involve fibronectin receptor binding to surface-bound plasma fibronectin (Wyler et al., 1985), and mannose-fucose receptor binding to mannose-containing oligosaccharides on LPG or gp63 (Blackwell et al., 1985).

Despite a great number of studies on the receptor-ligand interactions, the results are somewhat conflicting since they are influenced by the species and growth phase of the parasite, the source of the macrophages investigated (human versus mouse) and the culture conditions (presence or absence of serum).

In the future a critical area of study will be to define receptors and ligands involved in infection by Leishmania

amastigotes.

1.1.4.2. Strategies for survival of *Leishmania* within macrophages

1.1.4.2.1. Inhibition of the oxidative burst

Phagocytosis of *Leishmania* by macrophages involves specific receptors (Chang, 1981; Klempner *et al*., 1983). These receptors have been reported (Wright and Silverstein, 1983; Bryant *et al*., 1986) to differ in their capacity to elicit specific cellular responses, such as the triggering of the respiratory burst. Thus, the initial engagement of "appropriate" receptors, may play a critical role in determining the subsequent intracellular fate of the parasite. The development into metacyclic stage promastigotes is associated with the primary use of the CR1 and C3bi-binding site of CR3 that have been reported to be poor in triggering a respiratory burst and this may be critical for the subsequent survival of the parasite (da Silva *et al*., 1989).

The species and stage of *Leishmania* and source of macrophages also play an important role in the size of the oxidative burst stimulated (Bray and Alexander, 1987). Metacyclics stimulate a respiratory burst that is intermediate between amastigotes and non-infective promastigotes in magnitude (Mallinson and Coombs, 1989).

Leishmania also appear to be able to downregulate the oxygen-dependent killing mechanisms of macrophages. It has been shown (Remaley *et al*., 1984) that the surface acid phosphatase of *L. donovani* is capable of blocking the production of O_2^- and H_2O_2 by phagocytes. Interestingly, cellular and membrane activity of acid phosphatase of *L. donovani* correlates well with the virulence (Katakura and Kobayashi, 1988). Another potent inhibitor of the oxidative burst is LPG that interacts with the regulatory domain of protein kinase C, one of the key enzymes involved in the generation of oxidative metabolites (McNeely and Turco, 1987).

1.1.4.2.2. Scavenging of oxidative metabolites

Avoidance of exposure to oxygen metabolites may be part of the survival mechanism. However, some cells that do stimulate a respiratory burst manage to survive. *Leishmania* are provided with a number of substances that serve to neutralise or eliminate oxidative metabolites.

Superoxide dismutase and possibly catalase are thought to play a role in the detoxification of these radicals by the parasite (Murray, 1981; Haidaris and Bonventre, 1982). The role of glutathione in free radical scavenging is well documented in prokaryotes and eukaryotes (Meister and Anderston, 1983) and recently differences between this and the equivalent in trypanosomatids, trypanothione, have been highlighted but the role of trypanothione in the survival of *Leishmania*, is as yet unclear.

Recent experiments by Chan *et al*., (1989), have illuminated another important parasite-protective mechanism: the LPG of *Leishmania*, which scavenges oxygen radicals and prevents the killing of the parasite by hydroxyl radicals.

1.1.4.2.3. Inactivation of lysosomal enzymes and maintenance of a pH homeostasis within the phagolysosome

Once phagocytosed the parasites are enclosed in the parasitophorous vacuoles and lysosomal fusion follows with the subsequent formation of the phagolysosome, thus exposing the parasite to the microbicidal mechanisms of the lysosomal system. A number of phagolysosomal factors such as acid pH, osmotic stress or lytic enzymes contribute to the antiparasitic activities of the macrophages. *Leishmania* have developed a variety of escape mechanisms against these. LPG clearly inhibits the lysosomal hydrolytic enzyme β-galactosidase (El-On, *et al*., 1980) and erythrocytes coated with LPG are protected from lysis by the macrophage (Eilam *et al*., 1985). In addition to having a direct inhibitory effect on lysosomal enzymes, it is possible that LPG may act as a cell surface barrier preventing lysosomal enzymes from attacking the parasite (Turco, 1988). The gp63 molecule may also have parasite-protective function in the phagolysosome. Its

protease activity, with an optimum at pH 4, inactivates proteolytic host enzymes and thus may protect parasite proteins from phagolysosomal degradation (Chaudhuri _et_ _al_., 1989). It has also been suggested (Coombs and Pupkis, 1986) that in the amastigotes of some species of _Leishmania_ and in particular of the _L_. _mexicana_ complex, proteolysis involving a cysteine proteinase may result in changes in the lysosomal pH and hence lysosomal function, thereby being of especial importance to the parasites survival in macrophages. _L_. _donovani_ amastigotes are highly adapted to the acidic medium inside the phagolysosome, without blocking its acidification, and at the same time they are able to maintain a neutral intracellular pH (Mukkada _et_ _al_., 1985).

These biochemical processes may further explain the amazing capacity of amastigotes to survive and multiply within macrophages.

1.1.4.2.4. Modulation of the T-cell immune response

Macrophages are not only host cells for _Leishmania_ and antiparasitic effector cells but they also present antigen in the context of MHC class II molecules to T-cells that, together with interleukin I (IL-1), leads to T-cell activation (Bogdan _et_ _al_., 1990). _Leishmania_ are able to modulate the T-cell immune response by inhibition of T-cell activation. Infection of murine macrophages with _L_. _donovani_ has been shown to reduce both MHC class II expression and IL-1 secretion. In addition, _L_. _donovani_-infected macrophages are refractory to the activating potential of interferon-γ (IFN-γ) (Reiner _et_ _al_., 1988). However, this is not the case for all _Leishmania_ species (Cillary _et_ _al_., 1989) and the molecular basis of these differential features is yet unknown. _Leishmania_ are also able to modulate the T-cell immune response by induction of functionally distinct T-cell subsets in the host. The predominance of one or the other functionally distinct T-cell subsets and the respective lymphokines promotes either susceptibility (T_{H2}-cells) or resistance (T_{H1}-cells) to infection, as shown with _L_. _major_ in mice (Moll and Mitchell, 1988).

Current studies are concentrated on this area of research and

the results from these studies will have implications for the formulation of future vaccines and for immunobiology in general. From the known primary structure of the major surface glycoprotein (gp63) of *L. mexicana*, potential T-cell epitopes have been identified, synthesised and shown to be capable of stimulating the proliferation of mouse $CD4^+$ T_H cells. One peptide, PT3, specifically stimulates IL-2 secretion and T_{H1} cell subset proliferation (Jardim *et al.*, 1990). Vaccination with PT3 in the presence of adjuvant, partially protects mice from infection with either *L. mexicana* or *L. major* (Ash, 1990).

1.2. THE DISEASE IN HUMANS AND IN DOGS

The leishmaniases form a family of diseases which predominantly involve the parasitisation of the reticuloendothelial system by species of the genus *Leishmania*. The worldwide prevalence of the different human leishmaniases is unknown but thought to exceed 12 million cases (WHO, 1989). An incidence of 400,000 new infections per year has previously been reported (WHO, 1982). The three basic types of leishmanial disease have a geographical distribution determined by the occurrence of the appropriate insect vector. Biological and biochemical criteria suggest that *L. tropica*, *L. major* and *L. aethiopica* cause cutaneous leishmaniasis in the Old World, the *L. mexicana* and the *L. braziliensis* complexes cause cutaneous leishmaniasis and mucosal disease in the New World and the *L. donovani* complex causes visceral leishmaniasis. The leishmaniases can be broadly categorised epidemiologically as anthroponotic, with man as the only or main source of infection and transmission occurring mainly in settled communities, or zoonotic, with domestic animals (in settled communities) or wild mammals (in new urban developments, forestry areas, etc.) serving as the important source of infection. Clinically, several forms of infection are recognised in man, ranging from the single cutaneous ulcers caused by *L. major* to the hideously disfiguring and sometimes fatal mucocutaneous form in *L. braziliensis* infection, or to the

visceral infection, kala-azar, associated with _L. donovani_, which entails a high mortality rate if it is not successfully treated.

Dogs are also severely affected by visceral leishmaniasis (VL) caused by parasites of the _L. donovani_ complex. Their role as reservoirs or victims of other species of _Leishmania_ is less clear since few canid isolates have been adequately identified and most of the information discussed here refers to Mediterranean kala-azar (VL) caused by parasites of the _L. donovani_ complex and particularly _L. infantum_. The first dog infection by _Leishmania_ was reported (Nicolle and Comte, 1908) in Tunisia. Since then evidence has accumulated showing the diffusion of the infection in the Mediterranean area and its close relationship with human visceral leishmaniasis. There seems to be no doubt nowadays that canine infections are the major reservoir of the disease. Cats may also be infected with Mediterranean leishmanias but reports of clinical cases are rare (Holzworth, 1987). Wild carnivores and rodents like the fox and the black rat, have been suspected or incriminated as wild reservoirs in a number of Mediterranean countries and these could constitute the primary reservoir from which the secondary, synanthropic, canine reservoir could have developed. Generally, canine leishmaniasis foci maintain a constant level over a long period and epidemic outbreaks have never been reported (Adler, 1936). In Mediterranean foci, the frequency of infection in dogs has been reported to range between 3.7% (Bettini _et al_., 1980) to 23.9% (Pozio _et al_., 1981). The canine isolates in a number of Mediterranean countries have shown an isoenzyme pattern identical to that of reference strains causing canine and human VL of this area and have been typed as _L. infantum_. The infection rate in dogs does not correlate with the incidence of the disease in man in the Mediterranean countries (Pozio _et al_., 1981). In areas with a high infection rate among dogs the infection of human native inhabitants does not exceed 1-2% and includes mainly young children and babies (Levine, 1985; Molyneux and Ashford, 1983; Zuckerman and Lainson, 1977). The positivity of the Leishmanin or Montenegro skin test that detects cell mediated immunity, confirms that transmission to

man occurs as well. Since in some foci the vectors are known to be both zoophilic and anthropophilic it has been suggested that general resistance factors, such as nutrition or genetic resistance, could influence the course of the human disease. In a typical Mediterranean focus, patent cases represent 40-50% of the infected canine population, the remaining fraction being represented by latent pre-clinical forms (30-40%) or by resolutive cases (10-30%) (Lanotte et al., 1979; Pozio et al., 1981). Sand-flies get infected at significant rates only when fed on patent cases (Rioux et al., 1972). However, preclinical cases are of great epidemiological importance, since they represent a latent reservoir which if left untreated, evolve towards infective cases in the course of 1 year or more (Adler and Theodor, 1932). It is difficult to estimate the prevalence of imported leishmaniasis in dogs in non-endemic countries. In the United Kingdom, where the import of pets is strictly controlled, at least 7 cases were recognised among 4000 imported dogs, including animals imported from leishmania free countries (Longstaffe and Guy, 1986).

Proven or suspected vectors of L. infantum in man and dog in the Mediterranean area belong almost wholly to the genus Phlebotomus, subgenus Laroussius (WHO, 1984). Autochthonus leishmaniasis in sand-fly free areas has occurred sporadically in man (Longstaffe and Guy, 1986) and probably also in dogs (Zuckerman and Lainson, 1977). These infections may result from mechanical transmission, either direct from vertebrate to vertebrate or via insects. These modes of transmission seem to be rare, but have been documented (Longstaffe and Guy, 1986).

Canine leishmaniasis is characterised by a visceral distribution of parasites followed by a massive localisation in the parasitised macrophages in the skin. Depending on host resistance, clinical signs become evident within a period of a month to many years (Kammermann-Luscher, 1980; Lanotte et al., 1979).

Little is known about the pathogenesis of leishmaniasis in dogs. In man, the pathogenesis and clinical picture of the disease not only vary according to organotropism and other properties of the

infecting Leishmania species, but also depend upon the immunological competence of the host (Bryceson, 1979; Heyneman, 1982; Molyneux and Ashford, 1983). Since Leishmania is an obligatory intracellular parasite, host defence strongly depends upon T-cell activity. Without the support of T-cells, macrophages are not able to kill the amastigote stage of the parasite (Maul, 1980). Abundant B-cell activity but complete absence of cell mediated immunity (CMI) to leishmanial antigens characterises one pole of the clinical spectrum of leishmaniasis in man (Bryceson, 1979; Heyneman, 1982; Molyneux and Ashford, 1983). This so called "anergic state", allows unrestricted multiplication and dissemination of the parasites, initially with little organ damage. At the other end of the spectrum, CMI increases and B-cell activity decreases. A CMI which is inadequate but responsive to numerous parasitic antigens results in destructive leishmanial ulcers. With more effective CMI, processes are more localised, contain less parasites and may even tend to self cure. At the ultimate end of the spectrum, B-cell activity is virtually absent but delayed hypersensitivity is pronounced. Non-ulcerating granulomatous nodules with hardly any parasites characterises what is called allergic state (Bryceson, 1979; Heyneman, 1982). In some patients, medication seems to shift the immunopathologic state from the anergic to "allergic stage" without accomplishing definite cure. This may result in the development of so called post kala-azar dermal leismaniasis (PKADL) (Bryceson, 1979; Heyneman, 1982).

In dogs, leishmaniasis seems to develop mainly following the "anergic" model. Experimental infections with L. chagasi and L. donovani, caused rapid depletion of the T-cell regions and proliferation of B-cell regions in the lymphoid organs (Keenan et al., 1984b). In naturally infected dogs, the parasites disseminate all over the body despite high levels of circulating antibodies. Ulcers are common but granulomatous nodules seem to be rare (Kammermann-Luscher, 1980; Lanotte et al., 1979).

An enormous production of immunoglobulins is observed (Kammermann-Luscher, 1980) but rather harms than protects the

patient. Specific antibodies may enhance phagocytosis by macrophages, however, and it is precisely the macrophage that seems to be essential for the survival of the parasite. The production of antibodies unrelated to parasitic antigens have also been reported (Molyneux and Ashford, 1983) and these are thought to include autoantibodies that may be associated with the development of pathologic phenomena (Mischer and Belehu, 1982).

A potential hazard of disturbed T-cell regulation and exuberant B-cell activity is the generation of large amounts of circulating immune complexes (CIC). The production of antigen-antibody complexes is probably quite common in infectious diseases, but symptoms rarely result from their presence (Drutz and Graybill, 1982). In some circumstances, however, CIC deposition on the walls of blood vessels may cause vasculitis, polyarthritis or glomerulonephritis (Drutz and Graybill, 1982; Marcussen et al., 1989). CIC may also bind complement to blood cells and hence shorten their life span (Mischer and Belehu, 1982).

In visceral leishmaniasis in man, CIC can readily be demonstrated (Neva, 1985) and this has been connected with leucopenia, anaemia and thrombocytopenia in patients suffering from leishmaniasis (Mischer and Belehu, 1982). The kidneys of these patients may show pathologic changes secondary to CIC deposition (Neva, 1985), yet proteinuria and renal failure seem to be rare (Bryceson, 1979). Probably CIC play a major role also in the pathogenesis of canine visceral leishmaniasis and the renal failure and some other clinical manifestations of canine leishmaniasis, such as the bleeding tendency present in many leishmaniotic dogs, are CIC-induced. CIC may cause vasculitis and thrombocytopenia and, by way of renal failure, uraemia and hence platelet dysfunction. All of these impair haemostasis.

High concentrations of so called "cryoglobulins" may be present in the serum of patients with CIC. During cold weather these proteins may precipitate in the blood vessels in the extremities of the body and cause local ischaemia and necrosis (Stites, 1982). In dogs, emaciation and cachexia may occur despite normal or even

ravenous appetite. This has been ascribed to possible parasite-host competition for some essential nutrients. Tryptophan, an essential amino-acid, was significantly decreased in experimentally infected dogs (Keenan et al., 1984a).

Canine leishmaniasis has a variable and long incubation time and an insidious onset, and it is difficult to determine whether earlier anamnestic data are related to the disease. Most dog owners sought veterinary help because of a combination of problems such as listlessness, skin lesions, disturbed appetite, weight loss and chronic conjuctivitis or rhinitis. Skin problems are variable in character and extension but the most prominent feature is hyperkeratosis, presented as excessive scaling of the epidermis and thickening, depigmentation and chapping of the nozzle and the foot pads. The hair coat is dry and brittle with diffuse hair loss and appearance of ulcers is not uncommon (Figures 2a and 2b). Onychogryphosis (abnormally long curved nails) is considered very characteristic and present in the great majority of leishmaniotic dogs. The clinical course of the disease in dogs after a natural infection leads to death in 3-24 months if left untreated.

Canine leishmaniasis is diagnosed directly by microscopical observation of amastigotes in Giemsa-stained smears from lymph node aspirates. In case of negative results, inspection of stained bone marrow smears may still reveal parasites. If parasites cannot be detected by direct examination, the diagnosis may sometimes be established with cultivation of biopsy material from bone marrow or lymph nodes aspirates in specific media (Zuckerman and Lainson, 1977). Culture tests in Schneider's Drosophilla medium have been reported recently to be very successful in detecting parasites (von Reiter, 1985). A wide range of serological tests such as the indirect immunofluorescent test (IFT), can be used for the immunodiagnosis of leishmaniasis (Kammermann-Luscher, 1980; Zuckerman and Lainson, 1977). A positive reaction indicates that a dog has been infected but does not necessarily prove that the parasites are still present in the body. Once infected, however, over 90% of dogs will eventually develop clinical signs (Lanotte

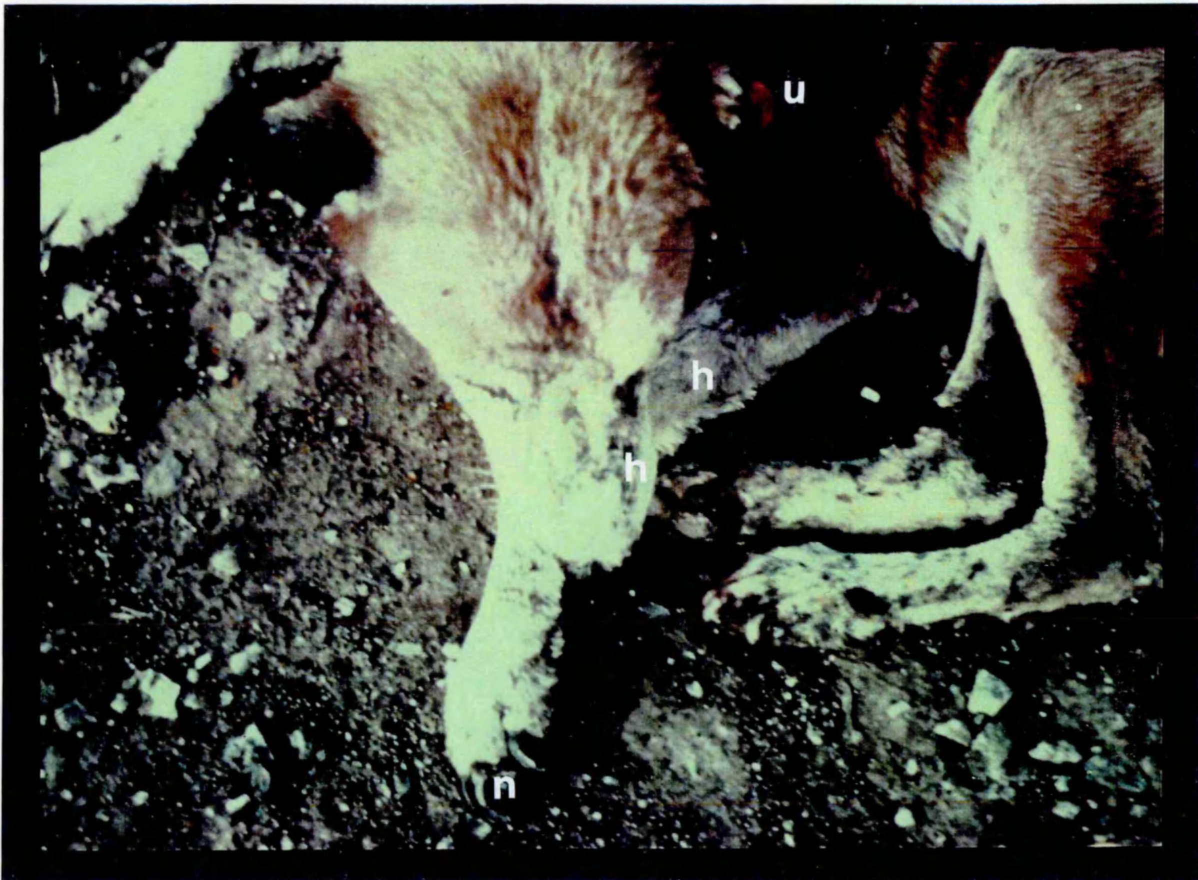


Fig. 2a. Canine leishmaniasis in Senegal: Note the ulcerated left pinna (u), relative overgrowth of nails (n) and facial and lower limb hyperkeratosis (h). In addition there was generalised loss of condition and periorbital alopecia.
(Photograph courtesy of WHO).

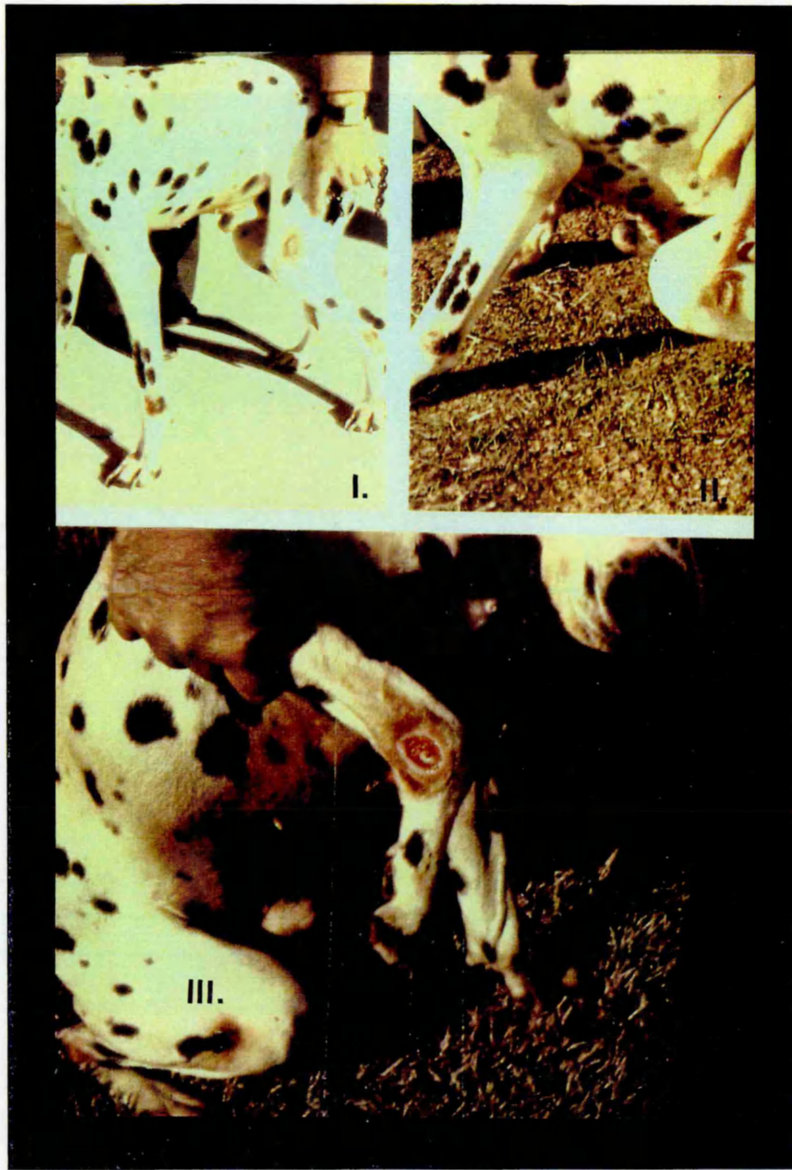


Fig. 2b. Canine leishmaniasis in Greece: I. & II. Initial stages of ulceration, III. Severe ulceration developed during a 4 week period. (Photograph courtesy of V. Contos).

et al., 1979). Hence a positive IFT in a clinically suspect dog is strong support for the preliminary diagnosis. Also, some of the initially seronegative animals may become seropositive after some weeks. The diagnosis of leishmaniasis can hardly be missed in dogs with characteristic features of the disease, provided one is familiar with the clinical picture. However, signs are often non specific and vague and may be confused with other skin diseases, myelo- and lympho-proliferative diseases and endocrine disorders. It is rather difficult to distinguish from canine systemic lupus erythematosus (SLE), since almost any sign present in leishmaniasis may also occur in SLE.

Treatment of leishmaniasis is at best difficult, usually involving toxic drugs based on heavy metals such as antimony. The lack of progress in development of better antileishmanial agents is illustrated by the fact that all the drugs in current use were introduced in the late 1940s. These are limited to four - sodium stibogluconate, meglumine antimoniate, pentamidine and amphotericin B. In general, response to antimonials varies according to the type of disease and various regimens are recommended in different geographical areas (Bryceson, 1979; WHO, 1984; Berman, 1988). Toxicity and variable activity attend the use of antimony in visceral leishmaniasis and many cases of muco-cutaneous leishmaniasis remain refractory to any therapy (Bryceson, 1979; Berman, 1988). The drugs used to treat human visceral leishmaniasis are poorly effective in the radical treatment of canine leishmaniasis. Antimonials, especially meglumine antimoniate (Glucantime^{R}) are considered the most effective drugs for the treatment of canine leishmaniasis (Kammermann-Luscher, 1980; Lanotte *et al.*, 1979; Longstaffe and Guy, 1986). Dosage regimens vary. Meglumine antimoniate (Glucantime^{R}) is usually injected im or iv at a dose rate of 100 mg/kg body weight per day for 2 or 3 courses of 10-14 days, each course separated by injection-free intervals of 10-14 days. Higher doses, up to 300 mg/kg every other day for 30-40 days as well as doses of 60-100 mg/kg daily for a course of 3 months have also been suggested (Euzeby, 1986). Dogs may

become stiff and painful when Glucantime(R) is injected deeply in the thigh and abscess formation or severe lameness due to muscle fibrosis may occur (Kammermann-Luscher, 1980; Longstaffe and Guy, 1986). Sodium stibogluconate (Pentostam R) is administered iv or im, in a course of 8 daily doses of 20-50 mg/kg which is repeated after a week's interval (Euzeby, 1986; von Reiter et al., 1985). Pentamidine (Lomidine R) is given at the dose level of 4 mg/kg with deep im injections, every other day and for a minimum of 15 injections (Euzeby, 1986). Many dogs become free of signs within a few weeks especially if treatment is undertaken at early stages (subclinical cases), however, a high incidence of relapses has been reported (Euzeby, 1986; Giauffret et al., 1976; Levine, 1973; Longstaffe and Guy, 1986). As these drugs are hepato-nephrotoxic, in clinical cases with parenchymal lesions, in order to avoid any additional parenchymal damage due to drug toxicity, the treatment profile includes antimonials for a week, 1 week without treatment and diamidines for 1 week (Euzeby, 1986). It is generally accepted that treatment leads only to a prolongation of the life time in most cases, radical cures are not obtained, and destruction of the infected dogs is recommended as the only effective measure to control epidemics in man (WHO, 1984). However, in Mediterranean foci where seropositive prevalence rates of canine leishmaniasis of 15-20% are common, the destruction of all serologically positive dogs is not possible. In a control study (Gradoni et al., 1988) in the isle of Elba, Italy, treatment with antimonials of prepatent cases and destruction of patent cases resulted in a two-third reduction of the disease frequency in dogs and it has been suggested (Gradoni et al., 1988) that wherever human VL, caused by *L. infantum* represents a serious health problem, continuous serological monitoring of the dog population followed by treatment and suppression activities should be integrated into those of the veterinary health services.

1.3. THE LEISHMANIASES: CONTROL

Control of human leishmaniasis depends on the particular syndrome and epizootiological situation, and measures are directed towards the organism, the vector and the reservoir host. Opportunity and feasibility for control of each of these components, singly or together, varies in different parts of the world. Control must be adapted to local situations in each endemic area once baseline data of the various components are available.

1.3.1. Control of vectors and reservoir hosts

Although the weakest link is the relatively fragile vector, the extensive technology required for its control is frequently unavailable. At best, control is feasible with the mature stages with variability and inaccessibility of the breeding and resting sites making it difficult. Also, every sand-fly colony requires it's own specific control strategy (Molyneux and Ashford, 1983). Control of reservoir hosts such as rodents and dogs (see section 1.2.), usually involves eradication of all the potential reservoirs. This measure accompanied by insecticide spraying proved effective in a few cases, notably in the USSR (Molyneux and Ashford, 1983). To support field studies of leishmaniasis ecology and epidemiology, new methods have been developed in order to identify the mammalian species on which a sand-fly has fed within the past 24-48 hours and research is also continuing on sand-fly pheromones that may prove useful in traps for sampling or controlling vectors (WHO, 1989).

1.3.2. Vaccination

There is, as yet, no good antileishmanial vaccine generally available for most of the diseases. There is extensive immunological crossreactivity between different species but they do not generate crossprotection. The development of vaccines against leishmaniasis continues to be a research priority by the WHO Special Programme for Research and Training in Tropical Diseases (TDR), with several candidate preparations entering trials in nonhuman primates. The

candidate vaccines in these trials are:

* Killed whole parasite preparations with or without BCG

* Promastigote fractions of L. infantum shown to be protective in mice and able to immunise dogs in L. infantum endemic areas

* Purified promastigote surface protease (gp63) incorporated into liposomes (Russel and Alexander, 1988) and used with or without muramylpeptide as adjuvant

* Promastigote lipophosphoglycan (LPG) which has proved protective in mice (Handman and Mitchell, 1985).

At the laboratory level protective Leishmania-specific T-cell clones, capable of transferring resistance to highly sensitive normal animals, have also been developed (WHO, 1989).

1.3.3.1. Chemotherapy

Antimonial compounds have formed the basis for the treatment of leishmaniasis since 1911. The pentavalent antimonials, sodium stibogluconate (Pentostam^{R}) and meglumine antimoniate (Glucantime^{R}) are still the drugs recommended for initial systemic therapy (Marsden and Jones, 1985; Berman, 1988). These drugs are composed of pentavalent antimony complexed to carbohydrates, however, the precise structure of these complexes are as yet unknown (Berman, 1988).

Their mechanism of action has not yet been fully elucidated. A recent work by Berman et al., (1987) has shown that Pentostam inhibits the glycolytic pathway and fatty acid oxidation in L. mexicana amastigotes. It has been suggested (Berman and Grogl, 1988) that the specific binding of Pentostam to polypeptides involved in the glycolytic and oxidative pathways may contribute to the cytotoxic effects of the drug. It has also been suggested (Berman and Grogl, 1988) that Pentostam may interact with the parasite nucleic acids or with low molecular weight parasite components. Topoisomerisation of DNA catalysed by type 1 DNA-topoisomerase in L. donovani was specifically inhibited by the pentavalent antimonials sodium stibogluconate and urea stibamine (Chakraborty and Majumber, 1988).

Antimonials are far from ideal drugs as several limitations are

associated with their use. They are not active orally and they require prolonged treatment. Also, they are not consistently effective, infrequent side effects such as cardiotoxicity and renal toxicity have been reported and they are excreted rapidly from the body. A 10 mgSb^V/kg body weight dose given intramuscularly produces a 10 μg/ml peak in the blood after 2 hours but over 80% of the antimony is excreted within 24 hours often leaving subtherapeutic plasma levels for much of the treatment (WHO, 1984). Recently the pharmacokinetics and toxicity of Pentostam have been reassessed (WHO, 1984) and this has shown that more frequent higher doses (10-20 mgSb^V/kg three times daily), help to ensure higher therapeutic plasma concentrations of antimony and are better tolerated than was first thought. The implementation of these findings has considerably reduced the failure rates of treatment.

The recommended second line drugs, pentamidine (in the treatment of visceral leishmaniasis), mepacrine and amphotericin B (in the treatment of Latin American cutaneous leishmaniasis), are even less acceptable requiring long-term parenteral administration and their toxicity is more pronounced.

There is a wide variability in the susceptibility not only of different species but also different isolates of the same species, to antimonials. Visceral leishmaniasis is usually responsive to antimonial therapy with cure rates of 70-100%, however, relapses and diffuse cutaneous forms of the disease are often difficult to treat due to apparently defective immunity (Marsden and Jones, 1985). Also, pharmacokinetic properties may account for the different responses of parasites in different tissue locations or between different individuals or host species.

Little experimental work has been done on mechanisms of resistance in *Leishmania* species and to antiprotozoan drugs in general. Preliminary data indicate that drug resistance may be a problem in leishmaniasis. Berman *et al*. (1982) have shown that variable susceptibility to pentavalent antimonials in infected macrophages *in vitro* correlates with the patient's response to treatment.

There is clearly a need for novel approaches to chemotherapy of these organisms. At present, there are at least two new promising areas in the liposome presentation of current antileismanial drugs and in novel chemical series (8-aminoquinoline and allopurinol-related compounds). However, it seems unlikely that effective new treatment will be available in the near future.

1.3.3.2. Biochemical strategies in search for new treatments

As with several other parasites, research aimed in finding new drugs has, in recent years, been focussed on defining metabolic differences between parasite and host that could represent good targets for chemotherapy. Unfortunately, relatively little is known about the biochemistry of the amastigotes of Leishmania and so only a few possible targets have been recognised. Rather more information is available on the promastigotes and this has been used to identify other possibilities. Leishmania species have several relatively parasite-specific biochemical pathways that are related to the metabolism of glucose, purines, pyrimidines, lipids and polyamines and these are or may be the biochemical basis of action(s) of the major clinical agents. The ones more relevant to this study are summarised here. Biochemical investigations have demonstrated several parasite specific features relevant to the mechanism of action of chemotherapeutic agents:

1. Glycolytic enzymes and some enzymes of fatty acid catabolism occur in organelles (glycosomes) in Leishmania but not in mammalian cells. Berman et al. (1985) reported that antimony inhibited both amastigote catabolism of glucose via glycolytic enzymes and catabolism of fatty acids and suggested that the mechanism of action of antimony may relate to alteration of glycosomal structure or function.

2. Leishmanias are incapable of synthesising purines de novo. Purine analogues can be utilised by the salvage pathways for purines in amastigotes in a greater extent than in mammalian cells and allopurinol and allopurinol ribonucleoside are metabolised into presumably toxic metabolites by these means. This has led to the

discovery that several purine and purine nucleoside analogues, including allopurinol and formycin B, have potent antileishmanial activity (Nelson et al., 1979; Carson and Chang, 1981). Initial trials using allopurinol against antimony-resistant cases of visceral leishmaniasis (Kager et al., 1981) showed some success. Clinical trials of the more active ribonucleoside analogues have also proved promising and are being followed up (Modabber, 1986). A multicenter trial of combined regimes of allopurinol and antimonials is being conducted in Colombia, Peru and Bolivia (WHO, 1989).

3. Amastigote sterol biosynthesis is akin to that of such fungi as Candida in that the major dimethylated sterol is of the ergostane series. The presence of fungus-like sterols as the primary leishmanial demethylated sterols provides a rationale for the antileishmanial activity of amphotericin B, ketoconazole and other antifungal agents that act on fungal sterols. As three orally active azoles (ketoconazole, itraconazole and fluconazole) are already licensed and a number of others are undergoing clinical evaluation, the potential use of azoles alone or in combination for the treatment of leishmaniasis should be investigated experimentally and clinically.

4. Some years ago interest in glutathione metabolism in trypanosomes was stimulated by observations that trypanosomes appeared to have a deficient capacity to withstand oxidative stress. This is due to the fact that in African trypanosomes, catalase and glutathione peroxidase are completely lacking or present only in trace amounts and these parasites appear to depend on reduced glutathione (GSH) to scavenge free radicals produced from hydrogen peroxide and superoxide anions (Meshnick et al., 1978). Many of the known trypanocidal drugs are thought to exert their toxic effects by generating highly reactive forms of oxygen such as H_2O_2, dioxygen anion and hydroxyl radical.

Glutathione is found in virtually all cells in millimolar quantities and is involved directly or indirectly in many important biological roles, including protein and DNA synthesis, metabolism

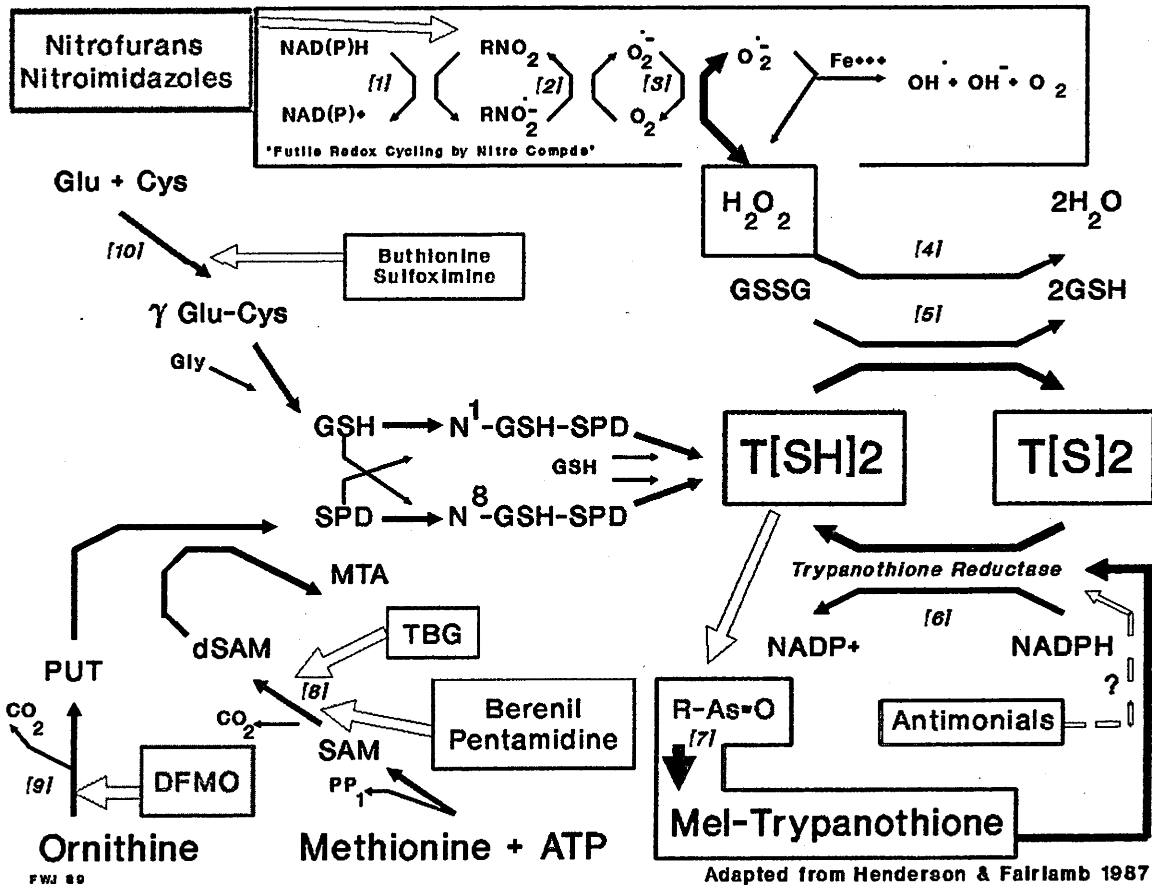
and protection of cells. African trypanosomes, however, are more susceptible to the effects of GSH depletion than the mammalian host cells, since administration of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, to mice infected with *Trypanosoma brucei* leads to prolonged survival times or cures (Arrick *et al*., 1981). In the presence of oxygen radicals and hydrogen peroxide, oxidised glutathione is formed. Oxidised glutathione (GS-SG) itself, can be toxic to cells by inducing disulfide exchange with key proteins and interfering with cellular metabolism and function (Meister and Anderson, 1983). Under normal conditions, oxidised glutathione does not accumulate in cells because it is reduced back to GSH by the NADPH-dependent enzyme, glutathione reductase (Williams, 1976). Thus this enzyme is of great importance in the maintenance of normal glutathione status within most cells and could present a potential target for the development of new drugs.

Recent studies (Fairlamb and Cerami, 1985) have shown that in trypanosomatids reduction of oxidised glutathione is unusual in that it is absolutely dependent on a low molecular weight co-factor which is a complex between GSH and the polyamine spermidine and contains an enzymatically reducible sulfhydryl group (or groups) which is essential for activity. This co-factor, initially isolated from the nonpathogenic haemoflagellate *Crithidia fasciculata* but also subsequently found in *T. b. brucei*, *T. cruzi* and *Leishmania mexicana*, was given the trivial name trypanothione and is unique to trypanosomatids.

In the light of these findings it is of great interest to determine to what extent trypanothione protects trypanosomatids from oxidative damage. If many of the existing trypanocidal drugs work by producing radicals or toxic metabolites, then a potent synergism might be predicted between these compounds and inhibitors of trypanothione metabolism.

An overall metabolic scheme for the biosynthesis of trypanothione [$T(SH)_2$] and its possible functions is illustrated diagrammatically in Figure 3. Trypanothione is maintained exclusively in the reduced form $T(SH)_2$, by means of the

Fig. 3. Metabolism and functions of trypanothione, showing possible sites of action of trypanocidal compounds.



Abbreviations:

DFMO = difluoromethylornithine

TBG = 1,3,5-triacetylbenzene tris(guanylhydrazone)

PUT = putrescine SPD = spermidine

GSH & GSSH = glutathione and its disulphide

GSH-SPD = glutathionylspermidine

$T(SH)_2$ = dihydrotrypanothione $T(S)_2$ = trypanothione disulphide

dSAM = decarboxylated S-adenosylmethionine

MTA = methylthioadenosine

SAM = S-adenosylmethionine

MEL-Trypanothione = trypanothione: melarsen adduct

NADPH-dependent enzyme, trypanothione reductase (reaction 6, Figure 3). $T(SH)_2$ reduces other disulphides, including glutathione (GS-SG) and cystine, by means of non-enzymatic thiol-disulphide exchange (reaction 5, Figure 3) (Fairlamb and Cerami, 1985). $T(SH)_2$ also is the electron donor for removal of H_2O_2 and other peroxides catalysed by trypanothione peroxidase (reaction 4, Figure 3). $T(SH)_2$, like GSH, would also be expected to act as a free radical scavenger to remove oxygen radicals such as OH^- (Henderson and Fairlamb, 1987). Figure 3 also shows points at which trypanocidal compounds are thought to act. With the exception of suramin, all the major classes of trypanocidal compounds are reported to have an inhibitory effect on this metabolic scheme. With the exception of DFMO, the target sites of these drugs are not known with certainty. It should be emphasised that some of these targets may not be the only ones with which these drugs interact.

It is difficult to see how DFMO could be synergistic with such disparate groups of compounds as arsenicals, nitroheterocyclics, diamidines and guanylhydrazones, and yet it had been shown to be so with African trypanosomes (Jennings, 1990). It is hypothesised that the polyamine-containing peptide dihydrotrypanothione, may provide a common biochemical link between polyamine metabolism, thiol metabolism and oxidative stress.

1.4. OBJECTIVES OF THIS PROJECT

The first successful treatment of chronic T. brucei CNS-infections in mice (Jennings, 1988b; 1991b) with a combination of DFMO and sodium stibogluconate (Pentostam[R]) led us to the decision to utilise the information which is accumulating on the synergistic effects of compounds which affect trypanothione metabolism in trypanosomes and to see if they act similarly against Leishmania spp..

The main objectives of this project were to evaluate trypanocidal compounds including DFMO, nitroheterocyclics, guanylhydrazones, arsenicals and diamidines, both alone and in

combination using a L. donovani mouse model and L. donovani promastigotes and amastigotes in culture, and to use the knowledge gained from these studies and also from studies on trypanosomes, in order to gain a better understanding of the mode of action of these drugs and to attempt to develop new therapeutic combinations as potential antileishmanial agents.

There is a body of evidence on the biological activities of each of the compounds or groups chosen for study.

1.4.1. DL-α-difluoromethylornithine, (DFMO), (Eflornithine[R])

DL-α-difluoromethylornithine (DFMO; Eflornithine[R]) is an enzyme activated irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme in the biosynthetic pathway of polyamine biosynthesis (reaction 9, Figure 3). Inhibition of this enzyme leads to a depletion of putrescine and spermidine and consequent cessation of macromolecular biosynthesis (Bacchi and McCan, 1987). Subsequent studies have shown that $T(SH)_2$ is also significantly depleted following exposure of T. brucei to DFMO (Fairlamb et al., 1987). Reduction in $T(SH)_2$ levels would therefore compromise the parasites ability to defend itself against oxidative stress by reducing the intracellular concentration of $T(SH)_2$ as a radical scavenger and by decreasing the intracellular activity of trypanothione peroxidase.

DFMO was developed by the Merrell Dow Research Institute as a potential anticancer agent; the company had carried out all the necessary preclinical toxicology and development. Although the antineoplastic effects of DFMO alone have been disappointing, the therapeutic value in combination with other drugs remains a strong possibility. DFMO is highly effective treatment of both early and late stages of T. b. gambiense trypanosomiasis, with dramatic efficacy noted even in arsenical-refractory cases, but it is relatively ineffective to the rhodesiense type of disease. Studies began in 1979 with experimental murine infections and with isolated parasites, and these studies have demonstrated the possibility of synergy with bleomycin Clarkson et al., 1983), suramin (Clarkson

et al., 1984) and 9-deazainosine (Bacchi *et al.*, 1987). The first two combinations were reported (Jennings, 1990) to give 100% cures after the infected mice were kept under a 2h light/4h dark regimen which insured a more even uptake of the drug (Gillet *et al.*, 1986). Additional studies (Jennings, 1988a; Jennings, 1988b) have shown that using a combination of DFMO/arsenicals (i.e. melarsoprol) in the late stage trypanosomiasis with CNS-involvement, can make it possible to reduce the dosage of the toxic arsenical drug and at the same time reduce the number of relapse infections.

The activity of DFMO against some *Leishmania* species has recently been shown in rodent models (Keithly and Fairlamb, 1988). Two percent DFMO in drinking water was active against *L. donovani* alone and showed synergy with bleomycin and an additive effect with sodium stibogluconate. The observation that DFMO alone also proved to be very effective against *L. guyanensis* is of great interest. Other probable potential uses of DFMO include treatment of cryptosporidiosis, malaria and hyperproliferative diseases (Bacchi and McCann, 1987).

1.4.2. Nitroheterocyclic compounds

Several nitroimidazoles are antiprotozoal compounds and have been used variously for the treatment of human trichomoniasis, giardiasis, amebiasis and Chagas' disease. Nifurtimox, nitrofurazone and MK 436 have been successfully used in experimental mouse infections with African trypanosomes.

The 2-substituted 5-nitroimidazoles (Cuckler *et al.*, 1970; Jennings *et al.*, 1980; Jennings and Urquhart, 1983) and 5-substituted 2-nitroimidazoles (Richle and Hofheinz, 1983; Zweygarth and Rottcher, 1987a), were shown to have a trypanocidal activity against *T. brucei* spp..

Nitrocompounds are thought to exert their cidal action against trypanosomes by "futile redox-cycling" (Docampo and Moreno, 1984). They undergo 1-electron reduction in enzyme catalysed reactions involving NADPH or NADH as electron donor (reaction 1, Figure 3). The enzymes catalysing these reactions are not known. In the

presence of dioxygen, the 1-electron reduced compound is reoxidised generating superoxide anion, (reaction 2, Figure 3). This is removed by means of superoxide dismutase (reaction 3, Figure 3), with the production of H_2O_2, which is in turn removed by trypanothione peroxidase (reaction 4, Figure 3). If superoxide anion and H_2O_2 accumulate, then radical species (presumably hydroxyl radical) are formed according to the metal-catalysed Haber-Weiss reactions. The highly reactive hydroxyl radical (or another unidentified radical species) is then thought to react with essential membrane lipids or DNA, leading to death of the cell. If this mechanism operates in trypanosomes, then a synergistic effect between nitroheterocyclics and DFMO is to be expected.

A report indicated that the mammalian enzyme glutathione reductase is capable of reducing nitro compounds using NADPH as electron donor (Carlberg and Mannervik, 1986). Henderson et al. (1988) also reported that trypanothione reductase can be induced to catalyse 1-electron reduction of functional groups including suitably substituted naphthoquinone and nitrofuran derivatives and nitroimidazoles.

1.4.3. Arsenicals

Until recently it has been generally accepted that aromatic arsenicals kill African trypanosomes by inhibition of the key glycolytic enzyme, pyruvate kinase (Flynn and Bowman, 1974). But as this conclusion is no longer acceptable, the mechanism by which melarsen oxide induces rapid lysis of trypanosomes, remains unclear.

Ehrlich was the first to recognise that trivalent arsenicals have an affinity for sulphydryl groups. Since that time, it has been demonstrated that trivalent arsenicals form considerably more stable complexes with dithiols rather than with simple monothiols (Fairlamb et al., 1989). Dihydrotrypanothione would therefore seem to be an ideal candidate for interaction with arsenical drugs. Such counteraction may be involved in the selective toxicity of aromatic arsenicals against African sleeping sickness trypanosomes (Fairlamb et al., 1989).

1.4.4. Diamidines

Pentamidine is currently used in the treatment of primary stage Gambian trypanosomiasis, antimony-resistant leishmaniasis and *Pneumonocystis carinii* pneumonia. The mechanism of action is unclear (Sands *et al*., 1985). It may compete with polyamines for binding to nucleic acids and may also bind to kDNA (Meshnick and Cerami, 1984). More recently, the diamidines pentamidine and diminazene aceturate (Berenil^R) have reported to inhibit S-adenosylmethionine decarboxylase in *T. brucei* (Bitonti *et al*., 1986), (reaction 8, Figure 3).

1.4.5. Guanylhydrazones

Guanylhydrazones also interfere with the polyamine biosynthesis at S-adenosyl-L-methionine decarboxylase (SAM-decarboxylase). SAM-decarboxylase was reported (Bacchi *et al*., 1979; Bitonti *et al*., 1986a) to be present in *T. b. brucei* bloodstream forms, as indicated by blockade of [^{14}C]methionine incorporation into spermidine in intact cells and by reversible inhibition of CO_2 production from [^{14}C]adenosylmethionine in cell extracts by methylglyoxal-bis (guanylhydrazone) (MGBG), and by MGBG inhibition of cell growth (Chang *et al*., 1978).

MGBG has only been used in limited experimental testing (Chang *et al*., 1978; Nathan *et al*., 1979) against relatively lethal trypanosome infections where efficacy was assessed by a prolongation of survival time.

Of several aromatic guanylhydrazones found to have antitrypanosomal activity (Ulrich *et al*., 1982; Ulrich and Cerami, 1984), one compound, 1,3,5-triacetylbenzene tris (guanylhydrazone) trihydrochloride i.e. [TBG], proved especially active against acute *T. brucei* infections in mice. TBG has also shown (Jennings *et al*., 1987; Jennings, 1988b) to be capable of curing *T. brucei* CNS-infections in mice if combined with DFMO and arsenicals.

2. MATERIALS AND METHODS

2.1. PARASITES: CULTIVATION AND MAINTENANCE

2.1.1. Amastigotes

Leishmania donovani (MHOM/ET/67/L82) amastigotes were maintained by serial sub-passage in female Golden Syrian hamsters (Bantin and Kingman Ltd., Grimsden, Hull, England). Amastigotes were released by gentle homogenisation of the spleen from an infected hamster, at room temperature, between two pieces of stainless steel gauze, in approximately 50 ml of PSGEMKA (20 mM sodium phosphate buffer, pH 7.3, 104 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM D-glucose, 1 mM ethylenediamintetraacetic acid [EDTA], 0.02% bovine serum albumin) until all but connective tissue was disrupted. The resulting suspension was then filtered through a No 1 Whatman filter paper to remove large debris and washed twice by centrifugation at 2500 x g for 10 minutes and resuspension in PSGEMKA buffer at 4^oC, before being used to infect 4-10 week old hamsters by intraperitoneal inoculation of 2-4 x 10^8 amastigotes in 0.4 ml per animal. Infected animals were killed within 2-5 months, the spleen was excised and used as a source of amastigotes for experimental work or for transformation to promastigotes (section 2.1.2.). The condition of the hamsters was a good indicator of the progress of the infection: hamsters remained healthy until a few days before death. Hamsters were routinely used at the first signs of ill health to obtain the maximal yield of parasites.

2.1.2. Promastigotes

Promastigotes of L. donovani were obtained by the transformation in vitro of amastigotes obtained from an infected animal. A small piece of infected tissue was aseptically removed and placed in 5 ml of transformation medium: HOMEM medium (Berens et al., 1976) supplemented with 20% (v/v) heat-inactivated foetal calf serum (HIFCS) and antibacterials (25 μg gentamycin sulphate/ml, 1 mg streptomycin sulphate/ml and 1000 units benzylpenicillin/ml). The infected tissue was then incubated at 25^oC for 48-96 hours during which transformation and replication of promastigotes occurred. For some experimental work, in vitro transformation of amastigotes was

used in order to assess the parasites viability.

Promastigotes were sub-passaged and cultured in HOMEM medium with 20% (v/v) HIFCS and 25 µg gentamycin sulphate/ml at 25°C. Cultures were normally initiated at a density of 1 x 10^5 promastigotes/ml and sub-passaged upon reaching early to mid-logarithmic phase of growth (density of approximately 10^6/ml).

2.2. HARVESTING PARASITES

2.2.1. Amastigotes

Amastigotes used for the infection of animals were isolated from the spleen of infected animals. This involved gentle homogenisation, at room temperature, between two pieces of stainless steel gauze in approximately 50 ml of PSGEMKA buffer as previously described (see section 2.1.2.).

Amastigotes used for the <u>in vitro</u> infection of peritoneal exudate cells (PECs), (see section 2.3.2.), were also obtained from the spleen of infected animals. The infected tissue was excised aseptically and placed in a sterile hand-held homogeniser with 10-15 ml of RPMI 1640 supplemented with 10% (v/v) HIFCS (see section 2.3.1.) and antibiotics. The amastigotes were released by several strokes of the homogeniser pestle and the residual large particulate matter removed by centrifugation (1000 x g for 5 minutes, at 4°C). The amastigotes remained in suspension free of large debris, they were then washed twice by centrifugation (2100 x g for 10 minutes, at 4°C) and kept at 4°C in cold RPMI medium before being used to infect the PECs.

2.3. MACROPHAGE-PARASITE INTERACTIONS <u>IN VITRO</u>

2.3.1. Macrophage maintenance

The medium used for macrophage culture was RPMI 1640, pH 7.2-7.3, supplemented with 10% (v/v) HIFCS, 50 µg benzylpenicillin/ml, 50 µg streptomycin sulphate/ml, 25 µg gentamycin sulphate/ml, 2 mM L-glutamine, 25 mM HEPES and 20 mM sodium hydrogen carbonate. The medium was adjusted to the appropriate pH using 1 M sodium

hydroxide.

Mouse resident peritoneal exudate cells (PECs) were obtained from female Balb/c mice by peritoneal lavage using 5 ml of complete medium (RPMI 1640, with all the supplements) per mouse. The number of mice used in each experiment varied between 2 and 4, depending upon the number of PECs required. The lavages from the mice were pooled and diluted with complete medium to give a cell density of 2.5×10^5 PECs/ml.

Eight-chamber Lab-Tek tissue culture slides were used for the maintenance of the PECs: 1×10^5 PECs in 0.4 ml were plated into each chamber. The cells were allowed to adhere overnight by incubation at 37°C, with a gas phase of 95% air, 5% CO_2. Non-adherent cells were removed the next day by vigorous washing with complete medium and the adherent cells remaining were then used. These cells were mainly macrophages but other cell types were present and such preparations will be referred to as the PECs.

2.3.2. Infection of the PECs

In calculating the ratios of parasites to PECs for experiments, the initial number of PECs plated out was taken to be the number of PECs present after washing, despite the removal of non-adherent cells. PECs were infected by adding appropriate numbers of _L. donovani_ amastigotes in the complete RPMI medium used for PEC culture. The number of parasites used for the infective period of 24 hours were at a ratio between 1 PEC: 2-3 amastigotes. At the end of the exposure period, the free parasites were removed by washing with RPMI medium and 0.4 ml of fresh RPMI medium was added to each well. The cells were incubated at 37°C and at the end of the experimental periods the PECs were air dried, fixed in absolute methanol and stained with Giemsa/May-Grünwald stain.

2.4. DETERMINING ANTILEISHMANIAL DRUG ACTIVITY

2.4.1. _In vivo_ model

The activity of potential antileishmanial agents against _L. donovani_ was determined using the visceral leishmaniasis model. In these

studies, 2 x 10^7 L. donovani amastigotes isolated from hamster spleens and resuspended in 0.2 ml PSGEMKA were inoculated intravenously, via the tail vein, into each female Balb/c mouse (Department of Zoology, University of Glasgow). The number of mice used in each experimental group was either 5 or 6. On day 14 post-infection, the appropriate treatment regimen was started and four days after the last day of treatment, the animals were sacrificed, after being anaesthetised, by cervical dislocation. Impression smears of the first prominent lobe of the liver, and in some cases also from spleen, were made, air dried and fixed in methanol before being stained for 20 minutes in 10% Giemsa's stain. The number of amastigotes per 100 infected cell nuclei were counted in duplicate smears for each experimental animal. In a number of experiments, the parasite load in bone marrow was also determined. In these cases, the left femur of the sacrificed animal was separated from the rest of the body after being exposed and cut free at the hip and the femoro-tibial joints. The femur was scraped clean of muscle and ligament and severed near its ends, a 26 g needle on a 1 ml heparinised syringe, containing 0.3 ml of 0.2% bovine serum albumin (BSA), was inserted through the cartilage at the knee joint of the femur and the plug of marrow was expelled into a plastic tube. The bone marrow cells were suspended using a Pasteur pipette and 2-3 drops of the bone marrow cell suspension were added to cuvettes of a cytocentrifuge (Cytospin 2, Shandon Southern Ltd., Runcorn, Cheshire). The cells were then spun on to a clean glass slide at 600 rpm for 5 minutes. Smears were allowed to air dry for 10 minutes before being fixed in absolute alcohol and stained with 50% May-Grünwald (pH 6.8) and 10% Giemsa (pH 6.8) for 10 minutes. The cell smears obtained tended to show an increasing cell density from the centre to the periphery of the smear. This, coupled with the fact that mast cells tend to aggregate, meant that care had to be taken to select representative areas of the smear in order to obtain more accurate parasite counts. 1000 cells were counted in each slide, moving from the periphery of the smear to the centre.

The results for each treated group were compared with the mean of the pooled results of an untreated control group, and were

expressed as either a percentage of the mean of the controls or as percentage reduction compared to the controls, so allowing the degree of antileishmanial activity to be quantified.

Drugs diluted or suspended in distilled water were made up freshly at the appropriate concentration so that they could be injected intraperitoneally (ip) in a ratio of 0.05 ml/5g body weight. DFMO was given orally in the drinking water, starting on day 5 after infection, usually for a period of 15 days. The mice were kept under a four hour dark and two hour light regimen, which ensured a more even intake of DFMO over each 24 hour period.

2.4.2. In vitro models

In vitro antileishmanial activity was determined by multipoint assays in which the drug concentration was reduced at two-fold intervals. The highest concentration tested for each drug was 100 µg/ml.

2.4.2.1. Promastigotes in vitro

The activity of a compound against promastigotes of L. donovani was assessed by monitoring its effect on promastigote growth. A promastigote suspension of 1×10^5 cells/ml in HOMEM growth medium was dispensed into each well of a 96 well microtitre plate, in a volume of 180 µl. Drugs were dissolved in water wherever possible, but several were dissolved in dimethyl sulphoxide (DMSO). In such cases controls were carried out to ensure that the final concentrations of this solvent had no effect on the parasites. Stock solutions of the compound being tested were normally filter sterilised (0.22 µm pore size filter) and diluted to give the necessary concentration so that the drugs were added as 20 µl volumes in each well to give a final volume of 200 µl, at the start of the experiment. In all cases 20 µl of the growth medium was added to control cultures. The plate was placed into a sealed plastic box with moist tissue paper on the bottom to maintain a humid atmosphere and incubated at 25°C, with air as the gas phase. The cell density of each culture was measured on day 6 and in some experiments daily for a period of five days, using an Improved

Neubauer Haemocytometer. The density and morphology of the parasites at various times during the experiments was observed by phase contrast microscopy of living parasites using a Leitz inverted microscope.

2.4.2.2. Amastigotes in vitro

In vitro investigations using PECs infected with L. donovani were carried out using standard conditions. Compounds under investigation were added during preparation of the standard medium. Drugs were dissolved in water wherever possible but several were dissolved in dimethyl sulphoxide (DMSO). In such cases controls were carried out to ensure that the final concentrations of this solvent had no effect on the parasites. Stock solutions of the compounds being tested were normally filter sterilised (0.22 μm pore size filter) and diluted in medium to give the necessary concentration so that the drugs were added as 0.4 ml volumes 24 hours after the infection of macrophages with amastigotes. In all cases 0.4 ml of the growth medium was added to control cultures. 48 hours after the addition of the drug solutions, the medium was replenished and the incubation continued for up to a further 72 hours before the experiment terminated. Cells were then fixed and stained (section 1.2.2.) and the number of amastigotes per 100 infected cells and the percentage of the cells infected determined by microscopic observation. The results for treated cells were expressed as a % of the mean of the controls, allowing the degree of antileishmanial activity to be quantified.

2.5. STATISTICAL ANALYSIS OF DATA

Statistical analyses were carried out by using the computer programs "Animal Designs" (developed by Dr. G. Gettinby, Department of Statistics and Modelling Science, Strathclyde University), and "SPSS". One way and two way analysis of variance tests in conjunction with the Neuman Keuls procedure were used to analyse the significance of data. The F value, on which the significance is based, is given with the degrees of freedom in subscript. The

probability (P) was taken as significant when $P<0.05$.

2.6. MATERIALS

The 5-nitroimidazole compounds, L611,744 [3α,4,5,6,7,8,9,9α-octahydro-3-(1-methyl-5-nitroimidazol-2-yl)cycloocta(D)isoxazole], MK 436 [3α,4,5,6,7,7α-hexahydro-3-(1-methyl-5-nitro-1H-imidazole-2-yl)-1,2-benzisoxazole] and L634,549 [cis-3α,4,5,6,7,7α-hexahydro-3-(1-methyl-5-nitro-1H-imidazole-2-yl)-1,2-benzisoxazole-6,7-diol], a dihydroxy analogue of MK 436, were supplied by Merck Sharp & Dohme Research Laboratories (Rahway, N. Jersey, U.S.A.). Fexinidazole [1-methyl-2-(4-methyl-thiophenoxy-methyl)-5-nitroimidazole, HOE 239] and diminazene diaceturate (Berenil[R]), pure sample, were supplied by Hoechst, Frankfurt, West Germany. Metronidazole and dimetridazole were given by RMB Ltd., England, whereas satranidazole and Go 10,213 [1-methane-sulphonyl-3-(1-methyl-5-nitro-14-imidazole-2-yl)-2-imidazolidinone], were a gift from Ciba-Geigy, Switzerland.

The 2-nitromidazole compounds, Ro 15-0216 [2-(dimethylamino)-α-(1-methyl-2-nitroimidazole-5-yl)-p-acetanisidide], Ro 15-6547 [α-(1-methyl-2-nitroimidazol-5-yl)-1-pyrolidine-p-acetanisidide], Ro 17-1051, (N-benzyl-2-nitro-1-imidazole acetamide; benznidazole) and Ro 16-5304, were given by Hoffmann-La Roche A.G., Switzerland.

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone, 98%), was supplied by Aldrich Chemical Company, U.S.A..

Trimelarsan(R) (Mel W[R], melarsonyl potassium) and Moranyl(R) (suramin sodique) were obtained from Specia, France.

The guanylhydrazones TBG [1,3,5-triacetylbenzene tris (guanylhydrazone) trihydrochloride], DBG [1,3-diacetylbenzene bis (guanylhydrazone) 2.HCL], 5-decNH-DBG [5-decanoamido-3-diacetylbenzene bis (guanylhydrazone).2HCL], AMPA-DBG [1,3-diacetyl-5-(2-amino-4-methylpyrimidine-6-ylamino) benzene], 5-amino-DBG [5-amino-1,3-diacetylbenzene bis (guanylhydrazone).2HCL] and TBHG [(1,3,5-triacetylbenzene tris (N-hydroxy-guanylhydrazone) tritosylate], were supplied by Profs. Ulrich and Cerami of the Rockefeller University, New York, U.S.A.

DL-α-difluoromethylornithine (DFMO, Eflornithine[R]), was supplied

by Merrell Dow Pharmaceuticals, Inc., Ohio, U.S.A.

Sodium stibogluconate (Pentostam^{R}), equivalent to 100 mg of pentavalent antimony/ml, was supplied by the Wellcome Foundation, Ltd., U.K..

Lomidine$^{(R)}$ injectable soln., equivalent to 6.259 g of pentamidine methanesulfonate/100 ml, was supplied by Rhone Merieux, Lyon, France.

The platinum [pt(II)-DAP-SSHS, pt(II)-DAP-SDI] and rhodium [Rh(III)-Ethyl-Xanthate, Rh(III)BT] drug complexes were supplied by Dr. D.G. Craciunescu, University of Madrid, Spain.

The DNA intercalators (diminazene derivatives) BSU-1041, BSU-1021, BSU-1006, BSU-1002, BSU-1037, BSU-1030, were supplied by Dr. T.C. Jenkins, ICR, Sutton.

The 8-hydroxyquinoline compounds, compound 1 (mwt. 524.4), compound 2 (mwt. 492.4), compound 3 (mwt. 506.4) and compound 4 (mwt. 519.3), were supplied by Dr. J.P. Dheyongera, University of the North, South Africa.

DL-buthionine-(S,R)-sulfoximine (BSO), No. B-2640, gentamycin sulphate, No. G-7632 and bovine serum albumin, No A-4503, were obtained from Sigma chemical company, St. Louis, Mo., USA. Other chemicals were obtained from BDH chemicals, Ltd., U.K.

The octyl degrol oil was supplied by Prof. N.B. Graham, Department of Pure and Applied Chemistry, Strathclyde University, Glasgow.

Benzylpenicillin sodium B.P., 600 mg, equivalent to 1,000,000 units, was obtained from Glaxo Laboratories Ltd., Greenford, England. Streptomycin sulphate B.P., equivalent to streptomycin base of 1 g, was obtained from Evans Medical Ltd., Greenford, England.

The principal components of the growth culture medium for Leishmania promastigotes, i.e. foetal calf serum, MEM Eagle suspension powder, MEM (50x) amino acids, MEM (50x) amino acids solution, RPMI 1640 were obtained from Gibco-Biocult, Scotland.

Lab-Teck 8-chamber tissue culture slides and Flow Pore D or single use 0.22 μm filter units, were obtained from Flow laboratories, England. Millex-GV non-pyrogenic, single use 0.22 μm filter units, were obtained from millipore, France.

3. RESULTS

3.1. PARASITE ISOLATION, GROWTH AND VIABILITY

3.1.1. Isolation of amastigotes

The method for isolating L. donovani amastigotes used in this study was a modification of that developed by Hart et al. (1981a). There was an approximately 80% recovery of the amastigotes in the final preparation of purified amastigotes. The yield of amastigotes obtained was sufficient allowing infection of animals and mouse peritoneal exudate cells (PECs) in culture, similar to that reported previously (Hart et al. 1981a) and with relatively little host cell contamination, although this in practice, was higher than that reported by Hart et al. (1981a).

3.1.2. Growth of promastigotes

Promastigotes of L. donovani cultured in HOMEM medium had an average doubling time of approximately 10.5 hrs during the log phase of growth. On average, the maximum cell density reached, was 2.4×10^7/ml.

3.1.3. Growth of amastigotes

Amastigotes of L. donovani growing in mouse peritoneal exudate cells (PECs) in RPMI 1640 medium, reached an average cell density of 811 amastigotes/100 infected macrophages at the end of the experiment (day 5 after infection). Amastigotes of L. donovani growing in Balb/c mice, reached an average of 158 parasites/100 host cell nuclei in liver, 39.3 parasites/100 host cell nuclei in spleen and 13.6 parasites/100 cell nuclei in bone marrow, 23 days after the infection.

3.1.4. Viability of parasites

Exclusion of the vital stain trypan blue was proved to be useful as a test for testing the viability of amastigotes before being used for infecting macrophages (PECs). No loss of amastigote viability was observed, as measured by this test and, in some

experiments, by their ability to transform to promastigotes. Flagellum motility was also a useful indication for viable promastigotes.

3.2. TRYPANOTHIONE METABOLISM AS A CHEMOTHERAPEUTIC TARGET FOR LEISHMANIASIS: THE EFFECT OF COMPOUNDS THOUGHT TO INHIBIT TRYPANOTHIONE METABOLISM *IN VIVO* AND *IN VITRO*

The main objective of this project was to evaluate compounds which are thought to affect trypanothione metabolism in trypanosomes against *L. donovani* and develop new therapeutic combinations as potential antileishmanial agents. In pursuing this, a number of trypanocidal compounds including arsenicals, 5-nitroimidazoles, 2-nitroimidazoles, guanylhydrazones, DL-α-difluoromethylornithine (DFMO) and diamidines, were tested *in vitro* and *in vivo* individually and in a series of combination experiments.

3.2.1. THE NITROIMIDAZOLES

3.2.1.1. The 5-Nitroimidazoles

Seven 5-nitroimidazole compounds; MK 436, fexinidazole (Hoe 239), L611,744, L634,549 (a dihydroxymetabolite of MK 436), metronidazole, dimetridazole and satranidazole (Go 10,213), were tested *in vitro* and *in vivo* using *L. donovani* promastigotes in culture (see section 2.4.2.) and *L. donovani*-infected Balb/c mice (see section 2.4.1.).

3.2.1.1.1. The 5-Nitroimidazoles in monotherapy experiments

3.2.1.1.1.(a) Effect of the 5-nitroimidazoles upon the growth of *L. donovani* promastigotes

The *in vitro* activities of the compounds tested are summarised in Table 3 and Figure 4. One way analysis of variance and then Neuman

Table 3

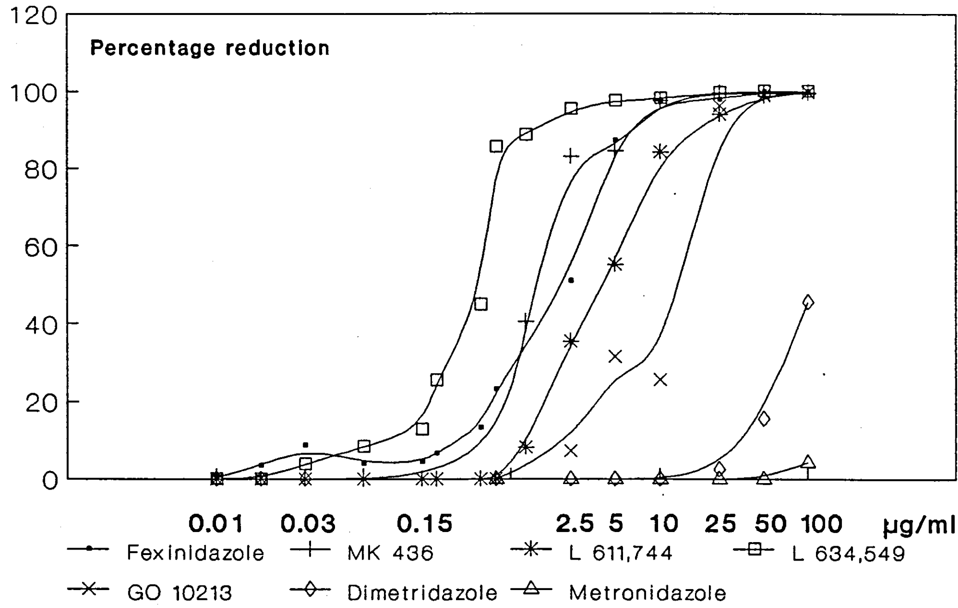
The effect of the 5-nitroimidazoles, at various concentrations, against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls				
	L611,744	MK 436	Dimetridazole	Go 10,213	Metronidazole
100	99.7 ± 0.4	99.5 ± 0.3	45.5 ± 7.9	99.8 ± 0.1	-9.9 ± 4.6
50	98.9 ± 0.1	99.5 ± 0.3	15.7 ±14.1	99.2 ± 0.4	-6.0 ± 4.9
25	94.1 ± 0.2	99.6 ± 0.4	2.5 ±12.7	96.3 ± 1.3	-5.6 ± 2.7
10	84.2 ± 0.7	97.6 ± 2.4	-7.6 ±15.2	25.6 ± 3.8	-4.7 ± 2.7
5	55.1 ± 6.7	84.6 ± 8.5	-4.0 ±32.3	31.5 ± 3.2	-3.5 ± 0.3
2.5	35.4 ± 3.9	83.0 ± 7.8	-1.2 ±10.8	7.2 ±15.7	-1.9 ± 2.6
1.25	8.1 ±10.7	40.6 ± 1.8	-11.0 ±11.1	-0.5 ± 0.9	-0.3 ± 0.4
Control**	2261.0 ± 241.8	2381.0 ± 602.5	2615.0 ± 353.5	2787.5 ± 45.9	2825.0 ± 247.5

Drug conc. µg / ml	Percentage reduction compared to controls	
	L634,549	Fexinidazole (Hoe 239)
100	100	100
50	100	99.8 ± 0.2
25	99.6 ± 0.4	97.8 ± 0.6
10	98.2 ± 2.5	97.4 ± 0.4
5	97.5 ± 3.5	87.2 ± 4.9
2.5	95.4 ± 5.9	50.9 ±11.4
1.25	88.6 ± 9.8	23.2 ± 4.1
0.5	85.6 ± 7.7	13.3 ± 8.9
0.25	44.8 ± 26.7	6.6 ±12.3
0.125	25.5 ± 6.9	4.4 ± 0.8
0.08	12.7 ± 7.6	3.9 ± 2.7
0.03	8.3 ±11.7	8.6 ± 7.2
0.018	3.8 ± 8.6	3.4 ± 8.1
0.008	-2.2 ± 4.2	0.5 ±22.9
Control**	2520,0 ± 458.9	2368.0 ± 328.8

**Mean number of parasites x 10^4/ml ± SD

Fig. 4. The effect of 5-nitroimidazoles against the promastigotes of _L. donovani_



Keuls multiple range test were used for data analysis. Promastigotes were incubated at 25°C in the presence of each compound at concentrations ranging from 1.25 μg/ml to 100 μg/ml, in complete HOMEM medium (see section 2.4.2.1.). Five of these compounds showed marked antileishmanial activity against promastigotes; the best, judged on a weight to weight basis, was L634,549 and in descending order of efficacy MK 436, fexinidazole, L611,744 and Go 10,213. Metronidazole and dimetridazole showed no appreciable activity. The activity of fexinidazole was monitored also over the 5 day incubation period and these data are summarised in Table 4 and Figure 5. There was no detectable activity during the first 24 hours of incubation but a marked growth inhibition was observed in 48 hours, at concentrations of 10 μg/ml and above ($F_{(7,15)}=37.7$, $P<0.05$).

3.2.1.1.1.(b) Effect of the 5-nitroimidazoles against *L. donovani* in mice

Balb/c mice were infected with *L. donovani* (see sections 2.1.1., 2.4.1.) and treated with L611,744 (12 consecutive daily doses), L634,549, fexinidazole and MK 436 (4 consecutive daily doses) at different dose levels. One way analysis of variance and the Neuman Keuls multiple range test were used to analyse the data. The treatment regimen and the percentage suppression in the liver parasite burden as compared with untreated controls, are shown in Table 5 and Figure 6 (for comparison, results using Pentostam are also shown). It can be seen that a 60-80% reduction can be achieved with all of them. The best result was with fexinidazole (Hoe 239) but the other nitroimidazoles were nearly as efficient. No statistically significant difference was observed between different dose levels tested when L634,549, MK 436 and fexinidazole were used.

Table 4

The effect of the 5-nitroimidazole fexinidazole, at various concentrations and time intervals, against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls				
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
100	35.1 ± 12.3	85.3 ± 8.9	96.2 ± 1.4	100	100
50	-7.0 ± 12.3	82.8 ± 6.4	93.9 ± 0	96.2 ± 4.6	100
25	-3.5 ± 12.3	71.9 ± 1.7	93.2 ± 2.8	95.6 ± 3.1	97.8 ± 0.6
10	-10.5 ± 42.1	68.6 ± 11.4	90.2 ± 2.3	96.3 ± 2.3	97.4 ± 0.4
5	-21.8 ± 22.4	50.0 ± 13.9	87.2 ± 3.1	81.3 ± 2.5	87.2 ± 5.0
2.5	-24.8 ± 7.4	37.1 ± 6.8	48.1 ± 11.8	45.8 ± 1.2	50.9 ± 11.4
1.25	-23.9 ± 40.0	9.0 ± 1.7	-30.1 ± 12.3	-9.3 ± 43.5	23.2 ± 4.1
Control**	61.0 ± 4.2	167.0 ± 5.7	457.5 ± 95.4	917.5 ± 95.4	1200.0 ± 120.0

** Mean number of parasites x 10^4/ml ± SD

Fig. 5. The effect of the 5-nitroimidazole fexinidazole, at various concentrations and time intervals, against L. donovani promastigotes.

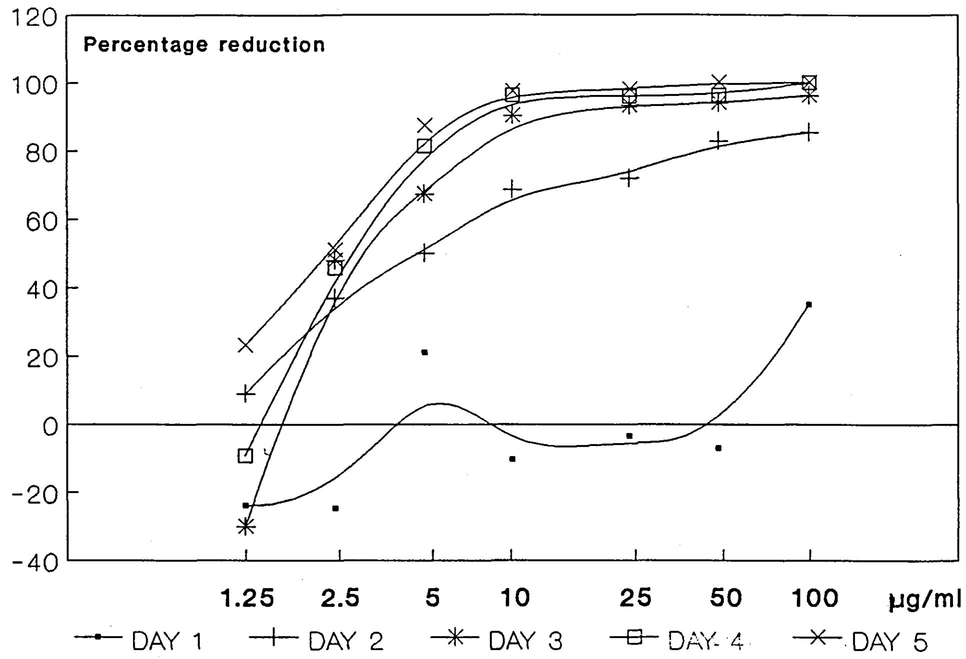


Table 5

The effect of the 5-nitroimidazoles L611,744, L634,549, fexinidazole and MK 436, given individually and at different dose levels, against <u>L. donovani</u> in mice.

TREATMENT REGIMEN

Infection (D0) → 7 days → [N][N][N][N][N][N][N][N][N][N][N][N] (D8–D19) → [K] (D23)

NITROIMIDAZOLE [N]	DOSE (mg/kg)	GROUP SIZE	% SUPPRESSION
L611,744	100	6	88.4 ± 10.2
	50	6	71.7 ± 13.8
	25	6	54.8 ± 14.7

Infection (D0) → 13 days → [N][N][N][N] (D14–D17) → [K] (D21)

NITROIMIDAZOLE [N]	DOSE (mg/kg)	GROUP SIZE	% SUPPRESSION
L634,549	200	6	76.3 ± 18.7
	100	6	59.0 ± 23.3
	50	6	47.8 ± 20.7
	25	6	40.8 ± 29.4

Infection (D0) → 13 days → [N][N][N][N] (D14–D17) → [K] (D21)

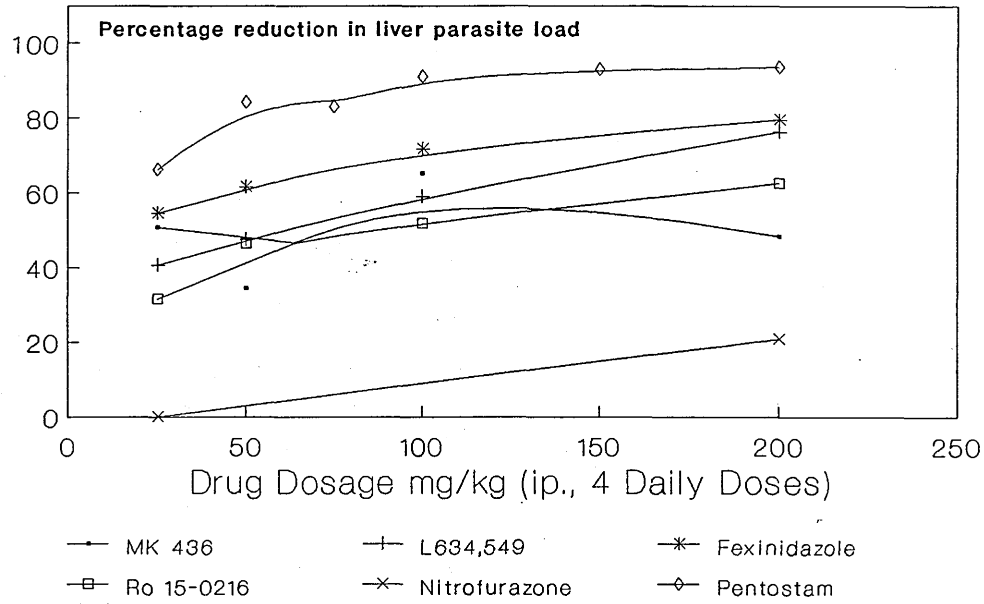
NITROIMIDAZOLE [N]	DOSE (mg/kg)	GROUP SIZE	% SUPPRESSION
Fexinidazole	200	6	79.5 ± 16.2
	100	6	71.7 ± 10.2
	50	6	61.5 ± 16.3
	25	6	54.6 ± 26.7

Infection (D0) → 13 days → [N][N][N][N] (D14–D17) → [K] (D21)

NITROIMIDAZOLE [N]	DOSE (mg/kg)	GROUP SIZE	% SUPPRESSION
MK 436	200	6	48.4 ± 20.5
	100	6	65.1 ± 15.8
	50	6	34.5 ± 33.2
	25	6	50.7 ± 29.5

[N] : 5-nitroimidazole [K] : Kill

Fig. 6. The effect of 5-nitroimidazoles given at various dose levels on L. donovani - infected Balb/c mice.



3.2.1.1.2. The 5-nitroimidazoles in combination experiments

3.2.1.1.2.(a) Effect of the 5-nitroimidazoles in combination with DFMO against L. donovani in mice

In combination experiments using DFMO, the DFMO was given as a 2% solution in the drinking water and mice were kept under a 4 hour dark, 2 hour light regimen that ensured a more even uptake of the drug. Infected mice were treated with the 5-nitroimidazoles fexinidazole, MK 436, L634,549 and L611,744 individually and in combination with DFMO. The treatment regimen and the percentage suppression of liver parasite burdens are shown in Table 6 and Figure 7. Fexinidazole ($F_{(7,15)}=37.7$, $P<0.05$), L634,549 $F_{(4,59)}=10.3$, $P<0.05$) and L611,744 showed similar activities whereas MK 436 was comparatively less active. Combination with DFMO produced only a marginal decrease in liver parasite burden which was statistically significant only when MK 436 was involved. Percentage decrease in parasite load using 5-nitroimidazoles monotherapy gave such high value that it is no wonder that DFMO addition did not show any potentiaton.

3.2.1.1.2.(b) The effect of L611,744 alone and in combination with DFMO and sodium stibogluconate (Pentostam^R) against L. donovani in mice

This nitroimidazole was studied in a greater detail. Two experiments were carried out and the treatment regimen and the percentage suppression in liver parasite load are given in Tables 7 and 8 and Figures 8 and 9. In the first experiment (Table 7, Figure 8), L611,744 was given ip for 12 consecutive days as a monotherapy and also in combination with DFMO. The nitroimidazole alone at 100 mg/kg reduced the parasite burden by 88% of that in the controls. Combination treatment with DFMO only marginally increased the effect to 95.4% at the highest concentration used. DFMO alone reduced the liver parasite load by 50%. Thus, under these conditions, DFMO appeared to be neither synergistic nor additive.

Table 6

The effect of 5-nitroimidazoles individually and in combination with DFMO against L. donovani in mice.

TREATMENT REGIMEN

Infection(D0) → 4 days →

→ D D D D D D D D D D D D D D D → K

D5 D19 D23

DRUG	% SUPPRESSION
DFMO	54.4 ± 10.6

Infection (D0) → 14 days →

→ N N N N → K

D15 D19 D23

DRUG	% SUPPRESSION
Fexinidazole	86.5 ± 2.9
MK 436	76.0 ± 6.3
L634, 549	86.7 ± 5.2
L611,744	87.1 ± 2.9

Infection (D0) → 4 days →

N N N N

→ D D D D D D D D D D D D D D D → K

D5 D19 D23

DRUG COMBINATION	% SUPPRESSION
Fexinidazole + DFMO	89.3 ± 5.9
MK 436 + DFMO	92.0 ± 3.7
L634, 549 + DFMO	86.9 ± 3.8
L611,744 + DFMO	93.1 ± 3.4

DFMO D : 2% soln. in the drinking water. Mice kept under 4 hours dark, 2 hours light regimen.
5-nitroimidazole N : 200 mg/kg K : Kill

Fig. 7. Effect of the 5-nitroimidazoles fexinidazole, MK 436, L634,549 and L611,744, alone and in combination with DFMO, on L. donovani - infected Balb/c mice.

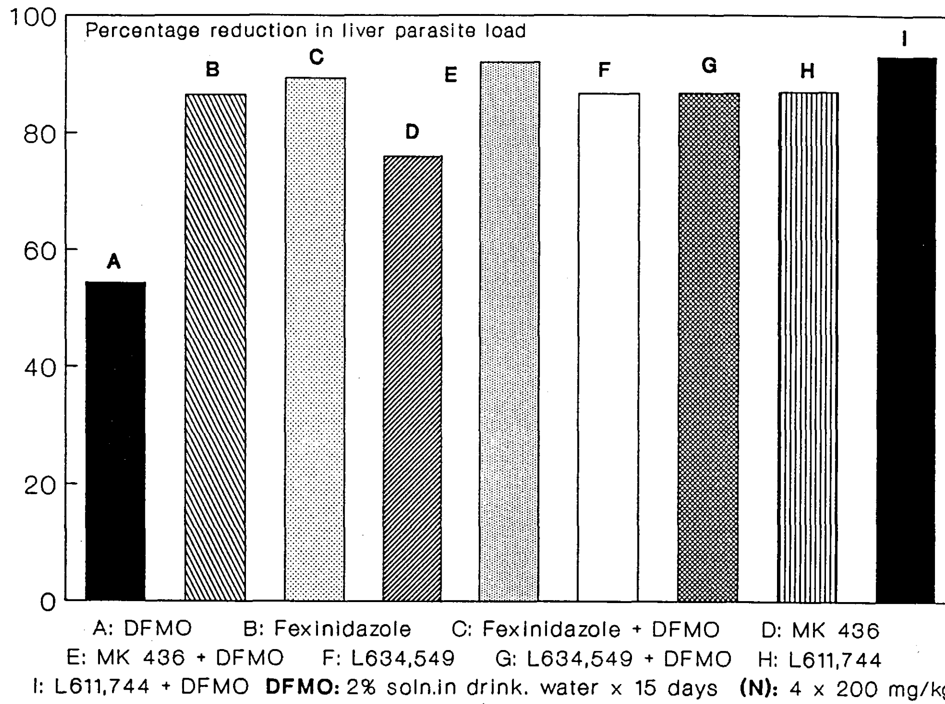


Table 7

The effect of the 5-nitroimidazole L611,744 individually and in combination with DFMO, against L. donovani in mice.

TREATMENT REGIMEN

Infection (D0) → 4 days →

D D D D D D D D D D D D D D D → K

D5 D19 D23

DFMO*	GROUP SIZE	% SUPPRESSION
2% soln.	6	48.0 ± 25.0

Infection (D0) → 4 days →

L L L L L L L L L L L L → K

D8 D19 D23

L611,744	GROUP SIZE	% SUPPRESSION
100 (mg/kg)	6	88.4 ± 10.2
50	6	71.7 ± 13.8
25	6	54.8 ± 14.7

Infection (D0) → 4 days →

L L L L L L L L L L L L

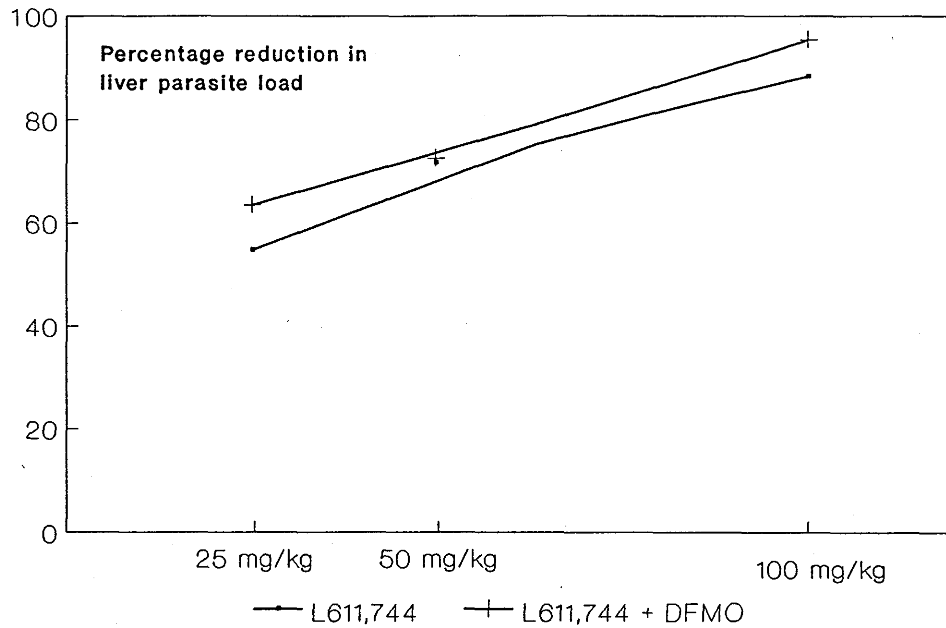
D D D D D D D D D D D D D D D → K

D5 D8 D19 D23

L611,744 + DFMO	GROUP SIZE	% SUPPRESSION
100 (mg/kg)	6	95.4 ± 6.2
50	6	72.6 ±15.6
25	6	63.6 ± 9.7

*In the drinking water. Mice kept under 4 hours dark, 2 hours light regimen. L : L611,744 K : Kill

Fig. 8. Effect of the 5-nitroimidazole L611,744, alone and in combination with DFMO, against L. donovani in Balb/c mice.



L611,744 (12 daily doses), DFMO 2% soln. in drinking water for 15 days

Table 8

The effect of the 5-nitroimidazole L611,744 individually and in combination with DFMO and Pentostam, against L. donovani in mice.

TREATMENT REGIMEN

Infection (D0) → 4 days →
D D D D D D D D D D D D D D → K
D5 ... D18 ... D22

DRUG	% SUPPRESSION
* DFMO 2% soln.	22.7 ± 25.3

Infection (D0) → 4 days → L L L L → K
D14 D17 D22

DRUG	% SUPPRESSION
L611,744	69.4 ± 14.0

Infection (D0) → 4 days →
L L L L (D14–D17)
D D D D D D D D D D D D D D → K
D5 D14 D17 D22

DRUG COMBINATION	% SUPPRESSION
L611,744 + DFMO	96.7 ± 6.2

Infection (D0) → 13 days → P P P P → K
D14 D17 D22

DRUG	% SUPPRESSION
Pentostam	-6.0 ± 34.0

Infection (D0) → 4 days →
P P P P (D14–D17)
D D D D D D D D D D D D D D → K
D5 D14 D17 D22

DRUG	% SUPPRESSION
Pentostam + DFMO	24.0 ± 17.3

Infection (D0) → 13 days →
L L L L
P P P P → K
D14 D17 D22

DRUG COMBINATION	% SUPPRESSION
L611,744 + Pentostam	74.0 ± 32.6

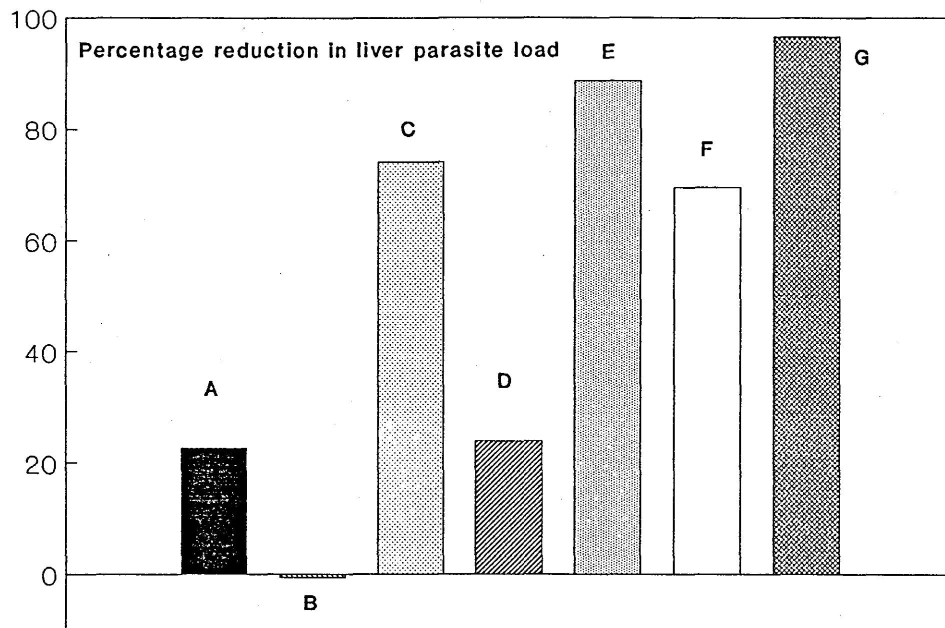
Infection (D0) → 4 days →
L L L L
P P P P (D14–D17)
D D D D D D D D D D D D D D → K
D5 D14 D17 D22

DRUG COMBINATION	% SUPPRESSION
L611,744 + DFMO + Pentostam	88.7 ± 8.0

* In the drinking water. Mice kept under 4 hours dark, 2 hours light regimen.

L : L 611,744 (200 mg/kg) P : Pentostam (5 mg/kg) K : Kill. Group size: 6

Fig. 9. The effect of DFMO alone and in various combinations with the 5-nitroimidazole L611,744 and Pentostam, on <u>L. donovani</u> - infected Balb/c mice.



A: DFMO B: Pentostam C: L611,744 D: DFMO + Pentostam E: DFMO + L611,744
F: Pentostam + L611,744 G: DFMO + Pentostam + L611,744

In the second experiment (Table 8, Figure 9), L611,744 was used in combination with Pentostam and DFMO. Surprisingly, in this experiment DFMO monotherapy showed no statistically significant effect on liver parasite load. In monotherapy using 4 consecutive daily doses of 200 mg/kg of L611,744, a 69% reduction in parasite burden was achieved. This was substantially improved when the nitroimidazole was combined with DFMO therapy, increasing the percentage reduction in liver parasite burden to 96%. Combination of L611,744 with Pentostam and DFMO resulted to almost 89% reduction in parasite load. In no case the parasites were totally cleared.

3.2.1.1.2.(c) Effect of the 5-nitroimidazole L634,549, diminazene diaceturate (Berenil^R) and melarsonyl potassium (Trimelarsan^R), alone and in combination, against <u>L. donovani</u> in mice

The treatment regimen and the percentage suppression in liver parasite burden are detailed in Table 9. The results which are illustrated in Figure 10 showed only a relatively minor effect on liver parasite burden for both Berenil and Trimelarsan when they were used as monotherapy, giving between 40-50% reduction ($F_{(9,113)}=47.1$, $P<0.05$). In combination with the 5-nitroimidazole L634,549, both compounds apparently produced a small additional effect over that produced by the nitroimidazole alone, however, this effect was not statistically significant. In no case were all the parasites cleared.

3.2.1.1.2.(d) The effect of the 5-nitroimidazoles MK 436, L634,549 and fexinidazole and suramin, alone and in combination, against <u>L. donovani</u> in mice

The treatment regimen and percentage suppression of parasites in liver are shown in Table 10. The results which are also illustrated in Figure 11 showed that suramin had a only a minor effect when used alone. When used in combination with the 5-nitroimidazoles, MK 436, L634,549 and fexinidazole its action appeared to reduce the effect of the nitroimidazole used alone.

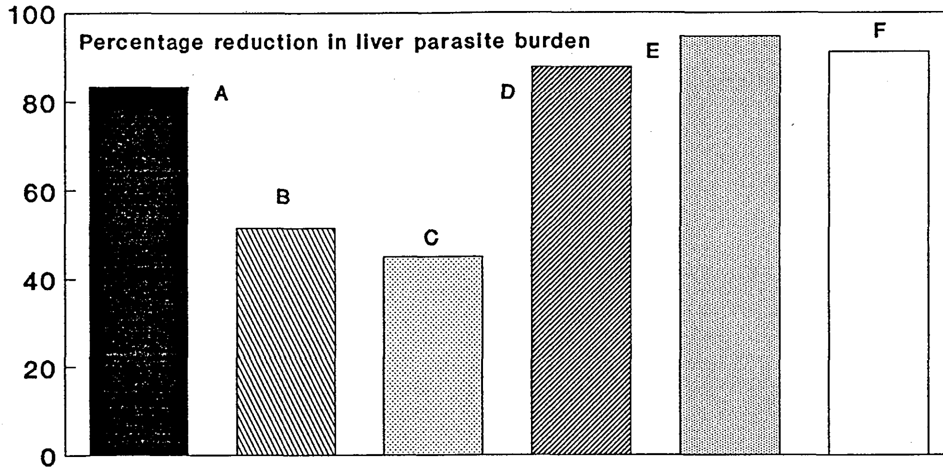
Table 9

The effect of L634,549, Berenil and Trimelarsan, individually and in combination, against L. donovani in mice.

DRUG/ COMBINATION	DOSE (mg/kg)	TREATMENT REGIMEN / GROUP SIZE	% SUPPRESSION
		Infection (D0) → 13 days → [N][N][N][N] (D14–D17) → [K] (D21)	
L634,549 [N]	200	6	83.4 ± 9.3
		Infection (D0) → 13 days → [B][B][B][B] (D14–D17) → [K] (D21)	
Berenil [B]	40	6	51.5 ± 18.2
		Infection (D0) → 13 days → [T][T][T][T] (D14–D17) → [K] (D21)	
Trimelarsan [T]	30	6	45.0 ± 16.6
		Infection (D0) → 13 days → [T][T][T][T] / [N][N][N][N] (D14–D17) → [K] (D21)	
L634,549 + Trimelarsan		6	87.8 ± 8.5
		Infection (D0) → 13 days → [B][B][B][B] / [N][N][N][N] (D14–D17) → [K] (D21)	
L634,549 + Berenil		6	94.7 ± 3.2
		Infection (D0) → 13 days → [B][B][B][B] / [T][T][T][T] / [N][N][N][N] (D14–D17) → [K] (D21)	
L634,549 + Berenil + Trimelarsan		6	91.2 ± 6.0

[K] : Kill

Fig. 10. Effect of the 5-nitroimidazole L634,549, Trimelarsan and Berenil, individually and in combination, on L. donovani - infected Balb/c mice.



A: L634,549 (L) B: Berenil (B) C: Trimelarsan (T)

D: (L) + (T) E: (L) + (B) F: (L) + (B) + (T)

L634,549: 4 x 200 mg/kg Trimelarsan: 4 x 30 mg/kg Berenil: 4 x 40 mg/kg

Table 10

The effect of MK 436, L634,549, fexinidazole and suramin, individually and in combination, against L. donovani in mice.*

TREATMENT REGIMEN

Infection → 13 days → [S] → [K]
D14 D21

DRUG	% SUPPRESSION
Suramin	33.7 ± 32.2

Infection (D0) → 13 days → [N][N][N][N] → [K]
D14 D17 D21

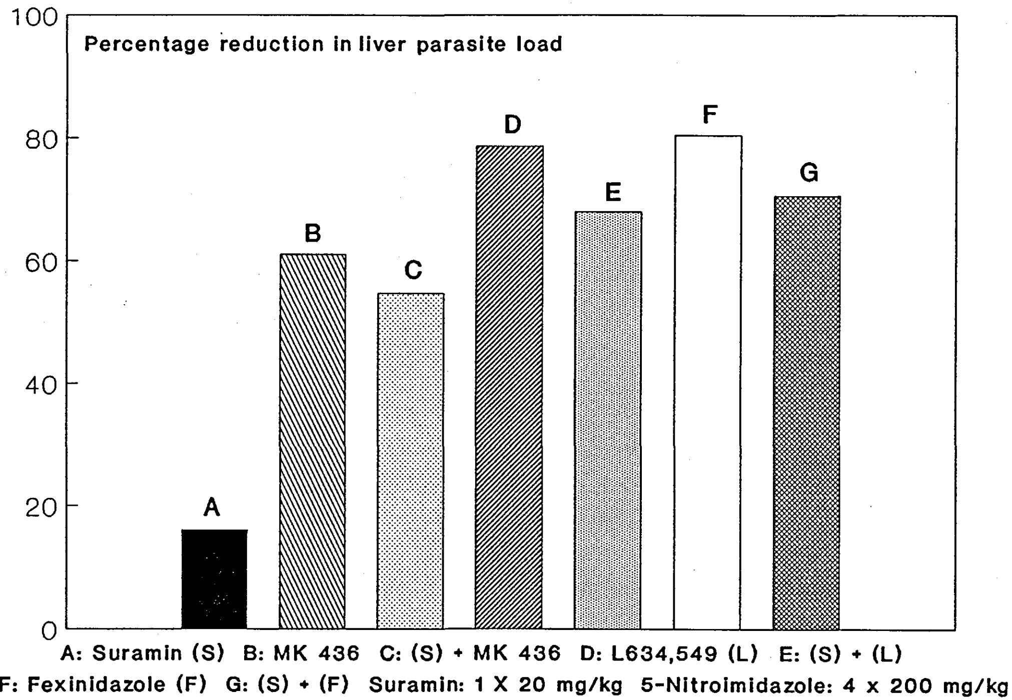
DRUG	% SUPPRESSION
L634,549	78.8 ± 7.5
MK 436	61.0 ± 20.9
Fexinidazole	80.4 ± 5.0

Infection (D0) → 13 days → [S] [N][N][N][N] → [K]
D14 D17 D21

DRUG COMBINATION	% SUPPRESSION
L634,549 + Suramin	67.9 ± 17.7
MK 436 + Suramin	54.3 ± 15.4
Fexinidazole + Suramin	70.6 ± 9.1

[S]: Suramin (20 mg/kg) [N] : L634,549, MK 436, Fexinidazole (200 mg/kg) *GROUP SIZE : 6

Fig. 11. The effect of the 5-nitroimidazoles L634,549, MK 436 and fexinidazole, individually and in combination with suramin, against L. donovani - infected Balb/c mice.



However, two way and one way analysis of variance and the Neuman Keuls multiple range test showed that there was no significant difference in the results obtained and the addition of suramin did not increase the effect of the nitroimidazole monotherapy.

3.2.1.1.2.(e) The effect of the 5-nitroimidazole MK 436 given at various dose levels alone and in combination with verapamil, against L. donovani in mice

It has been reported that verapamil affects drug uptake and efflux in a multiple drug resistant carcinoma cell line, resulting in drug accumulation within the cells (Fojo et al., 1985). In order to examine if it is possible to increase the effectiveness of the nitroimidazoles by combination with verapamil, mice infected with L. donovani were treated ip with four consecutive daily doses of the 5-nitroimidazole MK 436 at different dose rates, alone and in combination with verapamil. The treatment regimen and the percentage suppression in liver parasite burden are detailed in Table 11. The results, also illustrated in Figure 12, showed that there was no statistically significant difference between the groups treated with the nitroimidazole alone and the nitroimidazole/verapamil combination, with the exception of one in which MK 436 was given at the dose level of 4 x 50 mg/kg when an increase in effect was observed ($F_{(9,11)}=36.3$, $P<0.05$). The results suggest that verapamil does not significantly affect the concentration of the nitroimidazole MK 436 in the parasite.

3.2.1.2. THE 2-NITROIMIDAZOLES

3.2.1.2.(a) The effect of the 2-nitroimidazoles against the promastigotes of L. donovani

The 2-nitroimidazoles tested were Ro 15-0216, Ro 15-6547, Ro 16-5304 and Ro 17-1051 (benznidazole). The results are shown in Table 12 and Figure 13 and are from two experiments carried out in duplicate.

The 2-nitroimidazoles Ro 15-0216, Ro 15-6547, Ro 16-5304 showed

Table 11

The effect of the 5-nitroimidazole MK 436, given at various dose levels alone and in combination with verapamil, against L. donovani in mice.

TREATMENT REGIMEN

Infection → 13 days → [V|V|V|V] → [K]
D14 D17 D21

DOSE	% SUPPRESSION
30 mg/kg	12.8 ± 23.1

DOSE MK 436	% SUPPRESSION Infection (D0) → 13 days → [M|M|M|M] → [K] (D14 D17 D21)	% SUPPRESSION Infection (D0) → 13 days → [V|V|V|V / M|M|M|M] → [K] (D14 D17 D21)
200 mg/kg	81.9 ± 9.8	78.1 ± 6.0
100 mg/kg	61.8 ± 11.9	67.5 ± 9.4
50 mg/kg	36.3 ± 12.2	54.5 ± 10.9
25 mg/kg	25.5 ± 11.4	37.1 ± 17.6

[M]: MK 436 [V]: Verapamil 30 mg/kg GROUP SIZE: 6

Fig. 12. The effect of the 5-nitroimidazole MK 436 (MK), given at various dose levels alone and in combination with verapamil (V), on L. donovani - infected Balb/c mice.

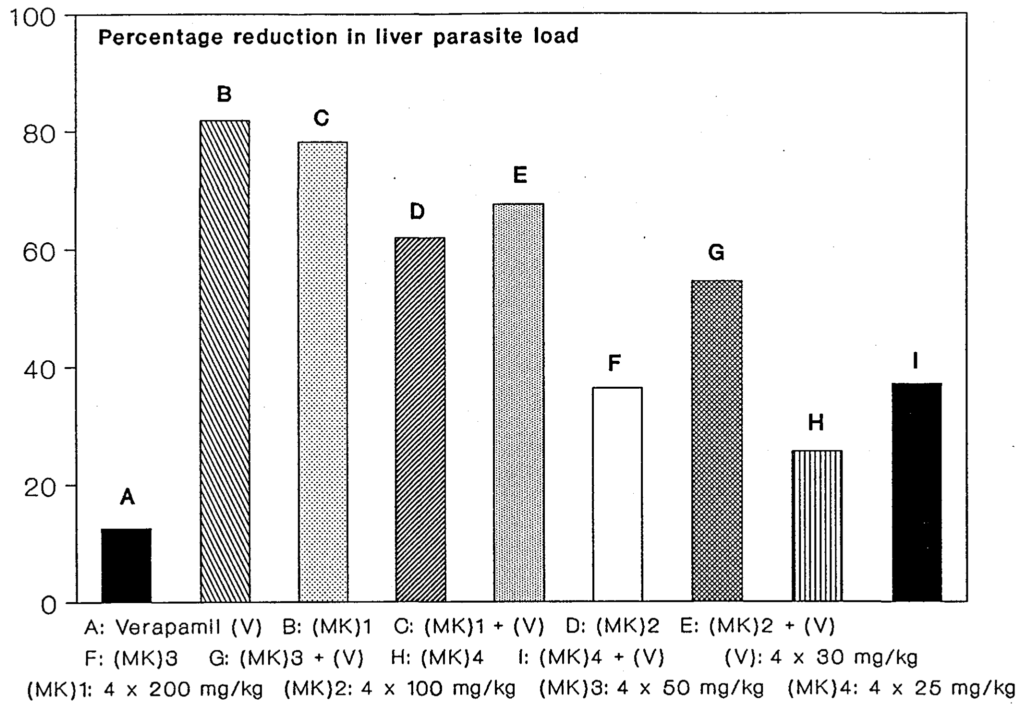


Table 12

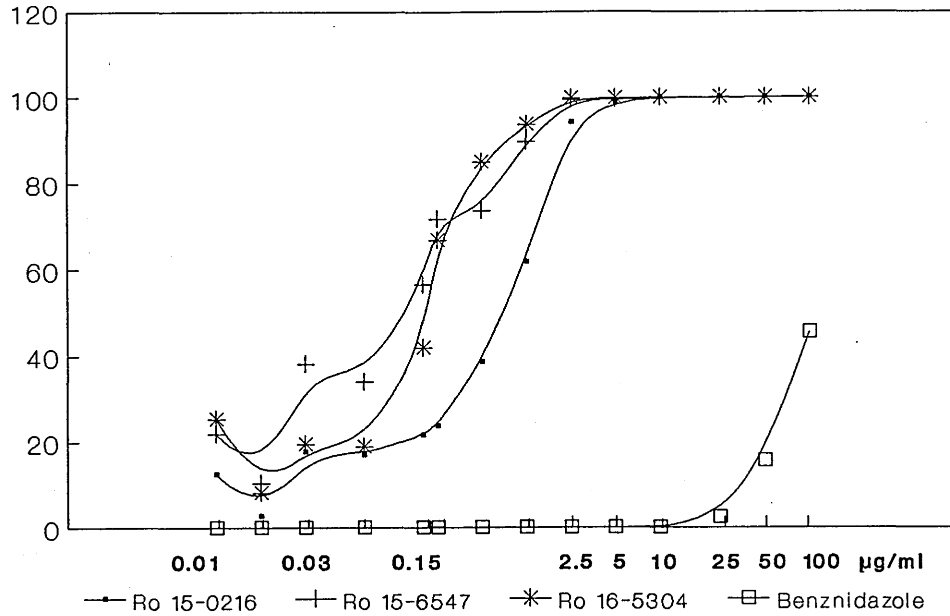
The effect of the 2-nitroimidazoles, at various concentrations, against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls			
	Ro 17-105	Ro 16-5304	Ro 15-0216	Ro 15-6547
100	40.0 ± 7.9	100	100	100
50	7.2 ± 2.6	100	100	100
25	4.9 ± 9.6	100	100	100
10	-6.7 ±10.9	99.9 ± 0.2	99.9 ± 0.1	99.9 ± 3.0
5	4.7 ± 1.1	99.7 ± 0.3	98.8 ± 0.9	99.5 ± 0.5
2.5	-7.4 ± 1.2	99.8 ± 0.1	94.1 ± 4.9	99.4 ± 0.7
1.25	3.3 ±15.5	93.6 ± 3.4	61.7 ±29.1	89.6 ± 5.1
Control**	2902.5 ± 180.3	2381.0 ± 602.5	2850.0 ± 212.1	1973.3 ± 83.1

Drug conc. µg / ml	Percentage reduction compared to controls		
	Ro 16-5304	Ro 15-0216	Ro 15-6547
0.5	84.9 ± 0.4	38.6 ± 6.6	73.5 ± 7.9
0.25	66.8 ± 3.0	23.8 ± 15.8	71.7 ± 6.4
0.125	41.9 ± 0.2	21.7 ± 16.8	56.4 ± 13.7
0.06	19.0 ± 2.3	17.0 ± 20.8	34.0 ± 14.6
0.03	19.6 ± 0.2	17.7 ±12.0	38.2 ± 5.2
0.016	8.2 ±12.3	2.7 ±12.0	10.5 ± 6.7
0.008	25.4 ± 3.9	12.5 ± 8.3	22.0 ± 21.5
Control**	1966.0 ± 114.8	2850.0± 212.1	1973.3 ± 83.1

Mean number of parasites x 10^4/ml ± SD

Fig. 13. The effect of 2-nitroimidazoles against the promastigotes of <u>L. donovani</u>



interesting antileishmanial activity *in vitro*, giving up to 100% reduction in promastigote numbers, at concentrations 2.5 μg/ml and above ($P<0.05$). Benznidazole showed some activity at 100 μg/ml, the highest level tested.

3.2.1.2.(b) *In vivo* activity of Ro 15-0216 in monotherapy

Infected mice were treated intraperitoneally with the 2-nitroimidazole Ro 15-0216 at different dose levels. The treatment regimen and the percentage suppression in liver parasite load are shown in Table 13. The nitroimidazole at the dose levels tested, produced between 40-76% reduction in liver parasite load, however, there was no significant difference between the varying dose levels used. The activity of Ro 15-0216 in *L. donovani*-infected Balb/c mice is also illustrated in Figure 6. It can be seen that its *in vivo* activity is less than the 5-nitro compounds.

3.2.1.2.(c) *In vivo* activity of Ro 15-0216 in combination with diminazene diaceturate (Berenil^R) and melarsonyl potassium (Trimelarsan^R, Mel W^R)

In this experiment, in order to overcome the putative problem of its rapid metabolism, the 2-nitroimidazole Ro 15-0216 was administered in the drinking water at different dose levels and the mice were kept under a 4 hours dark and 2 hours light regimen that ensured a more even intake of the drug. The treatment regimen and the results of using this alone and in combination with Trimelarsan and Berenil are given in Table 14 and Figures 14 and 15. Combination of Ro 15-0216 with Trimelarsan (4 x 30 mg/kg) was apparently less effective than giving the nitroimidazole alone, although the differences observed were not statistically significant (Figure 14). When Ro 15-0216 was combined with Berenil, (four daily doses of 40 mg/kg), a statistically significant difference was found only at the dose level of 20 mg Ro 15-0216/100 ml drinking water ($F_{(2,97)}=7.3$, $P<0.05$), although the combination effect being less than additive (Figure 15). In no case were the parasites totally

Table 13

The effect of the 2-nitroimidazole Ro 15-0216*, given individually and at different dose levels, against L. donovani in mice.[a]

TREATMENT REGIMEN

Infection → 13 days → [R|R|R|R] → [K]

D0 D14 D17 D21

DOSE (mg/kg)	% SUPPRESSION
200	76.3 ± 18.7
100	59.0 ± 23.3
50	47.8 ± 20.7
25	40.8 ± 29.4

[R] : Ro 15-0216 [K] : Kill [a]Group size: 6 *Injected ip.

Table 14

The effect of the 2-nitroimidazole Ro 15-0216*, individually and in combination with Berenil and Trimelarsan, against L. donovani in mice.[a]

TREATMENT REGIMEN

Infection (D0) → 4 days →
[R][R][R][R][R][R][R][R][R][R][R] → [K]
D5 D15 D19

Ro 15-0216 *	% SUPPRESSION
20 (mg/100ml)	45.9 ± 16.9
40	58.6 ± 10.7
80	62.9 ± 10.9

Infection (D0) → 4 days →
[*][*][*][*] → [K]
D8 D11 D19

DRUG	% SUPPRESSION
Berenil	60.5 ± 14.5
Trimelarsan	55.9 ± 11.2

Infection (D0) → 4 days →
[*][*][*][*]
[R][R][R][R][R][R][R][R][R][R][R] → [K]
D5 D8 D11 D15 D19

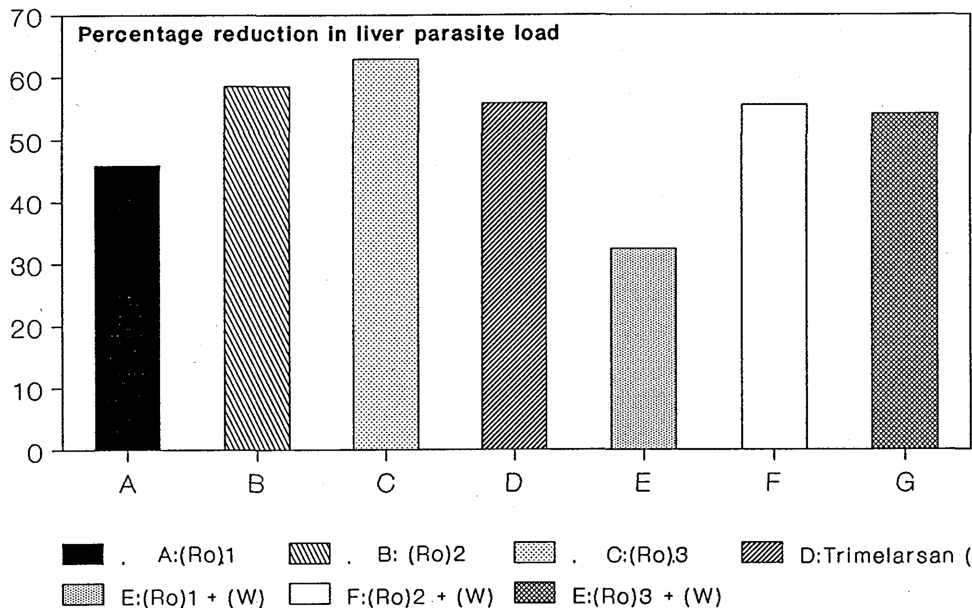
* Ro 15-0216 + Berenil	% SUPPRESSION
20 (mg/100ml) **	69.3 ± 8.2
40	57.6 ± 13.0
80	65.0 ± 12.5

* Ro 15-0216 + Trimelarsan	% SUPPRESSION
20 (mg/100ml)	32.3 ± 18.6
40	55.6 ± 12.8
80	54.2 ± 8.8

[*] : Berenil, 40 mg/kg, [*] :Trimelarsan, 30 mg/kg, [K] : Kill

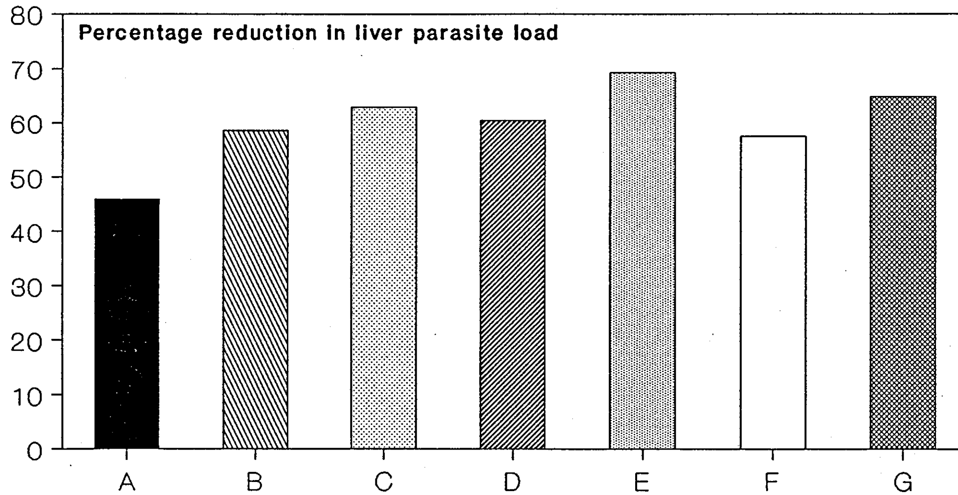
* In the drinking water. Mice kept under 4 hours dark 2 hours light regimen. [a] Group size 6 ** 5

Fig. 14. The effect of the 2-nitroimidazole Ro 15-0216 (Ro), given at various dose levels alone and in combination with Trimelarsan (W), on L. donovani-infected Balb/c mice.



(Ro)1 : 20 mg/100ml in drink. water x 11 days , **(Ro)2 :** 40 mg/100ml in drink. water x 11 days
(Ro)3 : 80 mg/100ml in drink. water x 11 days , **(W) :** 4 x 30 mg/kg

Fig.15. The effect of the 2-nitroimidazole Ro 15-021 (Ro), given at various dose levels alone and in combination with Berenil (B), on L. donovani - infected Balb/c mice.



A: (Ro)1. B: (Ro)2. C: (Ro)3. D: Berenil (B)
E: (Ro)1 + (B) F: (Ro)2 + (B) G: (Ro)3 + (B)

(Ro)1: 20 mg/100ml in drink. water x 11 days **(Ro)2:** 40 mg/100ml in drink. water x 11 days
(Ro)3: 80 mg/100ml in drink. water x 11 days **(B):** 4 x 40 mg/kg

cleared.

3.2.2. THE GUANYLHYDRAZONES

3.2.2.(a) The effect of guanylhydrazones against the promastigotes of *L. donovani*

A number of guanylhydrazones supplied by Profs. Ulrich and Cerami of the Rockefeller University were tested *in vitro* and the results are summarised in Table 15 and Figure 16. Four of these, TBG, 5-amino-DBG, 5-decNH- DBG and DBG, all showed good activity against promastigotes giving up to 100% reduction of promastigotes at concentrations 5 μg/ml and above whereas TBHG had no significant activity even at the highest level tested (100 μg/ml).

TBG's activity was monitored every day over the 5 day incubation period and these results are also shown in Table 15. This guanylhydrazone inhibited the growth of *L. donovani* at concentrations of 25 μg/ml and above during the first 24 hours of the incubation period and at relatively low concentrations (2.5-5 μg/ml) over the following 4 days, giving up to 100% reduction ($F_{(7,15)}=9.3$, $P<0.05$).

3.2.2.(b) The guanylhydrazones in combination experiments

Two of the guanylhydrazones, TBG and DBG, which had given promising results against promastigotes *in vitro* and which were available in sufficient quantity, were also tested *in vivo*, in combination with sodium stibogluconate (Pentostam^R) and DFMO. The treatment regimen and the percentage suppression in liver parasite load are detailed in Table 16. The results, which are also illustrated in Figures 17 and 18, showed that TBG produced no effect on liver parasite load of *L. donovani* when used alone. It also had little effect when used in combination with Pentostam and/or DFMO (Table 16, Figure 17). The results for DBG (Table 16, Figure 18), showed that this guanylhydrazone has some activity producing a 33.9% reduction in parasite load ($F_{(3,86)}=4.97$, $P<0.05$)

Table 15

The effect of guanylhydrazones, at various concentrations, against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls				
	DBG	5-decNH-DBG	AMPA-DBG	5-amino-DBG	TBHG
100	100	100	100	40.9 ± 9.1	31.4 ± 41.8
50	100	100	100	20.9 ± 0.8	6.5 ± 2.5
25	100	99.4 ± 0.9	99.9 ± 0.02	17.6 ± 6.9	-6.2 ± 23.8
10	86.7 ± 17.2	99.5 ± 0.9	97.8 ± 0.2	14.1 ± 13.5	-8.1 ± 22.3
5	53.4 ± 44.1	93.3 ± 7.8	97.1 ± 4.0	9.7 ± 7.9	-9.7 ± 5.4
2.5	20.4 ± 15.6	51.0 ± 5.2	84.6 ± 15.2	13.0 ± 8.6	-26.1 ± 40.4
1.25	13.2 ± 10.7	28.9 ± 20.2	19.0 ± 19.8	7.4 ± 1.5	-14.4 ± 27.9
0.5	11.0 ± 19.7	15.6 ± 3.2	13.7 ± 12.9	-	-
0.25	1.3 ± 33.2	-1.4 ± 10.4	15.8 ± 17.7	-	-
0.125	-0.9 ± 9.1	0.2 ± 10.7	0.3 ± 2.8	-	-
0.08	-6.4 ± 19.6	-11.1 ± 19.1	16.7 ± 11.7	-	-
0.03	7.8 ± 13.5	-11.9 ± 17.6	10.0 ± 0.1	-	-
0.018	-7.9 ± 24.6	-14.5 ± 15.7	10.0 ± 7.8	-	-
0.008	6.0 ± 26.5	-6.7 ± 0.51	25.7 ± 18.8	-	-
Control**	2820,0 ± 292.7	2447.5 ± 74.2	1666.0 ± 210.7	2882.5 ± 470.2	2790.0 ± 346.5

The effect of the guanylhydrazone TBG at different concentrations and time intervals against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls				
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
100	93.0 ± 0.9	99.3 ± 0.2	99.7 ± 0.2	100	100
50	85.8 ± 5.2	94.9 ± 0.8	99.9 ± 0.1	99.9 ± 0.1	100
25	74.2 ± 3.4	95.9 ± 0.2	98.8 ± 0	99.9 ± 0.1	99.9 ± 0.1
10	50.6 ± 8.3	86.6 ± 0.4	98.5 ± 0.5	99.8 ± 0.3	99.8 ± 0.6
5	16.0 ± 13.7	79.9 ± 3.7	97.5 ± 1.5	98.1 ± 0.4	99.2 ± 0.7
2.5	6.2 ± 12.4	62.6 ± 2.8	28.9 ± 14.6	80.9 ± 11.3	93.0 ± 1.8
1.25	19.3 ± 27.2	9.7 ± 3.2	1.8 ± 5.7	14.2 ± 51.0	43.8 ± 3.3
0.5	-58.9 ± 24.6	5.5 ± 16.4	24.5 ± 22.3	-15.6 ± 4.6	1.0 ± 25.0
Control**	43.0 ± 5.7	184.0 ± 19.8	347.5 ± 102.5	825.0 ± 226.0	1317.5 ± 3.3

** Mean number of parasites x 10^4/ml ± SD

Fig.16. The effect of guanylhydrazones against the promastigotes of L. donovani

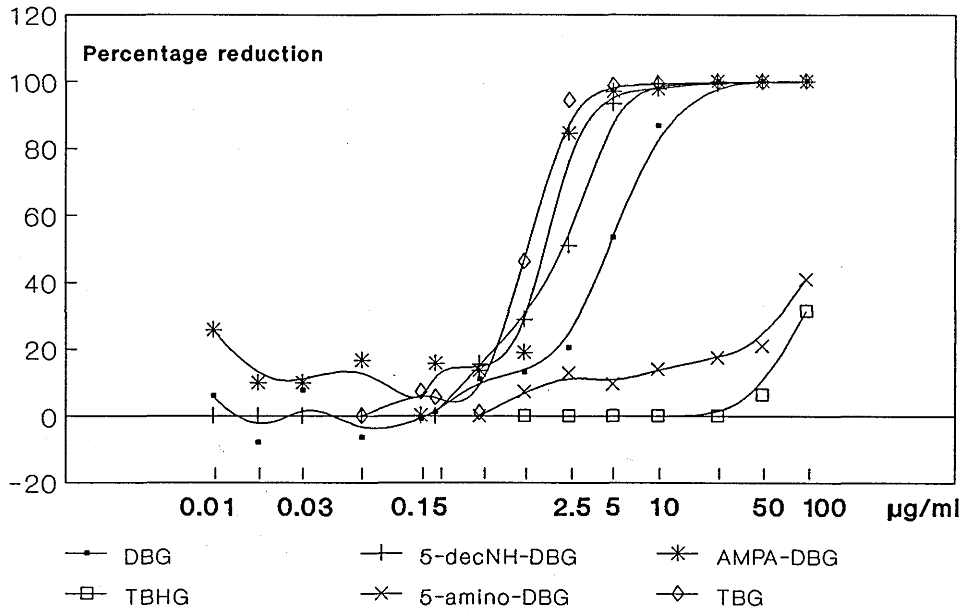


Table 16

The effect of TBG and DBG against L. donovani in mice*, individually and in combination with Pentostam and DFMO.

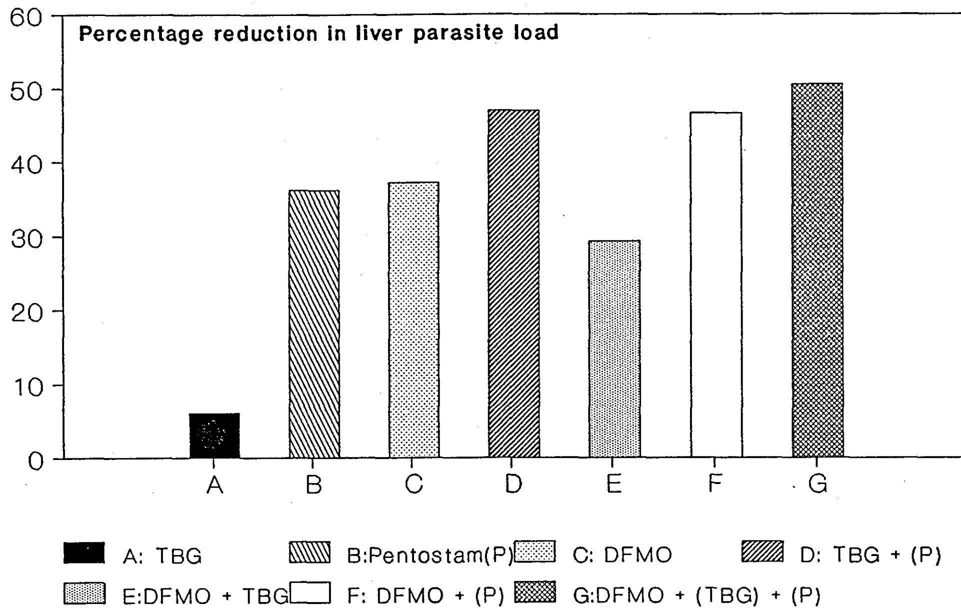
TREATMENT REGIMEN	DRUG / DRUG COMBINATION	% SUPPRESSION
Infection (D0) → 4 days → D (D5–D18) → K (D22)	DFMO	37.1 ± 12.0
Infection (D0) → 14 days → P, P (D15–D18) → K (D22)	Pentostam + TBG	46.9 ± 13.7
	Pentostam + DBG	38.9 ± 15.2
Infection (D0) → 14 days → P, P (D15–D18) → K (D22)	Pentostam	36.1 ± 12.3
Infection (D0) → 4 days → D (D5–D18); P, P (D15–D18) → K (D22)	Pentostam + DFMO	46.9 ± 10.9
Infection (D0) → 14 days → □ (D15–D18) → K (D22)	TBG	6.0 ± 16.6
	DBG	33.9 ± 16.9
Infection (D0) → 4 days → D (D5–D18); □ (D15–D18) → K (D22)	DFMO + TBG	29.2 ± 18.4
	DFMO + DBG	34.1 ± 19.5
Infection (D0) → 4 days → D (D5–D18); P, □, P (D15–D18) → K (D22)	Pentostam + DFMO + TBG	50.5 ± 13.0
	Pentostam + DFMO + DBG	55.4 ± 15.0

□ TBG 10 mg/kg □ DBG 10 mg/kg

[P] Pentostam 5 mg/kg, 1000 hrs & 1500 hrs each day [K] Kill * Group size 6

[D] DFMO 2% soln. in drinking water. Mice kept under 4 hours dark, 2 hours light regimen

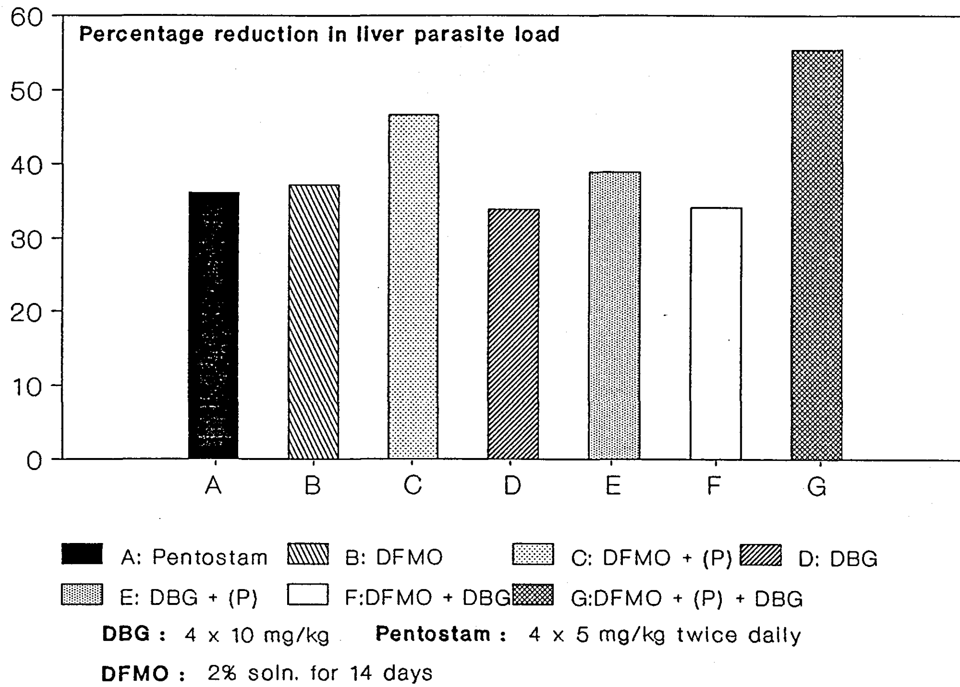
Fig. 17. The effect of guanylhydrazone TBG alone and when used in combination with DFMO and Pentostam, on L. donovani - infected Balb/c mice.



TBG : 4 x 10 mg/kg **Pentostam :** 4 x 5 mg/kg twice daily

DFMO : 2% soln. for 14 days

Fig.18. The effect of guanylhydrazone DBG alone and in combination with Pentostam and DFMO, on L. donovani - infected Balb/c mice.



at the dose level used although in combination it did not produce any additional effect over that produced by the other drug(s) alone. These results are in contrast to the in vitro activities of the two guanylhydrazones: DBG cleared the parasites only at high concentrations (5-100 µg/ml) whereas TBG was found to be active against promastigotes of L. donovani at concentrations as low as 1.25 µg/ml.

3.2.3. TESTING OF OTHER POTENTIAL INHIBITORS OF TRYPANOTHIONE METABOLISM

3.2.3.1. DL-α-DIFLUOROMETHYLORNITHINE (DFMO) (EFLORNITHINE^{R})

3.2.3.1.(a) Effect of DFMO against L. donovani promastigotes and amastigotes in vitro

The results of in vitro studies on DFMO are shown in Tables 17 and 18, Figures 19 and 20. Against promastigotes, at DFMO concentrations as low as 50 µg/ml, growth was completely blocked (Table 17, Figure 19) ($F_{(7,31)}=26.6$, $P<0.05$). DFMO showed some activity upon growth of L. donovani amastigotes in PECs which, however, it was not statistically significant (Table 18, Figure 20).

3.2.3.1.(b) Effect of DFMO against L. donovani in mice

Particular attention has been given to DFMO's antileishmanial activity in vivo and its efficacy in clearing tissues other than the liver. This new antiproliferative drug is especially effective in the treatment of human African trypanosomiasis but it has not been tested extensively for antileishmanial activity. DFMO is known to be metabolised very quickly and so has to be administered frequently and in large quantity. It was tested at 2% and 4% in the drinking water and for different lengths of time (7, 14, 30 days) using the L. donovani-Balb/c mouse model, and the parasite load in the liver, spleen and bone marrow was assessed. The treatment regimen and the percentage of parasites as compared to the untreated controls

Table 17

The effect of Trimelarsan, DFMO, Pentostam, buthionine sulfoximine (BSO), nitrofurazone, Lomidine and Berenil, at different concentrations, against L. donovani promastigotes.

Drug conc. µg / ml	Percentage reduction compared to controls			
	Trimelarsan	DFMO	Pentostam	BSO
100	6.1 ± 8.9	99.4 ± 0.4	16.2 ± 5.2	30.5 ± 3.9
50	-8.2 ± 12.0	99.1 ± 0.7	9.2 ± 2.7	21.2 ± 4.2
25	-1.8 ± 12.6	98.8 ± 0.6	6.6 ±14.1	20.8 ± 9.9
10	-13.7 ± 16.1	95.9 ± 3.9	9.0 ±12.3	27.2 ± 4.5
5	-12.5 ± 7.2	67.6 ± 36.1	2.5 ± 3.1	11.8 ± 4.3
2.5	-4.0 ± 13.5	38.5 ±40.9	0.3 ±14.6	12.6 ± 3.1
1.25	-13.9 ± 16.7	13.5 ± 1.6	-0.3 ±10.3	7.0 ± 4.2
Control**	1805.0 ± 183.8	2165.5 ± 272.2	2071.5 ± 163.5	2631.2 ± 227.6

Drug conc. µg / ml	Percentage reduction compared to controls		
	Berenil	Lomidine	nitrofurazone
100	100	100	100
50	100	100	100
25	99.7 ± 0.1	100	100
10	98.5 ± 0.8	100	100
5	97.0 ± 1.1	100	99.9 ± 0.1
2.5	98.1 ± 0.6	100	99.9 ± 0.1
1.25	87.9 ± 2.9	100	99.9 ± 0.1
0.5	53.9 ± 10.8	100	58.1 ± 16.1
0.25	11.5 ± 0.6	100	36.1 ± 42.1
0.125	8.7 ± 1.2	99.7 ± 0.2	45.5 ± 10.2
0.08	3.1 ± 3.5	50.3 ± 10.4	36.7 ± 18.6
0.03	0.5 ± 2.4	18.9 ± 27.4	33.9 ± 4.8
0.018	-9.7 ± 4.1	10.2 ± 15.2	18.7 ± 27.1
0.008	0.1 ± 9.2	4.5 ± 4.6	7.8 ± 26.0
Control**	2627,5 ± 222.7	2863.8 ± 280.7	1985.0 ± 269.6

** Mean number of parasites x 10^4/ml ± SD

Fig.19. The effect of Berenil, Trimelarsan, DFMO, Pentostam, Lomidine buthionine sulfoximine (BSO) and nitrofurazone, against promastigotes of L. donovani

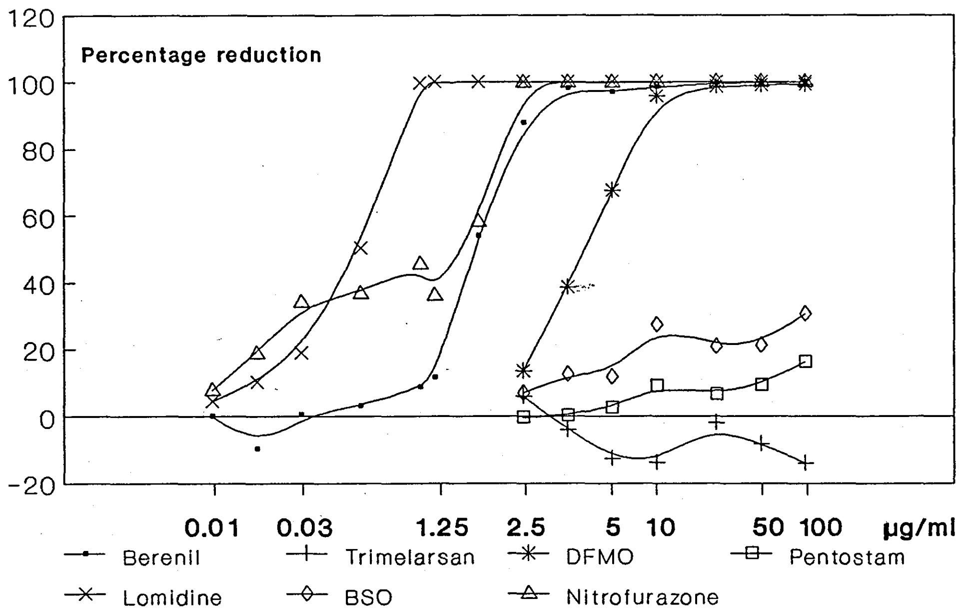


Table 18

The effect of DFMO, fexinidazole, Pentostam, Berenil, Trimelarsan and BSO, individually at various concentrations against L. donovani in PECs.

I. Number of cells infected

Drug conc. µg / ml	Number of cells infected as percentage of controls					
	Pentostam	DFMO	Fexinidazole	Berenil	Trimelarsan	BSO
100	4.3 ± 0.7	92.6 ± 13.6	*	*	*	*
50	5.4 ± 5.7	99.4 ± 6.6	*	*	67.6 ± 12.8	*
25	10.3 ± 7.3	110.2 ± 7.4	103.5 ± 14.6	*	98.1 ± 5.5	115.6 ± 3.9
10	8.6 ± 1.2	102.1 ± 9.0	102.6 ± 6.4	*	91.0 ± 6.3	119.4 ± 9.0
5	34.7 ± 10.9	111.2 ± 4.3	100.9 ± 2.6	52.1 ± 11.8	91.6 ± 8.6	107.7 ± 7.2
2.5	50.2 ± 16.9	112.5 ± 4.8	100.6 ± 4.7	70.6 ± 13.4	95.5 ± 9.4	97.6 ± 9.9
1.25	93.9 ± 6.2	101.6 ± 12.1	99.4 ± 9.9	82.7 ± 9.8	91.5 ± 7.9	109.2 ± 6.2
Control**	81.1 ± 5.0	85.1 ± 3.9	87.8 ± 8.6	76.9 ± 8.4	85.8 ± 7.8	76.3 ± 7.1

II. Number of amastigotes per 100 infected cells

Drug conc. µg / ml	Number of amastigotes / 100 infected cells, as % of controls					
	Pentostam	DFMO	Fexinidazole	Berenil	Trimelarsan	BSO
100	15.3 ± 8.8	69.1 ± 4.6	*	*	*	*
50	17.5 ± 6.1	73.0 ± 16.7	*	*	49.1 ± 7.0	*
25	20.4 ± 4.6	79.9 ± 3.2	57.7 ± 4.6	*	82.4 ± 13.1	81.4 ± 10.1
10	26.4 ± 26.4	81.5 ± 10.4	62.0 ± 7.2	*	89.2 ± 9.5	101.8 ± 1.0
5	43.8 ± 5.3	78.7 ± 9.9	74.2 ± 8.9	32.9 ± 9.5	83.9 ± 15.2	99.7 ± 7.6
2.5	37.1 ± 6.9	80.7 ± 8.0	96.7 ± 6.6	45.3 ± 9.0	85.2 ± 9.1	75.5 ± 16.9
1.25	70.2 ± 6.1	86.4 ± 15.5	77.7 ± 8.6	64.8 ± 12.8	83.3 ± 10.8	104.5 ± 5.4
Control**	983.9 ± 271.0	813.2 ± 30.2	705.3 ± 42.8	977.4 ± 205.0	873.0 ± 125.6	652.6 ± 116.6

* Drug concentration toxic to macrophages   ** Mean number of parasites ± SD

Fig.20. Effect of Pentostam, DFMO, Fexinidazole, Berenil, Trimelarsan and BSO, at various concentrations, against amastigotes of <u>L. donovani</u> in Balb/c mouse PECs

I. Percentage of infected PECs expressed as percentage of controls

Percentage of controls

140
120
100
80
60
40
20
0

1.25 2.5 5 10 25 50 100 µg/ml

Pentostam, DFMO, Fexinidazole, Berenil, Trimelarsan, BSO

II. Number of amastigotes / 100 infected cells expressed as percentage of controls

Percentage of controls

120
100
80
60
40
20
0

1.25 2.5 5 10 25 50 100 µg/ml

Pentostam, DFMO, Fexinidazole, Berenil, Trimelarsan, BSO

are summarised in Table 19. The data are also illustrated in Figure 21. The effect of DFMO on liver, spleen and bone marrow parasite load observed at the end of the administration period (DAI 20), was similar to that observed 4 days later (DAI 23). DFMO was found to have good activity against the liver parasites ($F_{(3,86)}$= 76.8, P<0.05), although they were not all eliminated even after prolonged drug treatment. The effect of DFMO administration was less profound on spleen parasite load ($F_{(3,88)}$=3.9, P<0.05) and a small effect was observed on DAI 39 on bone marrow parasite load ($F_{(3,77)}$=10.9, P<0.05). Administration of the 4% solution of DFMO was more beneficial than the 2% solution, in reducing the parasite load of spleen ($F_{(1,88)}$=23.7, P<0.05) but not of liver and bone marrow.

3.2.3.2. DIAMIDINES

3.2.3.2.1. Diminazene diaceturate (Berenil^{R})

3.2.3.2.1.(a) Effect of Berenil$^{(R)}$ against *L. donovani* promastigotes and amastigotes *in vitro*

The effect of Berenil, an inhibitor of S-adenosyl-L-methionine decarboxylase (Bitonti *et al*., 1986), upon growth of *L. donovani* *in vitro*, is shown in Tables 17 and 18, Figures 19 and 20. Against promastigotes (Table 17, Figure 19), growth was completely blocked at concentrations of 2.5 μg/ml and above ($F_{(7,15)}$=243.9, P<0.05). Against amastigotes infecting mouse PECs (Table 18, Figure 20), the inhibition was also marked but without giving a total clearance of the parasites and it was associated with toxicity to macrophages. At concentrations of 10 μg/ml and above, macrophages themselves were all killed ($F_{(4,13)}$=19.4, P<0.05).

3.2.3.2.1.(b) Effect of Berenil$^{(R)}$ against *L. donovani* in mice

Berenil was also tested at various dose levels against *L. donovani* in Balb/c mice. The treatment regimen and the percentage

Table 19

The effect of DFMO against L. donovani in mice,* after 7, 14 and 30 days of administration. Effect on liver, spleen and bone marrow parasite load.

TREATMENT REGIMEN

Infection (D0) → 7 days → DFMO for 7 days (D6–D12) → K (D16)

% SUPPRESSION :	LIVER	SPLEEN	B. MARROW
DFMO 2% soln.	34.1 ± 17.8	32.5 ± 27.7	21.2 ± 27.4
DFMO 4% soln.	48.6 ± 10.7	20.9 ± 31.1	1.8 ± 54.9

Infection (D0) → 7 days → DFMO for 14 days (D6–D19) → K (D20) | Infection (D0) → 7 days → DFMO for 14 days (D6–D19) → K (D23)

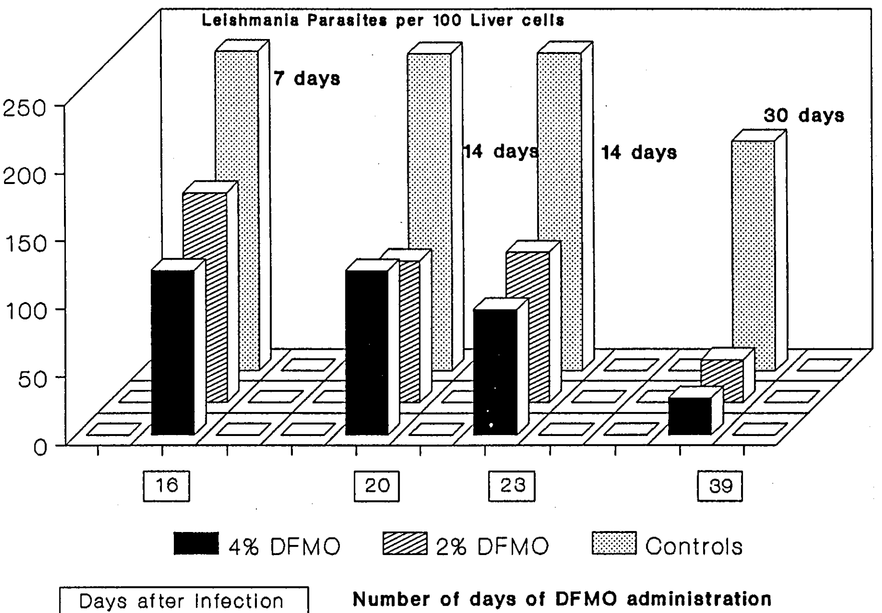
% SUPPRESSION :	LIVER (D20)	SPLEEN (D20)	B. MARROW (D20)	LIVER (D23)	SPLEEN (D23)	B. MARROW (D23)
DFMO 2% soln.	55.2 ± 10.1	36.5 ± 22.1	2.3 ± 37.6	52.5 ± 20.3	17.2 ± 39.5	63.4 ± 20.4
DFMO 4% soln.	48.1 ± 8.3	77.2 ± 14.3	24.9 ± 34.6	60.4 ± 11.4	53.6 ± 30.3	61.6 ± 17.2

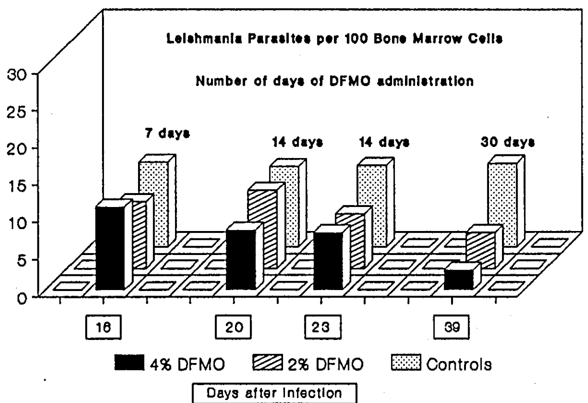
Infection (D0) → 7 days → DFMO for 30 days (D6–D35) → K (D39)

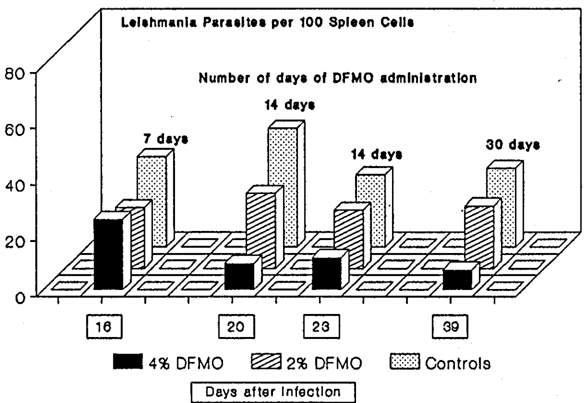
% SUPPRESSION :	LIVER	SPLEEN	B. MARROW
DFMO 2% soln.	81.1 ± 10.4	20.4 ± 49.6	57.1 ± 28.2
DFMO 4% soln.	83.8 ± 10.1	73.9 ± 26.0	75.7 ± 25.0

DFMO : 2%, 4% soln. in the drinking water
Mice kept under 4 hours dark, 2 hours light regimen. K : Kill * Group size 6

Fig. 21. The effect of DFMO against *L. donovani* in mice, after 7, 14 and 30 days of administration in the drinking water.







suppression of liver parasites as compared to untreated controls, are detailed in Table 20. The results, also illustrated in Figure 22, showed a marked 30-80% reduction in liver parasite load at drug concentrations up to 40 mg/kg ($F_{(6,75)}=124.3$, $P<0.05$), although concentrations above 40 mg/kg did not significantly increase the percentage suppression.

3.2.3.2.2. Pentamidine methanesulfonate (Lomidine[R])

3.2.4.2.2.(a) The effect of pentamidine against _L. donovani_ promastigotes

The effects of pentamidine upon growth of _L. donovani_ promastigotes are shown in Table 17 and Figure 19. Pentamidine inhibited growth at relatively low concentrations giving a 50-100% inhibition at concentrations of 0.08 μg/ml and above ($F_{(7,15)}=37.14$, $P<0.05$).

3.2.3.3. DL-BUTHIONINE-(S,R)-SULFOXIMINE (BSO)

3.2.3.3.(a) Effect of BSO against _L. donovani_ promastigotes and amastigotes _in vitro_

DL-buthionine-(S,R)-sulfoximine, an inhibitor of glutathione (GSH) synthesis (Arrick _et al_., 1981), was relatively inactive _in vitro_, showing some activity only against promastigotes when used at high concentrations, whereas amastigote growth in mouse PECs was unaffected. The results from the _in vitro_ testing of BSO are shown in Tables 17 and 18, Figures 19 and 20. It can be seen that against promastigotes, BSO had some activity at concentrations of 10 μg/ml and above ($F_{(7,15)}=5.6$, $P<0.05$) but it was inactive against amastigotes in PECs, showing only an apparent activity at 25 μg/ml which, however, was not statistically significant. In addition, it was toxic to macrophages at concentrations above 25 μg/ml.

3.2.3.3.(b) Effect of BSO against _L. donovani_ in mice

The _in vivo_ activity of BSO was better than its activity

Table 20

The effect of Berenil and buthionine sulfoximine (BSO) given individually at various dose levels, against L. donovani in mice.*

TREATMENT REGIMEN

Infection (D0) → 14 days → [B][B][B][B] → [K]
D15 D18 D22

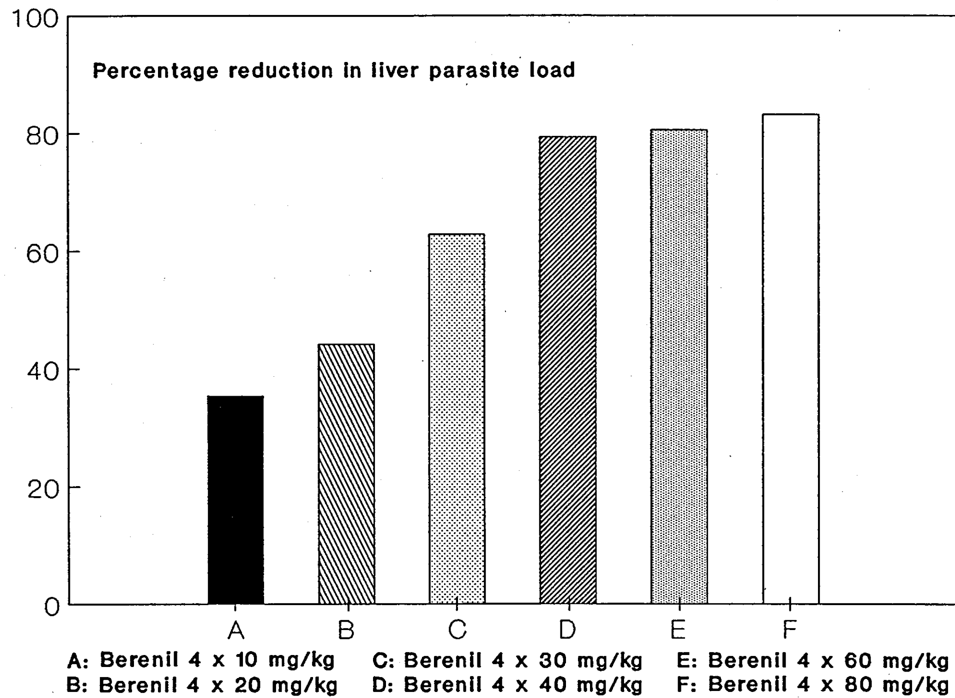
DOSE BERENIL	% SUPPRESSION
10 mg/kg	35.3 ± 6.2
20 mg/kg	44.1 ± 3.2
30 mg/kg	62.8 ± 13.2
40 mg/kg	79.4 ± 10.1
60 mg/kg	80.5 ± 6.9
80 mg/kg	83.1 ± 5.1

Infection (D0) → 14 days → [b][b][b][b] / [b][b][b][b] / [b][b][b][b] → [K]
D15 D18 D22

DOSE BSO	% SUPPRESSION
10 mg/kg	45.3 ± 9.9
20 mg/kg	73.0 ± 7.9
30 mg/kg	68.3 ± 8.0
40 mg/kg	64.8 ± 6.4

[B] : Berenil [b] : Buthionine sulfoximine (BSO) *GROUP SIZE : 6

Fig. 22. Effect of Berenil, given at various dose levels, against <u>L. donovani</u> in Balb/c mice.



in vitro, although it did not eradicate all the parasites even when used at high levels. It was tested at dose levels ranging between 10-40 mg/kg, three times daily and it produced a 45% to 70% reduction in liver parasite load ($F_{(4,55)}=126.7$, $P<0.05$). The treatment regimen and the results obtained are shown in Table 20 and Figure 23.

3.2.3.4. NITROFURANS

3.2.3.4.1. 5-nitro-2-furaldehyde semicarbazone, nitrofurazone

This compound normally associated with the treatment of T. cruzi and some bacterial infections, was also tested as it is now being used more frequently in the treatment of chronic sleeping sickness.

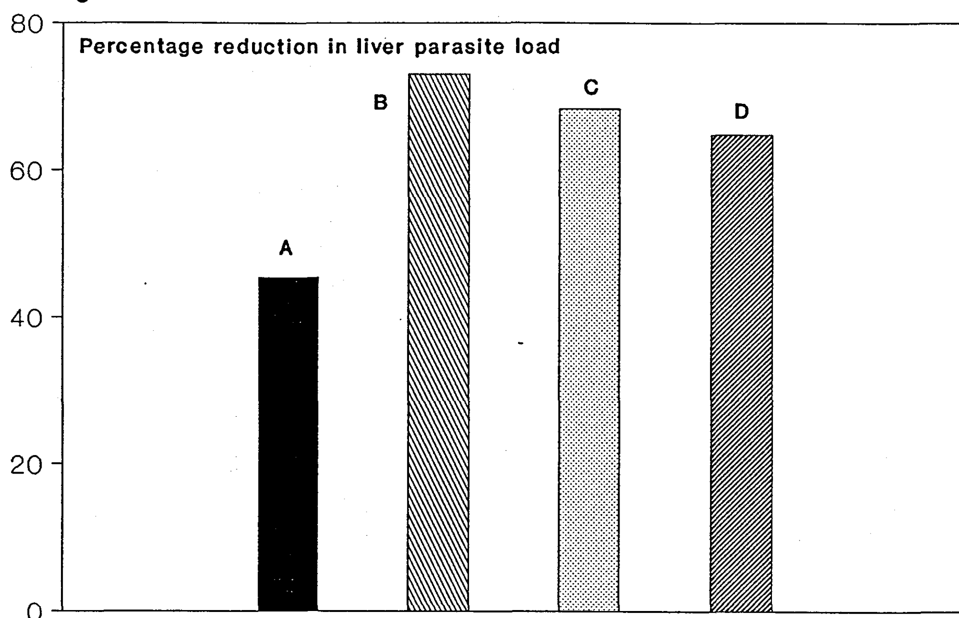
3.2.3.4.1.(a) In vitro activity

The in vitro activity of nitrofurazone has been investigated against promastigotes and the results are shown in Table 17 and Figure 19. At concentrations of 1.25 µg/ml and above this compound had shown marked activity giving up to 100% reduction ($F_{(8,17)}=8.55$, $P<0.05$), while at concentrations 0.08 µg/ml and below did not affect promastigote growth.

3.2.3.4.1.(b) In vivo activity

Nitrofurazone activity has been investigated as a monotherapy and in combination with Berenil and Trimelarsan. The treatment regimen and the percentage suppression in liver parasite load are given in Table 21 and Figure 24 (see also Figure 6). It can be seen that when it was given as 4 x 200 mg/kg (max. tolerated dose) there was a reduction in liver parasite load of about 25%. However, this reduction was not statistically significant. Berenil monotherapy again gave about 51% reduction ($F_{(5,65)}=10.5$, $P<0.05$), but when this was given in combination with the nitrofurazone this was reduced to less than 10%. Trimelarsan monotherapy gave a reduction in liver parasite load of 45%. There was also a marked decrease in

Fig. 23. Effect of buthionine sulfoximine, given at various dose levels, against L. donovani in Balb/c mice.



A: Buthionine sulfoximine: 4 x 10 mg/kg, three times daily
B: Buthionine sulfoximine: 4 x 20 mg/kg, three times daily
C: Buthionine sulfoximine: 4 x 30 mg/kg, three times daily
D: Buthionine sulfoximine: 4 x 40 mg/kg, three times daily

Table 21

The effect of nitrofurazone (5-nitro-2-furaldehyde semicarbazone), alone and in combination with Berenil and Trimelarsan, against <u>L. donovani</u> in mice.

TREATMENT REGIMEN

Infection → 13 days →

→ [N][N][N][N] → [K]

D14 D17 D21

DRUG	GROUP SIZE	% SUPPRESSION
Nitrofurazone	6	26.7 ± 24.3
Berenil	6	51.4 ± 22.3
Trimelarsan	6	45.0 ± 20.3

Infection (D0) → 13 days →

[*][*][*][*]

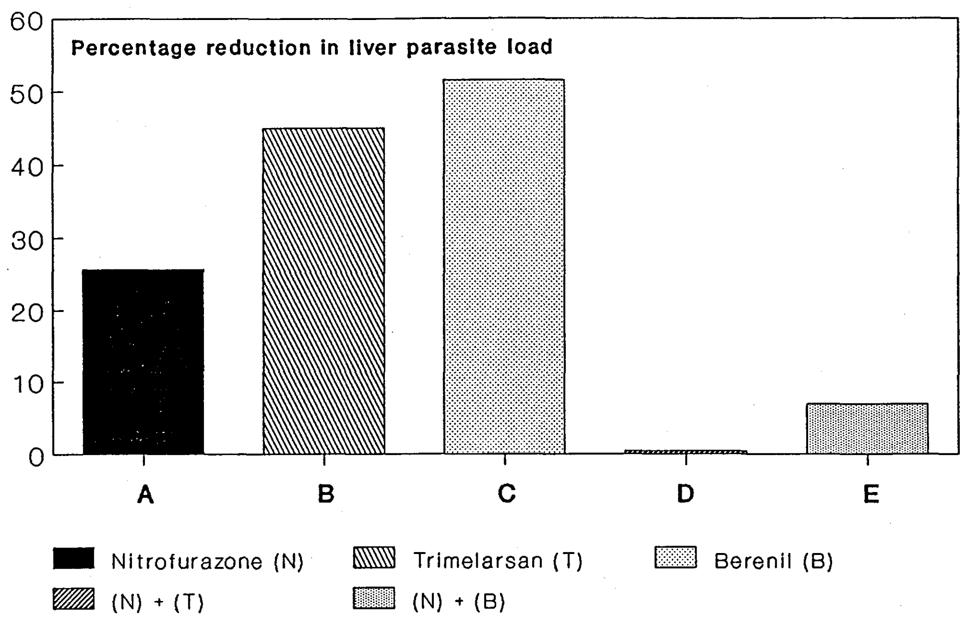
→ [N][N][N][N] → [K]

D14 D17 D21

DRUG COMBINATION	GROUP SIZE	% SUPPRESSION
Nitrofurazone + Berenil	4	6.8 ± 26.8
Nitrofurazone + Trimelarsan	5	0.5 ± 28.5

[N] : Nitrofurazone, 200 mg/kg [*] : Berenil, 40 mg/kg [*] : Trimelarsan, 30 mg/kg

Fig. 24. Effect of nitrofurazone, Trimelarsan and Berenil, individually and in combination, on L. donovani - infected Balb/c mice.



Nitrofurazone: 4 x 200 mg/kg Trimelarsan: 4 x 30 mg/kg Berenil: 4 x 40 mg/kg

efficacy of Trimelarsan when combined with nitrofurazone and the effect of Trimelarsan was completely reversed. The activities of these combinations were not significantly different when compared with the liver parasite load of controls. The above combination treatment regimens were also associated with mortality of some of the mice.

3.2.3.5. ANTIMONIAL COMPOUNDS

3.2.3.5.1. Sodium stibogluconate (Pentostam^{R})

3.2.4.5.1.(a) The effect of Pentostam$^{(R)}$ against L. donovani promastigotes and amastigotes

The in vitro activity of sodium stibogluconate (Pentostam^{R}) is shown in Tables 17 and 18, Figures 19 and 20. It can be seen (Table 17, Figure 19) that against promastigotes it was totally inactive. Against amastigotes growing in murine PECs (Table 18, Figure 20) showed some activity reducing the number of infected cells at concentrations of 5 μg/ml and above ($F_{(7,16)}$= 69.6, P<0.05) and the number of amastigotes/100 infected cells, at concentrations of 2.5 μg/ml and above ($F_{(7.16)}$= 13.9, P<0.05).

3.2.3.5.1.(b) In vivo activity of Pentostam$^{(R)}$

The treatment regimen and the percentage suppression in liver parasite load are given in Table 22 and Figure 6. It can be seen that a 40 to 93% reduction in liver parasite load can be achieved at dose levels ranging between 10-200 mg/kg ($F_{(8,99)}$=97.7, P<0.05). However, the results obtained after treatment at dose levels of 50 mg/kg and above were not significantly different to each other.

Table 22

The effect of Pentostam, given at various dose levels, against L. donovani in mice.*

TREATMENT REGIMEN

Infection (D0) → 13 days →
→ [P][P][P][P] → [K]
D12 D15 D19

DOSE	% SUPPRESSION
200 mg/kg	93.5 ± 3.5
150 mg/kg	92.9 ± 3.1
100 mg/kg	91.0 ± 4.3
75 mg/kg	82.9 ± 6.7
50 mg/kg	84.2 ± 7.0
25 mg/kg	66.2 ± 15.1
10 mg/kg	47.8 ± 21.2
5 mg/kg	3.5 ± 23.0

[P] : Pentostam (ip) [K] : Kill * Group size 6

3.2.4. DETECTION OF ADDITIVE OR SYNERGISTIC INTERACTIONS IN CHEMOTHERAPY BETWEEN COMPOUNDS THOUGHT TO INTERFERE WITH TRYPANOTHIONE METABOLISM IN VIVO AND IN VITRO

A main approach of this project was to evaluate combination therapy using compounds thought to interfere with trypanothione metabolism and for this reason, combination experiments involving nitroimidazoles and compounds known to interfere with trypanothione synthesis were carried out.

3.2.4.1. Effect of fexinidazole, DFMO, Pentostam(R), Berenil(R), and Trimelarsan(R), alone and in various combinations, against *L. donovani* promastigotes

Fexinidazole, DFMO, Pentostam, Berenil, and Trimelarsan have been tested, both alone and in various combinations, against *L. donovani* promastigotes in culture. This was carried out to see whether or not any of the compounds were synergistic against the parasite itself. The results, shown in Table 23 and Figure 25, are from one experiment, drugs were used at concentrations expected to give around 40% reduction in parasite numbers. It appeared that most of the compounds showed less than additive activity when in combination. There were no indications of synergistic effects. Trimelarsan apparently reduced the effect produced by the other drugs when it was used in combination. In no case were all the parasites killed.

3.2.4.2. Effect of fexinidazole, DFMO, Pentostam(R), Berenil(R), buthionine sulfoximine and Trimelarsan(R), alone and in various combinations, against *L. donovani* amastigotes growing in PECs

Experiments similar to those carried out with promastigotes were performed using the standard conditions for amastigote-initiated infections of PECs *in vitro*, as described in section 2.3.. The results of these studies are shown in Tables 18 and 24 and Figures 20 and 26. Of the compounds used, only Pentostam and Berenil showed

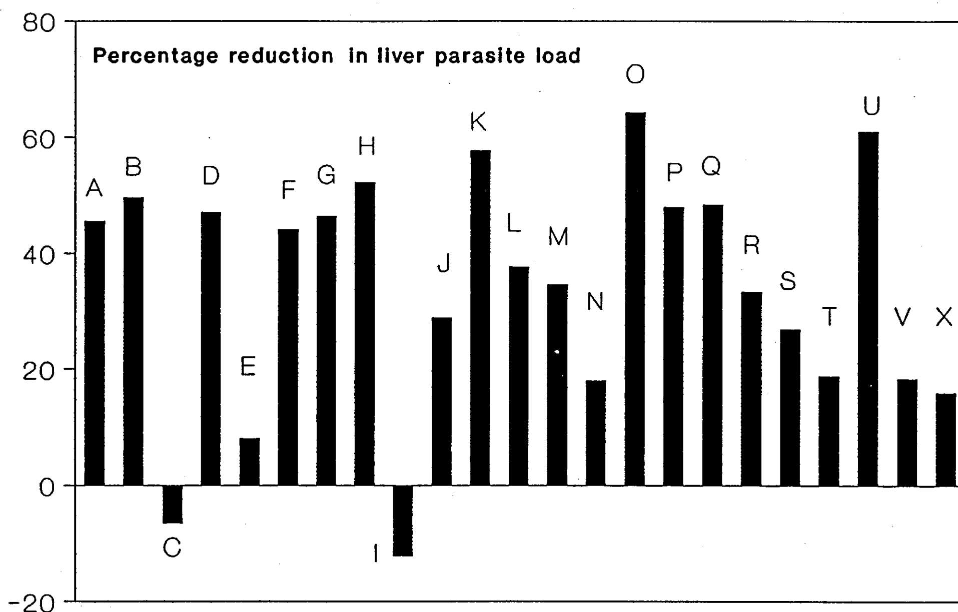
Table 23

The effect of DFMO, Pentostam, fexinidazole, Berenil and Trimelarsan, alone and in various combinations, against L. donovani promastigotes.

Drug / Drug Combination	% reduction compared to controls	Drug Combination	% reduction compared to control
[D]	49.6 ± 11.5	[B] + [F]	52.2 ± 3.0
[P]	-6.5 ± 17.2	[T] + [F]	-12.1 ± 19.0
[F]	45.5 ± 21.1	[D] + [F] + [B]	48.4 ± 13.2
[B]	47.0 ± 5.0	[B] + [P] + [F]	48.0 ± 18.4
[T]	8.0 ± 11.3	[D] + [F] + [T]	33.4 ± 17.2
[D] + [P]	34.6 ± 1.7	[B] + [F] + [T]	18.3 ± 8.6
[D] + [B]	64.2 ± 14.4	[B] + [F] + [T] + [P]	15.9 ± 1.7
[D] + [T]	57.7 ± 7.5	[D] + [F] + [B] + [T]	26.9 ± 15.5
[D] + [F]	44.1 ± 17.3	[D] + [P] + [B] + [F]	61.0 ± 9.2
[P] + [B]	37.7 ± 30.3	[D] + [B] + [F] + [T] + [P]	18.7 ± 20.0
[P] + [T]	17.9 ± 5.0		
[P] + [F]	46.4 ± 23.0	Mean number of parasites in controls x 10^4/ml ± SD	615.0 ± 312.7
[B] + [T]	28.8 ± 6.1		

[D] : DFMO (1.25 μg/ml) [F] : Fexinidazole (1.25 μg/ml)
[P] : Pentostam (100 μg/ml) [B] : Berenil (1.25 μg/ml) [T] : Trimelarsan (100 μg/ml)

Fig. 25. Effect of fexinidazole, DFMO, Berenil, Pentostam and Trimelarsan, alone and in various combinations, against L.donovani promastigotes.



A: Fexinidazole (1.25 µg/ml)
B: DFMO (1.25 µg/ml)
C: Pentostam (100 µg/ml)
D: Berenil (1.25 µg/ml)
E: Trimelarsan (100 µg/ml)
F: Fexinidazole + DFMO
G: Fexinidazole + Pentostam
H: Fexinidazole + Berenil
I: Fexinidazole + Trimelarsan
J: Berenil + Trimelarsan
K: Trimelarsan + DFMO
L: Berenil + Pentostam
M: Pentostam + DFMO
N: Trimelarsan + Pentostam
O: Berenil + DFMO
P: Berenil + Pentostam
Q: Fexinidazole + Berenil + DFMO
R: Fexinidazole + Trimelarsan + DFMO
S: Fexinidazole + Berenil + Trimelarsan + DFMO
T: Fexinidazole + Trimelarsan + Pentostam + DFMO + Berenil
U: Fexinidazole + Pentostam + Berenil + DFMO
V: Fexinidazole + Trimelarsan + Berenil
X: Fexinidazole + Pentostam + Berenil + Trimelarsan

good antileishmanial activity in this *in vitro* system, giving up to 85% at 100 μg/ml ($F_{(7,23)}$= 13.9, P<0.05) and 67% at 5 μg/ml ($F_{(4,13)}$=19.4, P<0.05) reduction of amastigotes respectively (Table 18, Figure 20). Berenil, however, proved to be toxic to macrophages at concentrations of 10 μg/ml or above. Toxicity to macrophages was also observed with fexinidazole (50-100 μg/ml), Trimelarsan (100 μg/ml) and buthionine sulfoximine (BSO) (50-100 μg/ml). DFMO was relatively inactive in this *in vitro* system. Table 24 and Figure 26 show the results from the drug combinations against the amastigotes in peritoneal exudate cells (PECs) at the end of the 5 day exposure period. The drug concentration used in each combination was the one that in monotherapy was found to give approximately 20% reduction of amastigotes compared with controls. Many of the combinations showed approximately additive effect. Exception were combinations involving Trimelarsan, which apparently reduced the effect produced by the other drug alone. Combinations involving five or more drugs proved toxic to macrophages. In no case were the parasites totally cleared.

3.2.4.3. Effect of DFMO, fexinidazole, Pentostam(R), Berenil(R) and Trimelarsan(R), alone and in various combinations, against *L. donovani* in mice

In order to maximise the detectability of synergistic and additive effects of the compounds used in combinations, it was decided to use relatively low drug concentrations in these combination experiments, such that each drug alone would be expected to give only approximately 20% reduction in liver parasite load. The compounds used were DFMO (1% soln. x 14 days), the 5-nitroimidazole, fexinidazole (4 x 10 mg/kg), Pentostam (4 x 5 mg/kg twice daily), Berenil (4 x 20 mg/kg) and Trimelarsan (4 x 20 mg/kg). The treatment regimen and the results produced, which are given in Table 25 and Figures 27a and 27b, showed that there was no more than an approximately additive effect in combinations involving DFMO, fexinidazole, Berenil and Pentostam. However, when Trimelarsan

Table 24

The effect of DFMO, Pentostam, fexinidazole, Berenil, Trimelarsan and BSO, alone and in various combinations, against L. donovani in PECs.

Drug / Drug Combination	Number of cells infected, % control	Control *	Amastigote number / 100 infected cells % control	Control *
[D]	91.4 ± 3.2	93.7 ± 6.1	77.9 ± 2.6	828.5 ± 88.9
[P]	93.2 ± 7.5	93.7 ± 6.1	76.5 ± 21.9	828.5 ± 88.9
[F]	99.6 ± 6.7	93.7 ± 6.1	65.4 ± 2.6	828.5 ± 88.9
[B]	80.5 ± 16.0	93.7 ± 6.1	74.1 ± 20.1	828.5 ± 88.9
[T]	98.2 ± 6.1	93.7 ± 6.1	81.9 ± 2.6	828.5 ± 88.9
[b]	87.6 ± 15.1	93.7 ± 6.1	76.1 ± 2.8	828.5 ± 88.9
[D] + [P]	99.8 ± 5.7	85.5 ± 2.1	60.4 ± 0.1	738.5 ± 33.3
[D] + [B]	102.3 ± 7.5	85.5 ± 2.1	72.6 ± 10.8	738.5 ± 33.3
[D] + [T]	105.9 ± 7.4	85.5 ± 2.1	106.4 ± 32.9	738.5 ± 33.3
[D] + [F]	100.0 ± 8.1	91.0 ± 7.0	55.4 ± 16.7	818.5 ± 113.9
[D] + [b]	102.5 ± 7.7	91.0 ± 7.0	59.8 ± 6.8	818.5 ± 113.9
[P] + [B]	90.1 ± 15.3	91.0 ± 7.0	44.6 ± 9.7	818.5 ± 113.9
[P] + [T]	97.0 ± 1.4	98.3 ± 2.0	79.3 ± 9.9	771.3 ± 140.9
[P] + [F]	78.0 ± 5.2	98.3 ± 2.0	48.7 ± 7.8	771.3 ± 140.9
[P] + [b]	81.7 ± 10.2	98.3 ± 2.0	53.0 ± 4.9	771.3 ± 140.9
[B] + [T]	96.6 ± 4.5	98.3 ± 2.0	101.3 ± 27.2	771.3 ± 140.9
[B] + [F]	92.6 ± 3.1	98.3 ± 2.0	54.4 ± 11.1	771.3 ± 140.9
[B] + [b]	93.6 ± 7.2	98.3 ± 2.0	58.9 ± 2.0	771.3 ± 140.9
[T] + [F]	97.2 ± 9.0	95.7 ± 5.7	78.6 ± 14.2	759.4 ± 32.5
[T] + [b]	93.0 ± 6.6	95.7 ± 5.7	60.2 ± 8.1	759.4 ± 32.5
[F] + [B]	82.0 ± 7.0	95.7 ± 5.7	66.1 ± 14.7	759.4 ± 32.5
[D] + [F] + [B]	99.6 ± 6.8	93.7 ± 6.4	56.0 ± 5.4	828.5 ± 88.9
[D] + [B] + [b]	105.9 ± 7.4	85.5 ± 2.1	67.5 ± 3.7	738.5 ± 33.3
[D] + [F] + [B] + [b]	97.7 ± 7.5	95.7 ± 5.7	57.8 ± 3.2	759.4 ± 32.5
[D] + [F] + [B] + [P]	51.6 ± 11.0	95.7 ± 5.7	33.0 ± 6.0	759.4 ± 32.5
[D] + [F] + [B] + [b] + [P]	61.1 ± 4.0	95.7 ± 5.7	35.0 ± 6.4	759.4 ± 32.5
[D] + [F] + [B] + [b] + [P] + [T]	**	98.3 ± 2.0	**	771.3 ± 140.9

* Mean ± SD ** Drug combination toxic to macrophages. Few parasites present

[D] DFMO 100 µg/ml [B] Berenil 1 µg/ml

[P] Pentostam 1.25 µg/ml [T] Trimelarsan 25 µg/ml

[F] Fexinidazole 5 µg/ml [b] Buthionine sulfoximine (BSO) 5 µg/ml

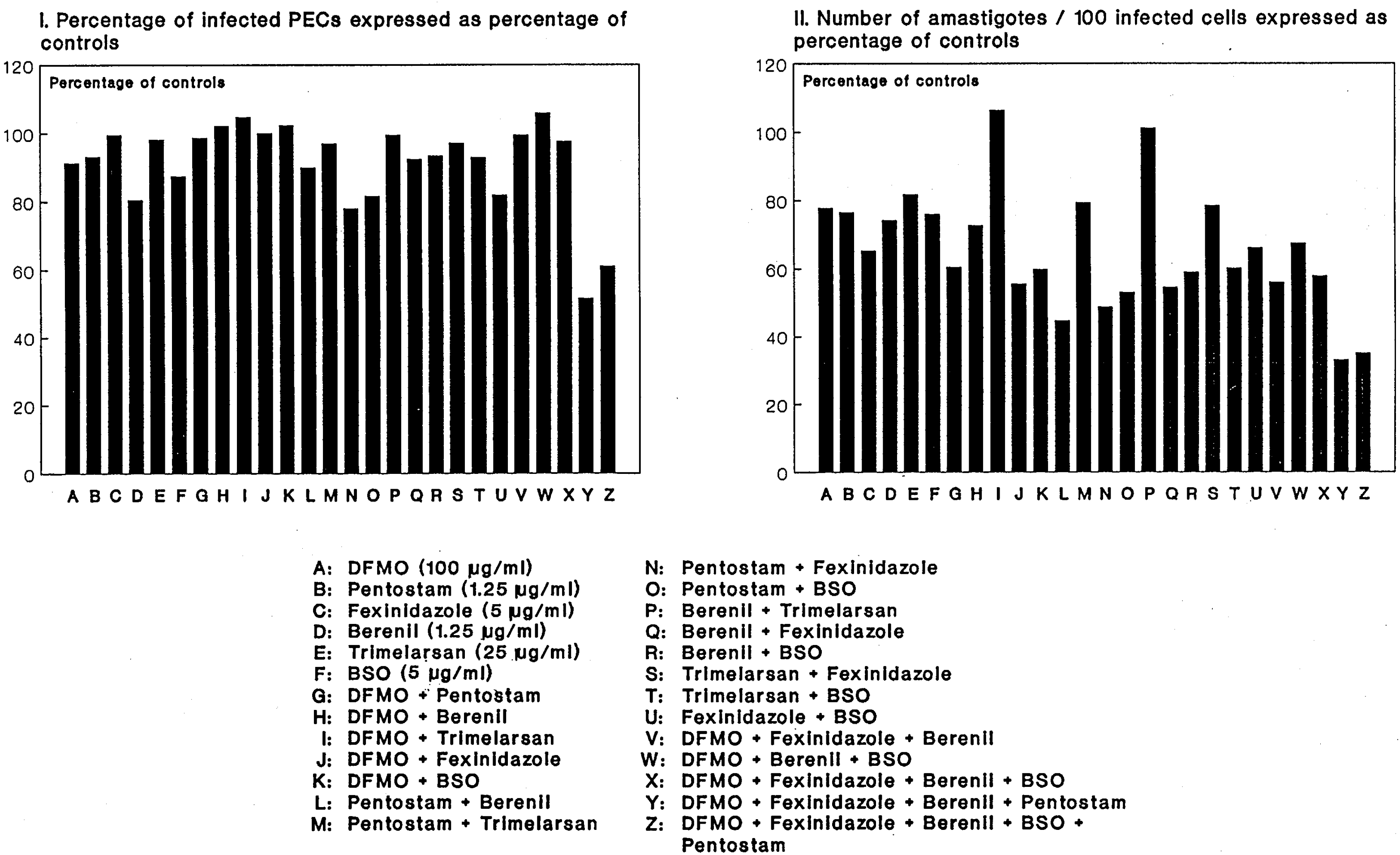


Fig. 26. Effect of Pentostam, DFMO, fexinidazole, Berenil, Trimelarsan and BSO, alone and in various combinations, against amastigotes of L.donovani in Balb/c mouse PECs.

Table 25

The effect of DFMO, fexinidazole, Pentostam, Berenil and Trimelarsan, individually and in various combinations, against L. donovani in mice.*

MONOTHERAPY

TREATMENT REGIMEN

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] → [K]
D6 D16 D19 D23

DRUG	% SUPPRESSION
DFMO [D]	21.3 ± 12.7

Infection → 15 days → [P P P P / P P P P] → [K]
D0 D16 D19 D23

DRUG	% SUPPRESSION
Pentostam [P]	25.8 ± 12.1

Infection → 15 days → [□ □ □ □] → [K]
D0 D16 D19 D23

DRUG	% SUPPRESSION
Fexinidazole □	27.9 ± 14.5
Berenil □	35.9 ± 17.8
Trimelarsan □	31.5 ± 17.6

DUAL COMBINATIONS

Infection (D0) → 15 days → [□ □ □ □ / □ □ □ □] → [K]
D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Berenil □ + Fexinidazole □	48.7 ± 8.8
Trimelarsan □ + Fexinidazole □	28.0 ± 8.6
Berenil □ + Trimelarsan □	34.6 ± 16.1

Infection (D0) → 15 days → [P P P P / P P P P] → [K]
D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + Fexinidazole □	40.8 ± 14.7
Pentostam [P] + Berenil □	55.7 ± 24.1
Pentostam [P] + Trimelarsan □	23.8 ± 16.8

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with □ □ □ □ D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
DFMO [D] + Fexinidazole □	46.2 ± 8.2
DFMO [D] + Berenil □	38.4 ± 10.0
DFMO [D] + Trimelarsan □	33.8 ± 11.4

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with P P P P / P P P P D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + DFMO [D]	40.8 ± 14.7

MULTIPLE COMBINATIONS

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with F F F F / B B B B D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
DFMO [D] + Fexinidazole [F] + Berenil [B]	36.8 ± 12.3

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with P P P P / F F F F / P P P P D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + DFMO [D] + Fexinidazole [F]	60.6 ± 12.6

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with T T T T / P P P P / F F F F / P P P P D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + DFMO [D] + Fexinidazole [F] + Trimelarsan [T]	37.9 ± 11.9

Infection (D0) → 6 days → [D D D D D D D D D D D D D D] (with F F F F / P P P P / B B B B / P P P P D16–D19) → [K]
D6 D16 D19 D23

DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + DFMO [D] + Fexinidazole [F] + Berenil [B] +	78.9 ± 8.6

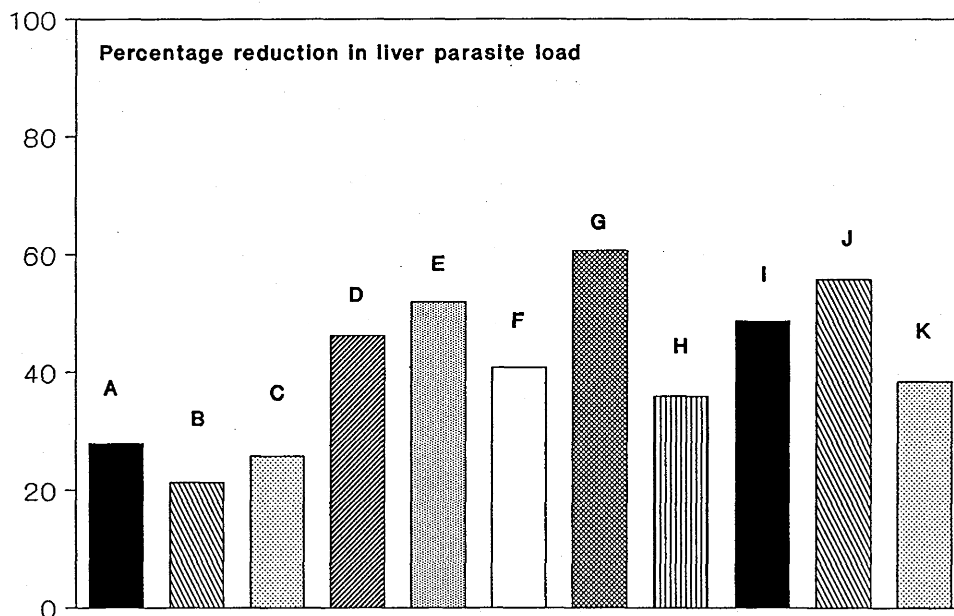
Contd.

Table 25 (contd.)

MULTIPLE COMBINATIONS (contd.)	
Infection (D0) → 6 days → D6–D23: D (DFMO) daily D6 to D19; P, B, P, F, T on D16 to D19 → K (D23)	
DRUG COMBINATION	% SUPPRESSION
Pentostam [P] + DFMO [D] + Fexinidazole [F] + Berenil [B] + Trimelarsan [T]	33.4 ± 12.9

[ ] Berenil 10 mg/kg [ ] Trimelarsan 20 mg/kg [ ] Fexinidazole 10 mg/kg
[P] Pentostam 5 mg/kg, 1000 hrs & 1500 hrs each day [K] Kill *Group size 6
[D] DFMO 1% soln. in drinking water. Mice kept under 4 hours dark, 2 hours light regimen

Fig. 27a. The effect of DFMO, fexinidazole, Pentostam and Berenil, alone and in various combinations on L. donovani - infected Balb/c mice.



A: Fexinidazole (4 x 10 mg/kg)

B: DFMO 1% soln in drinking water

C: Pentostam (5 mg/kg twice daily for 4 days)

D: DFMO + Fexinidazole

E: DFMO + Pentostam

F: Pentostam + Fexinidazole

G: DFMO + Pentostam + Fexinidazole

H: Berenil (4 x 20 mg/kg)

I: Berenil + Fexinidazole

J: Berenil + Pentostam

K: Berenil + DFMO

Combinations significantly different to each other, at the .05 level:

(B) different to (G), (K), (E), (I), (D) and (F), ($F_{(9,108)}$= 10.5)

(A) different to (G), (K), (E), (I) and (D), ($F_{(9,108)}$= 10.5)

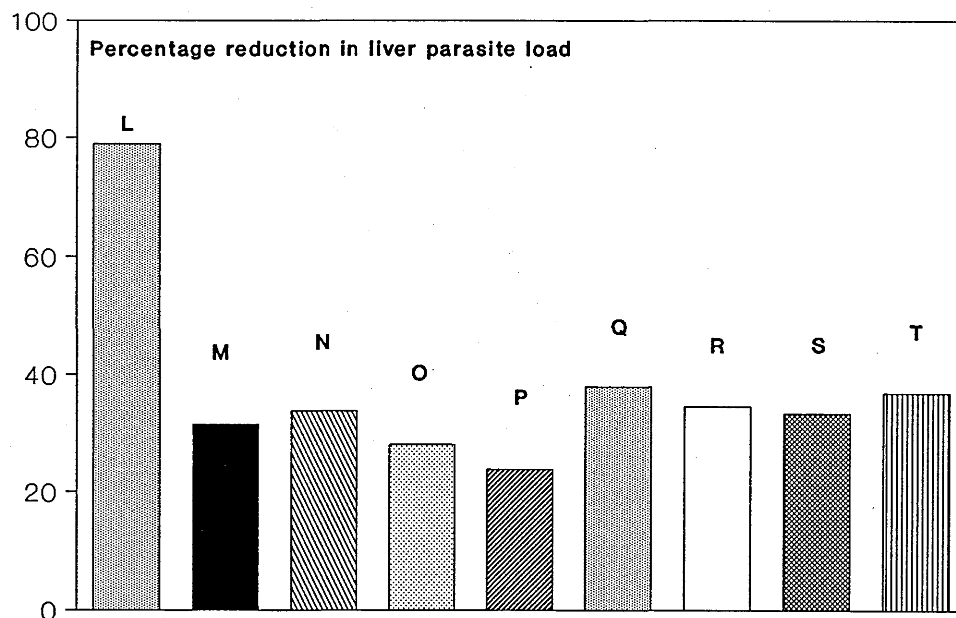
(C) different to (G), (K), (E), (I), (D), (J), (L) and (E), ($F_{(9,108)}$= 10.5)

(F) different to (G), ($F_{(9,108)}$= 10.5)

(H) different to (G), (J) and (L), ($F_{(7,86)}$= 4.7)

(K) different to (G), (J), (L) and (F), ($F_{(7,86)}$= 4.7)

Fig. 27b. The effect of DFMO, Trimelarsan, fexinidazole, Berenil and Pentostam, in various combinations, on L. donovani - infected Balb/c mice.



L: DFMO + Fexinidazole + Pentostam + Berenil

M: Trimelarsan (4 x 20 mg/kg)

N: DFMO 1% soln + Trimelarsan

O: Trimelarsan + Fexinidazole (4 x 10 mg/kg)

P: Trimelarsan + Pentostam (4 x 5 mg/kg, Twice Daily)

Q: Pentostam + Fexinidazole + DFMO + Trimelarsan

R: Trimelarsan + Berenil (4 x 20 mg/kg)

S: DFMO + Pentostam + Fexinidazole + Trimelarsan + Berenil

T: Berenil + DFMO + Fexinidazole

Combinations significantly different to each other at the .05 level:

(L) different to (P), (O), (M), (S), (N), (R), (Q) and (T) ($F_{(9,106)}$= 25.4).

was used, it appeared to reduce the effect produced by all the other drugs. The overall conclusion from these experiments was that although combinations involving DFMO, fexinidazole, Berenil and Pentostam have interesting activity against L. donovani in mice, they were not apparently synergistic.

Thus, the results at this stage had shown that the combinations were less effective against L. donovani than had been hypothesised. There are several possible explanations for this and the potential of drug combinations as antileishmanials had been pursued in other ways.

3.3. IN VIVO EXPERIMENTS INVOLVING THE DRUGS OF CURRENT USE, SODIUM STIBOGLUCONATE (PENTOSTAM^R) AND PENTAMIDINE (LOMIDINE^R)

Sodium stibogluconate and pentamidine were tested in vivo in some detail, by using them individually and in combination experiments. It was hoped that in this way it would be possible to maximise the effectiveness of drugs already available for use in humans.

3.3.1. Effect of sodium stibogluconate (Pentostam^R) and pentamidine (Lomidine^R) on liver parasite load, after 5, 6, 7 and 8 days of administration, against L. donovani in mice

The results of this experiment are detailed in Table 26 and Figure 28. The parasite loads of the two control groups, as determined on day 27 and day 29 after infection, were not significantly different. Pentamidine, at the dose level and length of treatment used, had no effect on liver parasite load and showed toxic side effects, particularly with prolonged treatment. Pentostam itself produced a 60-87% reduction in liver parasite load ($F_{(8,85)}=99.8$, $P<0.05$). Under the conditions used, combination of Pentostam with pentamidine did not result in any additional effect over that produced by Pentostam alone.

Table 26

The effect of Pentostam[**] and pentamidine[***] on liver parasite load, after 5, 6, 7 and 8 days of administration, against L. donovani in mice.[*]

TREATMENT REGIMEN

Infection (D0) → 18 days → [DRUG for 5 days: D19–D23] → K (D27)

DRUG	% SUPPRESSION
Pentostam	58.8 ± 6.7
Pentamidine	0.1 ± 16.3

Infection (D0) → 18 days → [DRUG for 6 days: D19–D24] → K (D27)

DRUG	% SUPPRESSION
Pentostam	61.1 ± 7.8
Pentamidine	3.6 ± 13.1

Infection (D0) → 18 days → [DRUG for 7 days: D19–D25] → K (D29)

DRUG	% SUPPRESSION
Pentostam	69.3 ± 10.3
Pentamidine	****

Infection (D0) → 18 days → [DRUG for 8 days: D19–D26] → K (D29)

DRUG	% SUPPRESSION
Pentostam	87.4 ± 5.8
Pentamidine	****

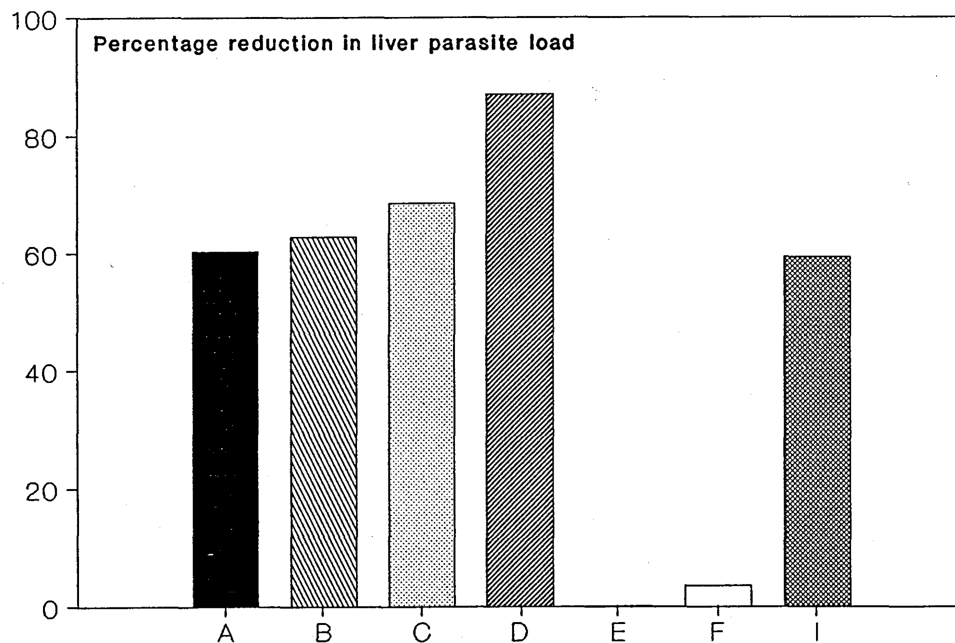
Infection (D0) → 18 days → [Pentostam for 5 days: D19–D23] → one day interval → [pentamidine for 2 days: D24–D26] → K (D29)

DRUG COMBINATION	% SUPPRESSION
Pentostam + pentamidine	60.2 ± 19.4

K : Kill   * Group size 6   **** All the mice died during treatment

** 5 mg/kg TWICE DAILY *** 25 mg/kg TWICE DAILY, with the exception of the first day of treatment when it was given only once.

Fig. 28. Effect of Pentostam (5 mg/kg, twice daily) and pentamidine (25 mg/kg, twice daily), given for different lengths of treatment time (5, 6, 7, 8, days), against L. donovani in Balb/c mice.



A: Pentostam 5 x 5 mg/kg twice daily
B: Pentostam 6 x 5 mg/kg twice daily
C: Pentostam 7 x 5 mg/kg twice daily
D: Pentostam 8 x 5 mg/kg twice daily
E: Pentamidine 1 x 25 mg/kg + 4 x 25 mg/kg twice daily
F: Pentamidine 1 x 25 mg/kg + 5 x 25 mg/kg twice daily
G: Pentamidine 1 x 25 mg/kg + 6 x 25 mg/kg twice daily *
H: Pentamidine 1 x 25 mg/kg + 7 x 25 mg/kg twice daily *
I: Pentostam 5 x 5 mg/kg twice daily + Pentamidine 2 x 25 mg/kg

* All the mice died before the completion of treatment

3.3.2. Effect of sodium stibogluconate (Pentostam^R), pentamidine (Lomidine^R) and DFMO, on liver parasite load, alone and in combination, against L. donovani in mice

Another possibility that was investigated was the combination of DFMO with Pentostam^R and pentamidine. These results are shown in Table 27 and Figure 29. Pentamidine did not significantly reduce the liver parasite load and in combination with DFMO did not produce any additional effect over that produced by DFMO itself. When in combination with Pentostam, it reduced the effect produced by the other drug alone. In contrast, Pentostam gave a 25% reduction when tested alone and the combination of Pentostam with DFMO was additive in effect ($F_{(9,110)}=13.7$, $P<0.05$). Surprisingly, when pentamidine was combined together with Pentostam and DFMO, this resulted in reduction of the effect produced by the combination of the latter two drugs.

3.3.3. Effect of sodium stibogluconate (Pentostam^R), pentamidine (Lomidine^R) and buthionine sulfoximine (BSO), on liver parasite load, alone and in combination, against L. donovani in mice

These results are shown in Table 27 and Figure 30. It can be seen that a 50% reduction in liver parasite load was achieved when buthionine sulfoximine was used alone with a dose regimen involving 3 daily doses (see also section 3.2.3.3.b). However, in combination with DFMO and pentamidine, a decrease in its efficacy was observed ($F_{(9,110)}=13.7$, $P<0.05$).

Table 27

The effect of DFMO, Pentostam, pentamidine and buthionine sulfoximine (BSO), individually and in combination, against L. donovani in mice.

TREATMENT REGIMEN	DRUG / DRUG COMBINATION	% SUPPRESSION
Infection (D0) → 6 days → D D D D D D D D D D D D D D (D6 – D19) → K (D23)	DFMO	21.3 ± 11.7
Infection (D0) → 15 days → P P P P / P P P P (D16 – D19) → K (D23)	Pentostam	25.8 ± 12.1
Infection (D0) → 15 days → p p p p (D16 – D19) → K (D23)	Pentamidine	8.1 ± 14.1
Infection (D0) → 15 days → b b b b / b b b b / b b b b (D16 – D19) → K (D23)	BSO	49.8 ± 9.8
Infection (D0) → 6 days → D D D D D D D D D D D D D D (D6 – D19); P P P P / P P P P (D16 – D19) → K (D23)	Pentostam + DFMO	52.0 ± 6.6
Infection (D0) → 6 days → D D D D D D D D D D D D D D (D6 – D19); p p p p (D16 – D19) → K (D23)	DFMO + pentamidine	23.4 ± 11.5
Infection (D0) → 6 days → D D D D D D D D D D D D D D (D6 – D19); P P P P / p p p p / P P P P (D19 – D19) → K (D23)	Pentostam + DFMO + pentamidine	26.1 ± 11.4
Infection (D0) → 15 days → P P P P / p p p p / P P P P (D16 – D19) → K (D23)	Pentostam + pentamidine	17.2 ± 14.2
Infection (D0) → 6 days → D D D D D D D D D D D D D D (D6 – D19); b b b b / b b b b / b b b b / p p p p (D16 – D19) → K (D23)	DFMO + pentamidine + BSO	42.4 ± 11.5

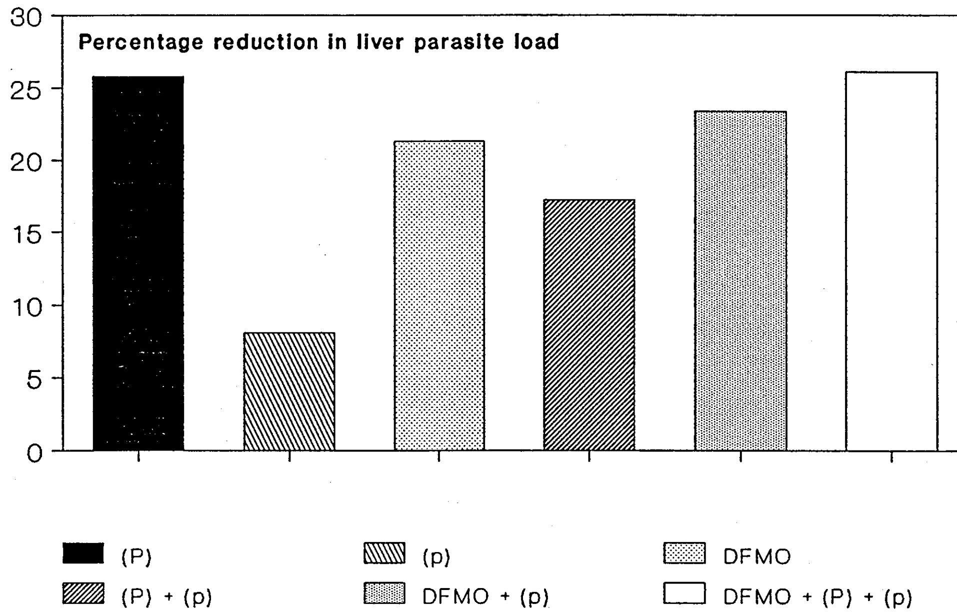
[p] pentamidine 25 mg/kg

[P] Pentostam 5 mg/kg, 1000 hrs & 1500 hrs each day [K] Kill * Group size 6

[D] DFMO 1% soln. in drinking water. Mice kept under 4 hours dark, 2 hours light regimen

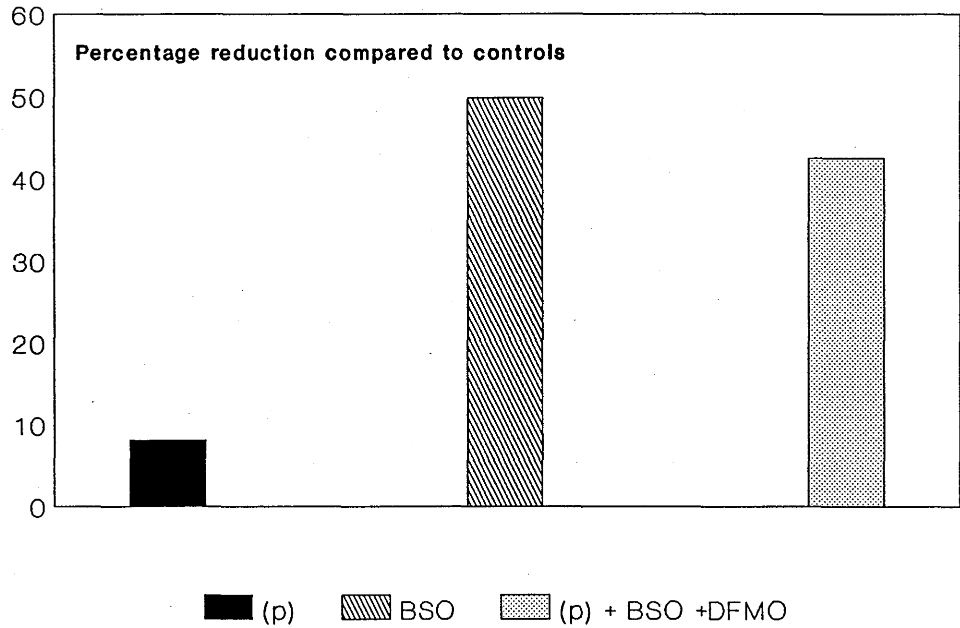
[b] BSO 10 mg/kg 1000 hrs, 1300 hrs & 1500 hrs each day

Fig. 29. Effect of DFMO, Pentostam and pentamidine, individually and in combination, against L. donovani in Balb/c mice.



Pentostam (P) : 4 x 5 mg/kg Twice Daily pentamidine (p) : 4 x 25 mg/kg
DFMO: 1% soln. in drinking water for 14 days

Fig. 30. Effect of buthionine sulfoximine, alone and in combination with pentamidine and DFMO, against L. donovani in Balb/c mice.



DFMO 1% soln. in drinking water for 14 days pentamidine (p) : 4 x 25 mg/kg
buthionine sulfoximine (BSO) : 4 x 10 mg/kg Three Times Daily

3.4. A STUDY ON THE PREVENTION OF THE RELAPSE OF VISCERAL LEISHMANIASIS IN THE MOUSE MODEL, USING COMPOUNDS THOUGHT TO INTERFERE WITH TRYPANOTHIONE METABOLISM

One of the drawbacks of the currently used drugs in the chemotherapy of visceral leishmaniasis, is the relapse of the disease after an apparently successful treatment. This is believed to be due to the fact that parasites located in sites other than the liver, such as the spleen and the bone marrow, are difficult to eradicate. Thus, it was of interest to test the efficacy of the new compounds and combinations in clearing the parasites not only from liver but also from spleen and bone marrow. In order to test this possibility it was decided first to develop an experimental relapse model of leishmaniasis in the Balb/c mouse which could be used to test the efficacy of the most promising drug combinations. Particular attention was given to DFMO to assess the potential of the drug for preventing relapse of visceral leishmaniasis. The results of this experiment are described in subsection 3.2.3.1.(b).

3.4.1. Development of a relapse model in Balb/c mice

The treatment regimen and the results produced are given in Table 28 and Figure 31. It can be seen that treatment with sodium stibogluconate solution (Pentostam^{R}) greatly reduced the number of parasites found in liver but to a lesser extent the parasite load in the spleen and bone marrow, as determined on day 23 after infection (DAI 23). In general, in the Pentostam-treated groups, there was a progressive increase in parasite load between DAI 51 and and DAI 80 in all organs screened other than the bone marrow, with the number of parasites present in the spleen and bone marrow not being significantly different from the numbers in the controls on DAI 51 and DAI 80. One way and two way analysis of data and the Neuman Keuls multiple range test were used to analyse the data and the results from this analysis are given below.

Results from liver: The % suppression obtained on DAI 51,

Table 28

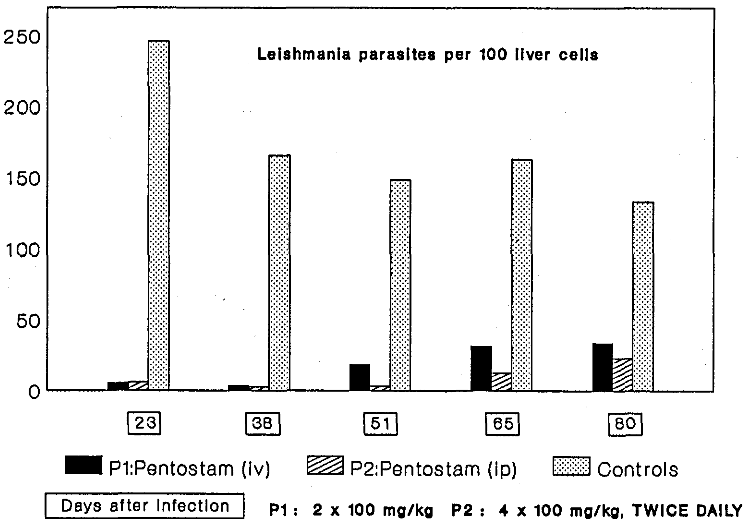
The effect of Pentostam (iv/ip) against L. donovani in mice[a], after 23, 38, 51, 65, and 80 days of infection on liver, spleen and bone marrow parasite load.

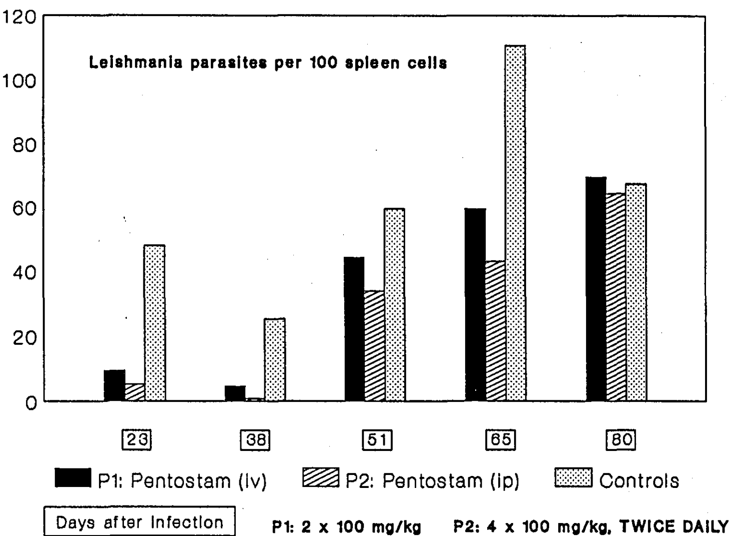
TREATMENT REGIMEN		% SUPPRESSION		
		LIVER	SPLEEN	B. MARROW
Infection (D0) → 16 days → Pentostam iv/ip (D16 – D17/D19) → [K] D23	Pentostam (iv) *	97.8 ± 0.7	80.3 ± 12.3	66.3 ± 20.8
	Pentostam (ip) **	97.6 ± 0.5	88.8 ± 6.6	50.9 ± 26.9
Infection (D0) → 16 days → Pentostam iv/ip (D16 – D17/D19) → [K] D38	Pentostam (iv) *	97.7 ± 2.5	81.6 ± 16.5	-17.4 ± 58.1
	Pentostam (ip) **	98.3 ± 1.5	96.6 ± 4.9	78.5 ± 17.8
Infection (D0) → 16 days → Pentostam iv/ip (D16 – D17/D19) → [K] D51	Pentostam (iv) *	87.4 ± 8.9	25.7 ± 45.6	22.4 ± 38.2
	Pentostam (ip) **	97.6 ± 1.2	43.1 ± 32.6	82.8 ± 12.8
Infection (D0) → 16 days → Pentostam iv/ip (D16 – D17/D19) → [K] D65	Pentostam (iv) *	80.5 ± 4.1	46.2 ± 53.8	65.0 ± 29.2
	Pentostam (ip) **	92.1 ± 4.5	61.0 ± 21.1	62.1 ± 11.2
Infection (D0) → 16 days → Pentostam iv/ip (D16 – D17/D19) → [K] D80	Pentostam (iv) *	74.7 ± 12.3	-2.8 ± 33.5	-14.6 ± 60.3
	Pentostam (ip) **	82.7 ± 9.5	4.69 ± 32.6	53.0 ± 34.3

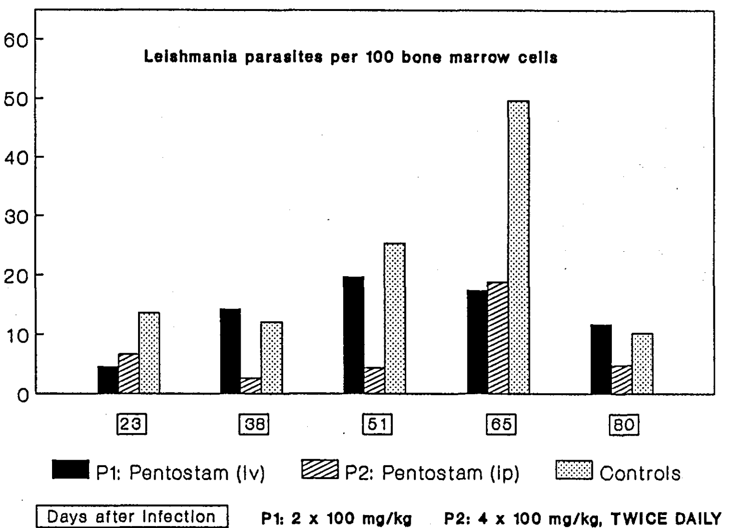
*Pentostam iv : 2 x 100 mg/kg, **Pentostam ip : 4 x 100 mg/kg TWICE DAILY

[K] : Kill [a] Group size :4 for D23 & D38, 5 for D51, D65 and D80

Fig. 31. Development of a relapse model of leishmaniasis in the Balb/c mouse: Effect of Pentostam given iv and ip, 16 and 19 days after infection (DAI), on liver, spleen, and bone marrow parasite load, 23, 38, 51, 65, and 80 DAI.







DAI 65 and DAI 80 with ip treatment was significantly higher than that obtained after iv treatment ($F_{(4,82)}=24.5$, $P<0.05$). Iv and ip treatment effects were significantly different when compared with the untreated controls ($F_{(9,82)}=34.3$, $F_{(9,84)}=40.8$, $P<0.05$). Interestingly, an increase in the number of parasites was observed on day 51 after infection in the sodium stibogluconate (Pentostam^R) treated groups.

Results from spleen: The percentage suppression resulted with ip treatment was significantly higher than that obtained with iv treatment ($F_{(1,80)}=5.1$, $P<0.05$). Iv and ip parasite loads determined on DAI 23 and DAI 38, were significantly different when compared with the untreated controls ($F_{(9,18)}=14.6$, $F_{(9,82)}=18.7$, $P<0.05$), but they reached the control levels on DAI 51 and onwards.

Results from bone marrow: The percentage suppression resulted with ip treatment was significantly higher than that obtained with iv treatment ($F_{(1,67)}=8.58$, $P<0.05$). Treatment effects were significantly different when compared with the untreated controls on DAI 51 (ip) and DAI 65 (iv, ip) ($F_{(9,68)}=10.7$, $F_{(9,70)}=19.1$, $P<0.05$).

In most organs of control groups screened, there was an increase in parasite load after DAI 51. An exception was the parasite load in liver, where a decrease was observed.

3.4.2. The effect of DFMO, sodium stibogluconate (Pentostam^R), fexinidazole, diminazene diaceturate (Berenil^R) and buthionine sulfoximine (BSO) in combination, against L. donovani in mice: Effect on liver, spleen and bone marrow parasite load

In this experiment the drugs were used at high concentrations in order to test the potential of the combinations to remove all parasites and prevent relapse. The treatment regimen and the results from use of the drug combinations are given in Table 29 and Figures 32, 33, 34 and 35. It can be seen that these combinations reduced significantly the number of parasites, giving up to 99% reduction in parasite load, but without giving a total clearance of parasites

Table 29

The effect of DFMO, Pentostam, fexinidazole, Berenil and buthionine sulfoximine (BSO) in combination, against L. donovani in mice*. Effect on liver, spleen and bone marrow parasite load on day 23 and 39 or 70 after infection.

TREATMENT REGIMEN	Kill	% SUPPRESSION LIVER	SPLEEN	B. MARROW
Pentostam + DFMO				
Infection (D0) → 5 days → D6–D19: D daily (D6–D19); P, P twice daily (D16–D19)	K D23:[a]	99.3 ± 0.7	94.7 ± 4.8	93.9 ± 2.8
	K D39:[a]	98.6 ± 1.9	55.2 ± 49.3	94.2 ± 2.8
DFMO + BSO + Fexinidazole				
Infection (D0) → 5 days → D6–D19: D daily (D6–D19); b, b, F, b daily (D16–D19)	K D23:	54.2 ± 18.0	78.3 ± 16.7	83.2 ± 8.7
	K D39:[b]	81.5 ± 12.9	32.5 ± 35.4	81.3 ± 58.3
DFMO + BSO + Berenil				
Infection (D0) → 5 days → D6–D19: D daily (D6–D19); b, b, B, b daily (D16–D19)	K D23:	66.5 ± 13.8	62.6 ± 30.0	59.3 ± 40.6
	K D39:[c]	99.7 ± 0.5	4.1 ± 12.6	94.4 ± 26.3
BSO + Fexinidazole + Berenil				
Infection (D0) → 5 days → D16–D19: b, b, B, F, b daily	K D23:	88.6 ± 7.8	91.6 ± 5.8	85.4 ± 5.5
	K D70:	90.5 ± 11.5	13.8 ± 16.2	66.0 ± 21.7

P Pentostam 5 mg/kg, 1000 hrs & 1500 hrs each day

D DFMO 2% soln. in drinking water: 1. x 14 days (D6-D19, mice sacrificed on D23),
2. x 30 days (D6-D35, mice sacrificed on D39),

Mice kept under 4 hours dark, 2 hours light regimen.

b BSO 10 mg/kg 1000 hrs, 1300 hrs & 1500 hrs each day

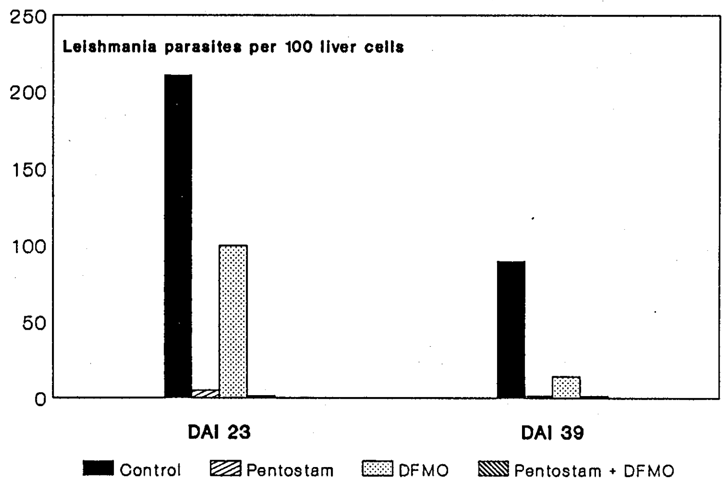
F Fexinidazole 200 mg/kg, 1000 hrs each day

B Berenil 40 mg/kg, 1000 hrs each day

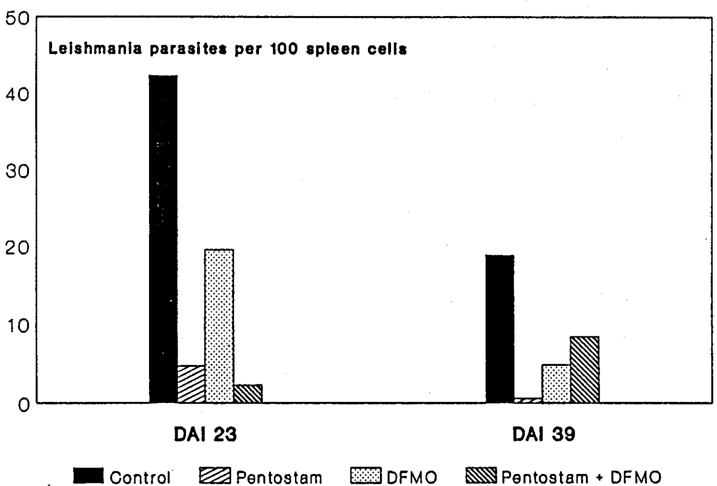
K Kill * Group size 6, a: 5 & b, c: 4 a & c: bacterial infection

Fig.32. The effect of DFMO (4% soln. x 14 days, DAI 6-DAI 19, x 30 days, DAI 6-DAI 35) in combination with Pentostam (4 X 100 mg/kg, TWICE DAILY, DAI 16-DAI 19), on liver, spleen and bone marrow parasite load, on DAI 23 and DAI 39.

Liver



Spleen



Bone Marrow

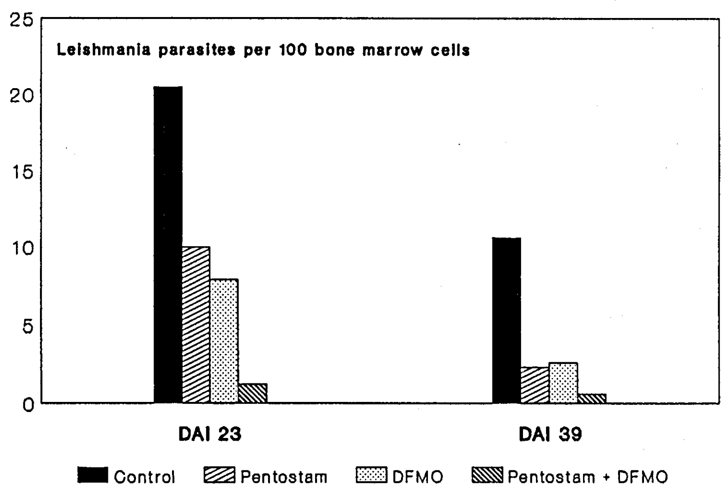
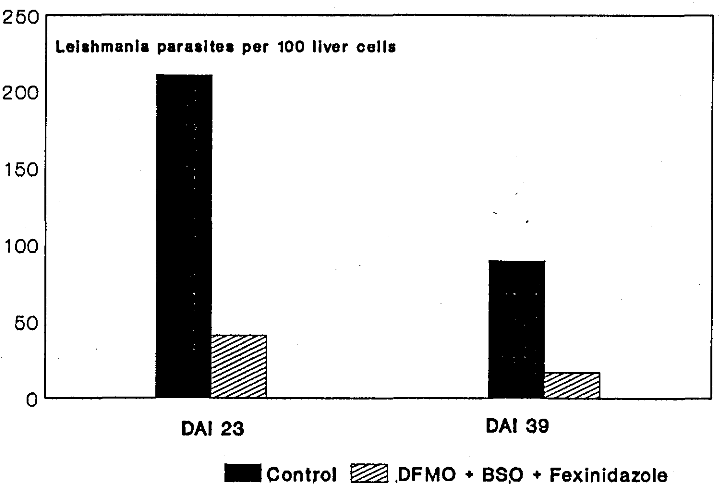
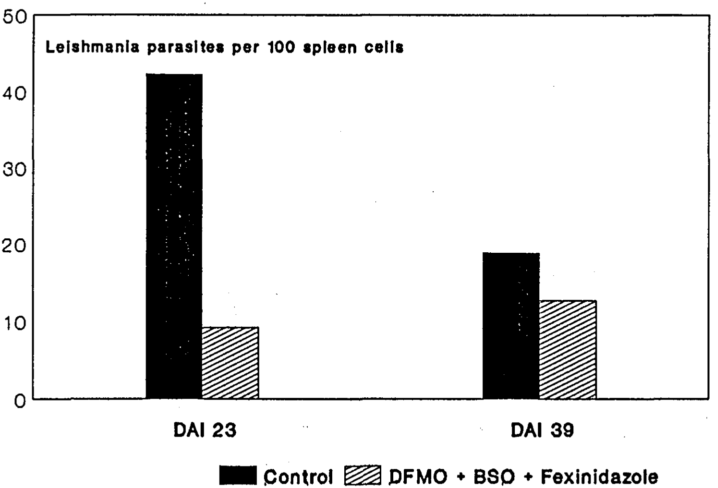


Fig.33. The effect of DFMO, (4% soln. x 14 days, DAI 6-DAI 19, x 30 days, DAI 6-DAI 35), BSO (4 x 20 mg/kg, THREE TIMES DAILY, DAI 16-DAI 19), and fexinidazole, (4 x 200 mg/kg, DAI 16 -DAI 19) in combination, on liver, spleen and bone marrow parasite load, on DAI 23 and DAI 39.





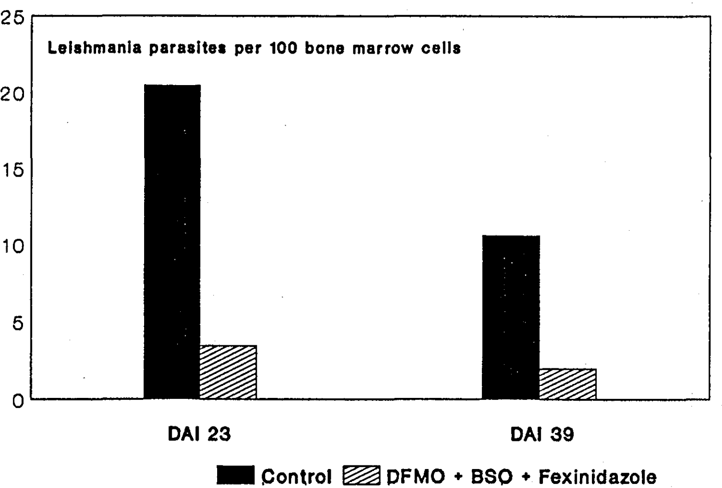
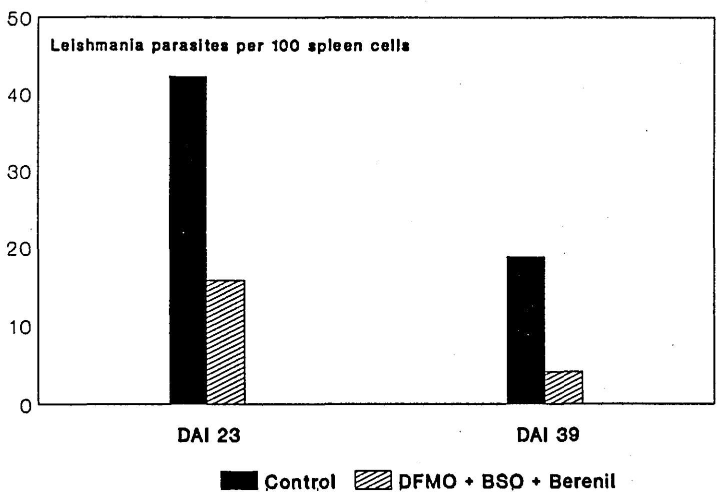
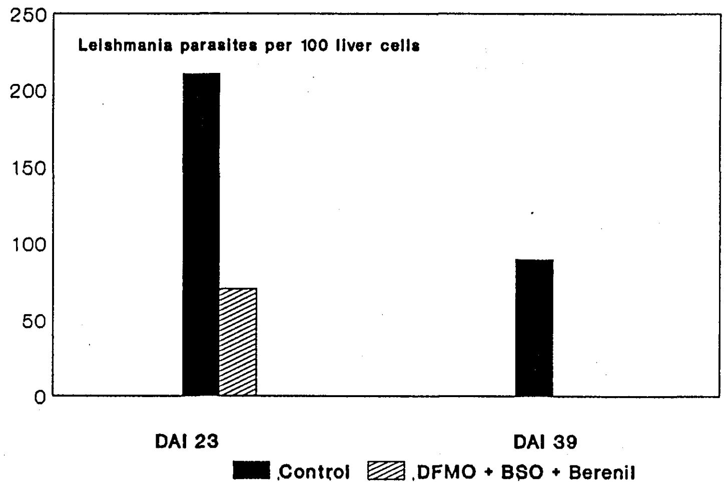


Fig. 34. The effect of DFMO (4% soln. x 14 days, DAI 6-DAI 19, x 30 days, DAI 6-DAI 35), BSO (4 x 40 mg/kg, THREE TIMES DAILY, DAI 15-DAI 19) and Berenil (4 x 40 mg/kg, DAI 15-DAI 19) in combimation, on liver, spleen and bone marrow parasite load, on DAI 23 and DAI 39.





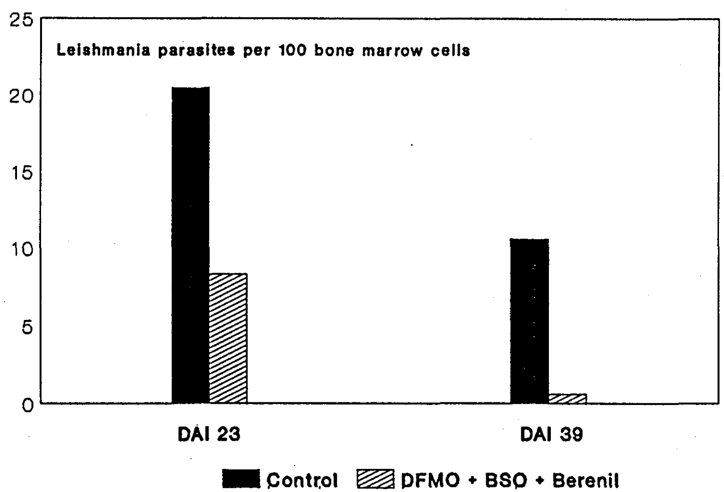
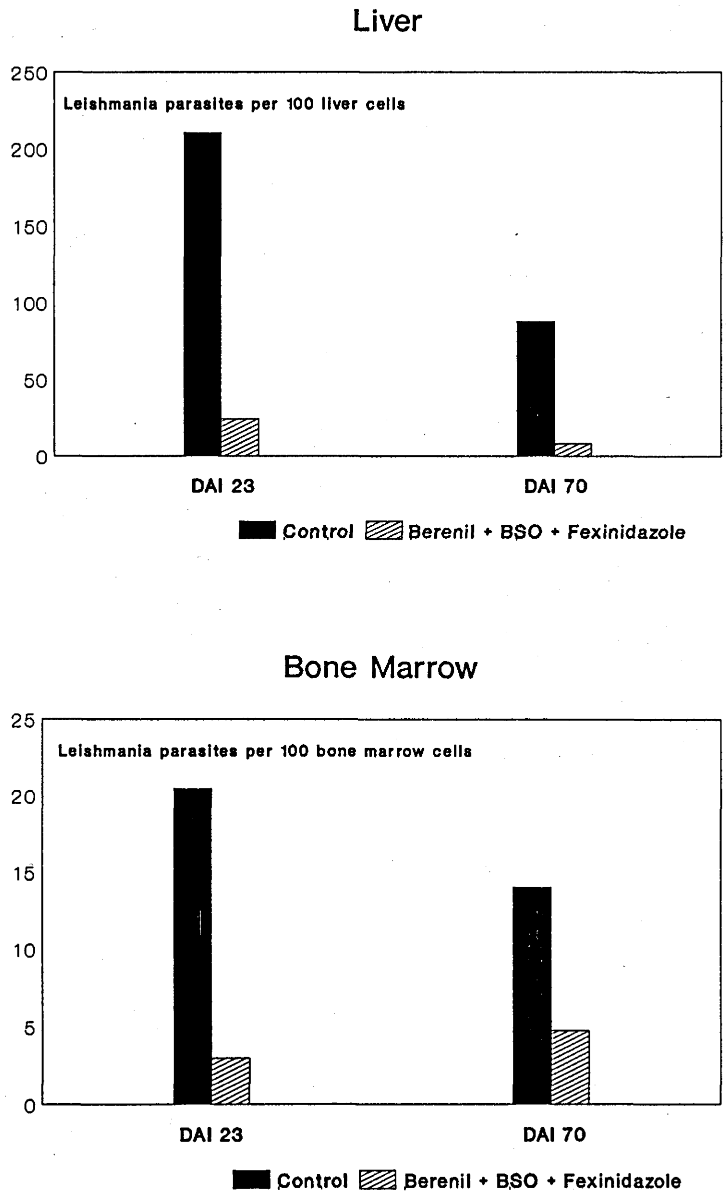


Fig. 35. The effect of Berenil (4 x 40 mg/kg, DAI 16-DAI 19, BSO (4 x 20 mg/kg, THREE TIMES DAILY, DAI 16-DAI 19) and fexinidazole (4 x 200 mg/kg, DAI 16-DAI 19), in combination, on liver, spleen and bone marrow parasite load, on DAI 23 and DAI 70.



Spleen

Leishmania parasites per 100 spleen cells

60
50
40
30
20
10
0

DAI 23
DAI 70

Control
Berenil + BSO + Fexinidazole

present in the tissues tested. The best results were obtained with the combination of Pentostam with DFMO. The data were analysed with two way analysis of variance and the Neuman Keuls multiple range test and the results of this analysis are given below.

Effect on liver: Liver parasite loads determined on day 23 after infection (DAI 23) were significantly different to those determined on DAI 39 or DAI 70 ($F_{(3,77)}$=11.8, P<0.05). Also, the effects of the combinations were significantly different to each other ($F_{(3,77)}$=18.9, P<0.05), with the exception of the combination involving DFMO, BSO and fexinidazole which was similar in effect to the combination of DFMO, BSO and Berenil.

Effect on spleen: Spleen parasite loads determined on DAI 23, DAI 39 and DAI 70, were not significantly different from each other. Of the combinations tested, only that involving DFMO, BSO and Berenil was different in effect when compared with the others ($F_{(3,74)}$=6.1, P<0.05).

Effect on bone marrow: Bone marrow parasite loads, determined on DAI 23, DAI 39 and DAI 70, were not significantly different from each other. Of the combinations tested only, that involving DFMO, BSO and Berenil was different in effect when compared with the others ($F_{(3,32)}$=2.64, P<0.05).

Thus, the results obtained, presented in section 3.4. above, have showed that many of the compounds thought to inhibit the synthesis or interfere with the activity of trypanothione, have antileishmanial activity. However, none of the combinations appeared to be synergistic under the conditions used even though they appeared to be more effective than Pentostam itself.

3.5. THE EFFICACY OF A DRUG-CARRIER SYSTEM, OCTYL DEGROL OIL, IN THE CHEMOTHERAPY OF VISCERAL LEISHMANIASIS

In one attempt to overcome the rapid metabolism of sodium stibogluconate (Pentostam^{R}) and achieve effective drug levels for longer periods, a new experimental compound, octyl degrol oil, reported to be effective in allowing slow release of drugs (Prof. N.B. Graham, Department of Pure and Applied Chemistry, Strathclyde University, Glasgow), was investigated. Here, representative results of a preliminary study are presented. Two experiments were carried out in order to test the possibility that the material could be beneficial in the chemotherapy of leishmaniasis: the drug was administered as suspension in the oil at a ratio of 0.01 ml/g of body weight subcutaneously, (I) at various dose levels and (II) for different numbers of doses. For a direct comparison of the octyl degrol oil system with the free sodium stibogluconate, the efficacy of the free drug was assessed in parallel using the experimental model of leishmaniasis. The experimental procedure for the infection of mice and the determination of the antileishmanial activity was as described earlier (section 2.). The treatment regimen and the results obtained are given in Table 30 and Figures 36 and 37.

In the first experiment (Table 30, Figure 36), infected mice were treated with a single dose of sodium stibogluconate (Pentostam^{R}) at dose levels ranging from 200 mg/kg to 25 mg/kg. Statistical analysis of the results showed that the drug in suspension with the oil gave significantly higher suppression of parasite numbers than the free drug, at each dose level tested ($F_{(1,88)}=3.7$, $P<0.05$), indicating that the oil had a beneficial effect. However, there was no significant difference in effect between the different dose levels tested.

In the second experiment, Pentostam$^{(R)}$ was administered for different numbers of doses (Table 30, Figure 37) given at two days intervals so that the last dose was on day 20 after infection (DAI). Statistical analysis of data showed that treatment effects

Table 30

The effect of Pentostam, as free drug and with octyl degrol oil, against L. donovani in mice*.

I. Different dose levels of Pentostam

TREATMENT REGIMEN

Infection (D0) → 13 days → [P][P][P][P] (D14 – D17) → [K] (D21)

PENTOSTAM	DOSE (mg/kg)	GROUP SIZE	% SUPPRESSION
FREE	200	6	50.0 ± 13.9
	100	6	47.5 ± 16.6
	50	6	57.4 ± 19.4
	25	6	56.0 ± 21.6
WITH OCTYL DEGROL OIL	200	6	69.7 ± 20.2
	100	6	74.5 ± 16.0
	50	6	53.5 ± 25.7
	25	6	46.8 ± 14.6
OCTYL DEGROL OIL CONTROL		6	10.5 ± 25.1

II. Different length of treatment time with Pentostam

Infection (D0) → 10 days → [O][O][O][O][O][O][O][O][O][O] (D11, D14, D17, D20) → [K] (D24)

DRUG	% SUPPRESSION
Octyl Degrol Oil	-49.1 ± 79.2

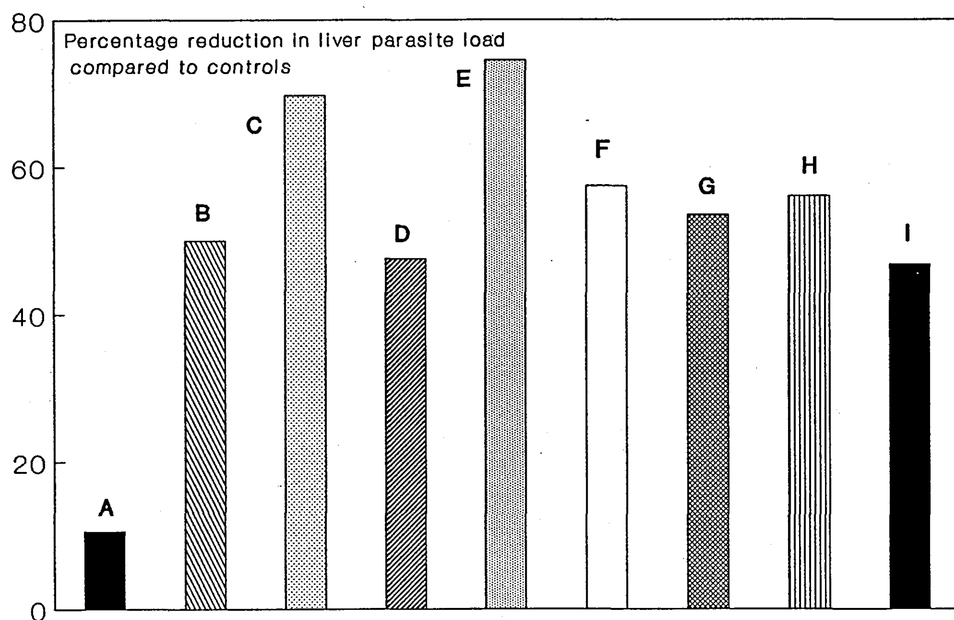
Regimen	DRUG	% SUPPRESSION	Regimen	DRUG COMBINATION	% SUPPRESSION
Infection (D0) → 10 days → [P] (D11, D14, D17, D20) → [K] (D24)	Pentostam	8.8 ± 43.1	Infection (D0) → 10 days → [P/O] (D11, D14, D17, D20) → [K] (D24)	Pentostam + Octyl Degrol Oil	24.4 ± 34.5
Infection (D0) → 10 days → [P] (D14, D17, D20) → [K] (D24)	Pentostam	15.0 ± 41.7	Infection (D0) → 10 days → [P/O] (D14, D17, D20) → [K] (D24)	Pentostam + Octyl Degrol Oil	52.8 ± 43.7
Infection (D0) → 10 days → [P] (D17, D20) → [K] (D24)	Pentostam	-4.6 ± 46.4	Infection (D0) → 10 days → [P/O] (D17, D20) → [K] (D24)	Pentostam + Octyl Degrol Oil	32.6 ± 14.9
Infection (D0) → 10 days → [P] (D20) → [K] (D24)	Pentostam	-10.0 ± 25.0	Infection (D0) → 10 days → [P/O] (D20) → [K] (D24)	Pentostam + Octyl Degrol Oil	45.0 ± 24.0

[P]: Pentostam 25 mg/kg

[O]: Octyl Degrol Oil .01 ml/g

[K]: Kill *Group size: 6

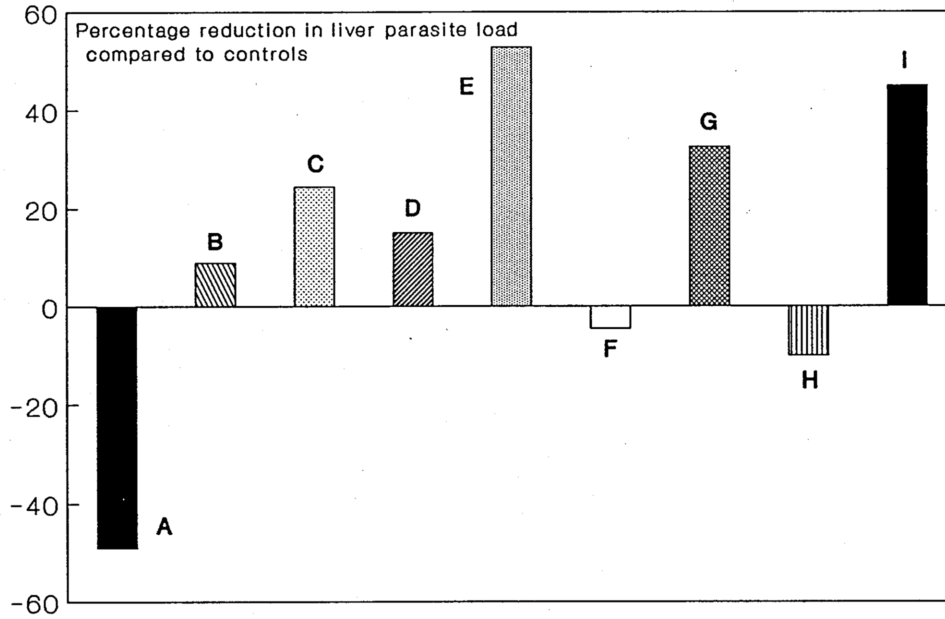
Fig. 36. Comparison of Pentostam (P) and (P) / Octyl Degrol Oil (O) on L. donovani - infected Balb/c mice. I. Various dose levels of Pentostam.



A: Octyl Degrol oil (O) B: (P)I C: (P)1 + (O) D: (P)2 E: (P)2 + (O)
F: (P)3 G: (P)3 + (O) H: (P)4 I: (P)4 + (O)

(P)1 : 1 x 200 mg/kg **(P)2 :** 1 x 100 mg/kg **(P)3 :** 1 x 50 mg/kg **(P)4 :** 1 x 25 mg/kg

Fig. 37. Comparison of Pentostam (P) and (P) / Octyl Degrol oil (O) on L. donovani - infected Balb/c mice. II. Various numbers of doses of Pentostam.



A: Octyl Degrol oil (O) B: Pentostam (P)1 C: (P)1 + (O) D: (P)2
E: (P)2 + (O) F: (P)3 G: (P)3 + (O) H: (P)4 I: (P)4 + (O)
(P)1 : 4 x 25 mg/kg **(P)2 :** 3 x 25 mg/kg **(P)3 :** 2 x 25 mg/kg **(P)4 :** 1 x 25 mg/kg

using the drug in oil, were significantly better than the effects produced by the free drug ($F_{(1,88)}$=23.3, P<0.05). However, no significant difference was observed between the different lengths of treatment time at the dose level tested.

3.6. ASSESSMENT OF THE ANTILEISHMANIAL ACTIVITY OF DNA INTERCALATORS, PLATINUM AND RHODIUM DRUG COMPLEXES AND 8-HYDROXYQUINOLINE COMPOUNDS, AGAINST PROMASTIGOTES OF L. DONOVANI

A number of experimental compounds, available for testing as potential antitrypanosomals, were screened as potential antileishmanials against promastigotes of *L. donovani*. These included a number of DNA intercalators (diminazene derivatives), platinum and rhodium drug complexes and 8-hydroxyquinoline compounds. The microscopically observed effects of these compounds upon the growth of *L. donovani* promastigotes are summarised in Table 31, Figures 38, 39 and 40. The extent of growth inhibition, as assessed after 5 days of incubation, was dependent upon drug concentration and group and/or compound used. With the exception of BSU-1041, all the DNA intercalators showed interesting antileishmanial activity (Table 31, Figure 38). BSU-1021 ($F_{(7,15)}$= 802.9, P<0.05), BSU-1006 ($F_{(7,15)}$)=1798, P<0.05), BSU-1030 ($F_{(7,15)}$=7041.9, P<0.05) and BSU-1002 ($F_{(7,15)}$=29.1, P<0.05) appeared to be very active, giving 100% reduction at concentrations 5 μg/ml and above. In contrast, BSU-1041 showed some activity at concentrations ranging between 10-100 μg/ml ($F_{(7,15)}$=7.8, P<0.05). Growth of *L. donovani* was relatively unaffected by the platinum and rhodium drug complexes even at the highest concentration tested (100 μg/ml) (Table 31, Figure 39). Only Rh(III)-BT showed some activity, giving only 25% inhibition of growth at 100 μg/ml. All the 8-hydroxyquinoline compounds showed very good antileishmanial activity giving up to 100% reduction. Compound 1 ($F_{(7,15)}$=1084, P<0.05) and compound 2 ($F_{(7,15)}$=109.8, P<0.05) showed similar activities giving 100% reduction at concentrations 2.5 μg/ml and

Table 31

The effect of a number of DNA intercalators, platinum and rhodium drug complexes and 8-hydroxyquinolines, at various concentrations, against L. donovani promastigotes.

I. DNA INTERCALATORS

Drug conc. µg / ml	Percentage reduction compared to controls					
	BSU-1041	BSU-1021	BSU-1006	BSU-1002	BSU-1037	BSU-1030
100	28.5 ± 11.4	100	100	100	97.0 ± 0.7	100
50	22.4 ± 0.7	99.5 ± 0.2	100	100	96.7 ± 1.2	100
25	18.2 ± 1.5	99.2 ± 0.2	100	100	88.8 ± 0.3	100
10	17.3 ± 6.6	98.7 ± 0.2	100	100	33.4 ± 0.7	100
5	14.1 ± 0.4	96.6 ± 2.4	98.7 ± 0.7	78.5 ± 7.9	15.2 ± 1.5	99.4 ± 0.1
2.5	11.5 ± 0.4	65.0 ± 3.3	34.4 ± 1.8	38.6 ± 12.1	8.7 ± 1.0	40.7 ± 3.2
1.25	1.9 ± 1.1	29.1 ± 0.5	0.7 ± 4.6	11.3 ± 25.3	-0.7 ± 1.0	26.1 ± 0.8
Control**	2968.0 ± 7.3	2833.5 ± 98.3	2949.0 ± 99.7	2814.0 ± 72.1	2938.0 ± 17.0	3041.0 ± 98.9

II. PLATINUM AND RHODIUM DRUG COMPLEXES

Drug conc. µg / ml	Percentage reduction compared to controls			
	pt(II)-DAP-SSHS	pt(II)-DAP-SDI	Rh(III)-Ethyl-Xanthate	Rh(III)-BT
100	2.9 ± 1.8	-0.4 ± 3.2	0.7 ± 1.3	23.5 ± 10.7
50	1.9 ± 1.6	2.5 ± 10.5	-0.02 ± 1.5	7.1 ± 19.4
25	3.9 ± 5.1	1.6 ± 2.1	1.0 ± 5.3	3.7 ± 7.5
10	3.4 ± 0.7	2.1 ± 0.5	-1.9 ± 2.4	1.1 ± 0.4
5	3.1 ± 3.4	4.3 ± 9.6	-1.2 ± 0.3	0.1 ± 0.4
2.5	2.3 ± 2.8	-1.2 ± 0.6	-4.0 ± 2.0	-0.9 ± 0.4
1.25	3.0 ± 0.1	-1.4 ± 0.1	-3.7 ± 1.0	-0.7 ± 1.0
Control**	2402.0 ± 68.0	2354.0 ± 4.0	2342.0 ± 2.1	2362.0 ± 36.1

III. 8-HYDROXYQUINOLINES

Drug conc. µg / ml	Percentage reduction compared to controls			
	Compound 1	Compound 2	Compound 3	Compound 4
100	100	100	100	100
50	100	100	100	100
25	100	100	100	100
10	100	100	93.7 ± 4.4	93.3 ± 2.3
5	100	100	5.6 ± 4.3	5.4 ± 4.8
2.5	90.3 ± 11.9	93.7 ± 9.7	1.0 ± 1.7	4.5 ± 2.8
1.25	2.57 ± 2.1	11.6 ± 12.5	-1.1 ± 1.9	2.3 ± 4.3
Control**	1755.0 ± 325.0	1949.0 ± 430.0	1947.0 ± 470.0	1762.0 ± 386.0

** Mean number of parasites x 10^4/ml ± SD

Fig. 38. The effect of DNA intercalators against promastigotes of L. donovani.

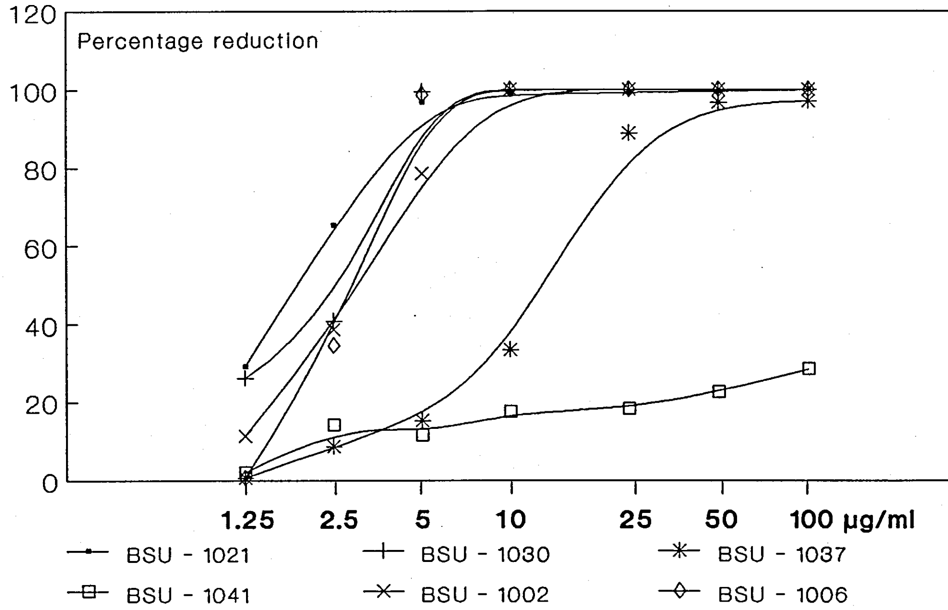
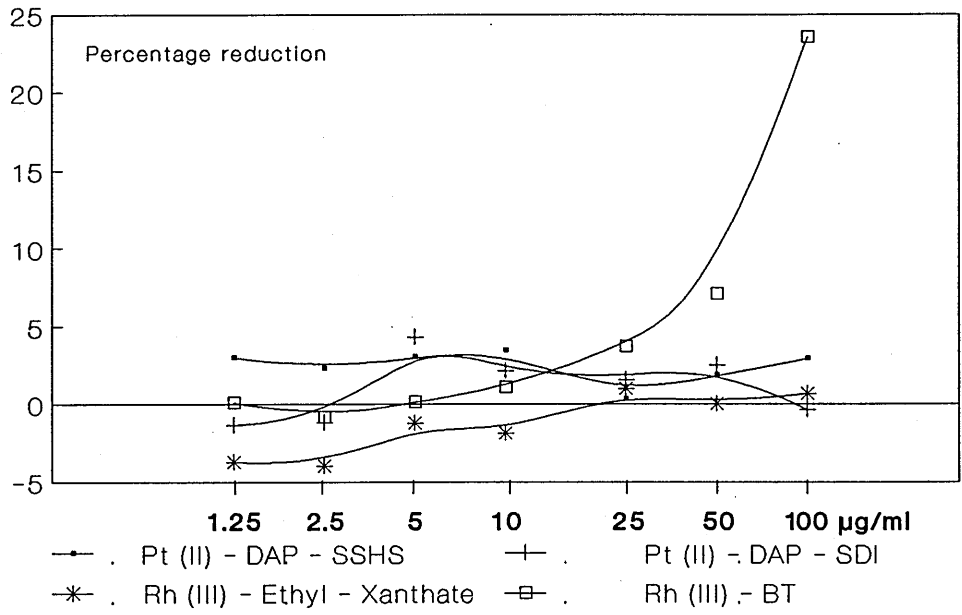
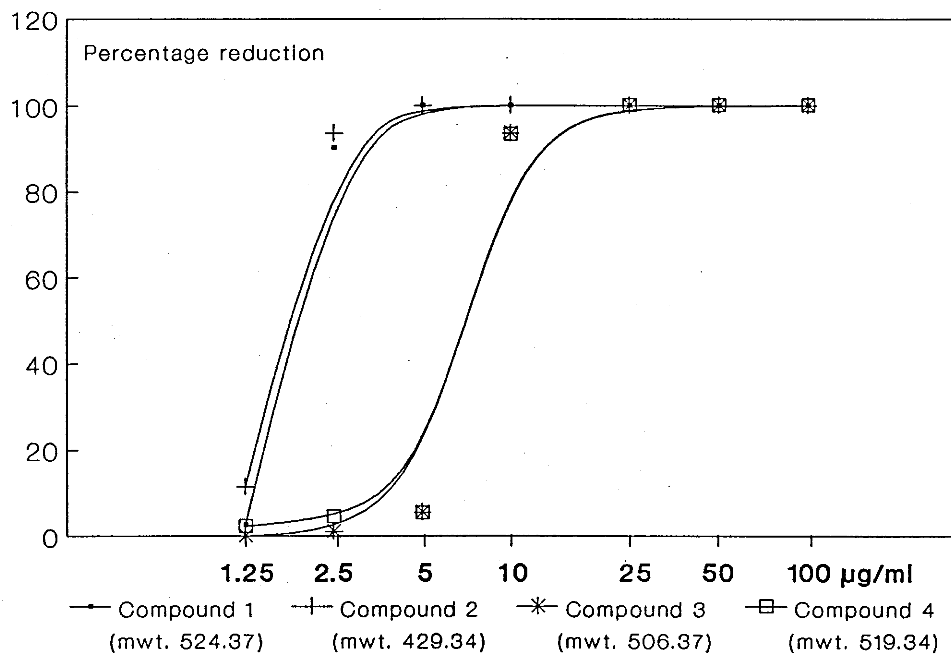


Fig. 39. The effect of platinum and rhodium drug complexes against the promastigotes of L. donovani.



above whereas compound 3 ($F_{(7,15)}=45.2$, $P<0.05$) and compound 4 ($F_{(7,15)}=51.1$, $P<0.05$) were also giving 100% reduction at concentrations of 10 μg/ml and above (Table 31, Figure 40).

Fig. 40. The effect of 8-hydroxyquinoline compounds against the promastigotes of L. donovani.



4. DISCUSSION

4.1. EXPERIMENTAL METHODS USED

Promastigotes similar to the stages of *Leishmania* that occur in the sand-fly can be cultivated easily in a wide range of media, with high yields of organisms and for this reason have been used extensively for biochemical, immunological and drug screening experiments (Hendricks and Childs, 1980). They do not, however, represent the parasite form present in the mammalian host and their drug susceptibility, as reported in many early chemotherapy screens, contrasts markedly with that of the amastigote, most importantly to the clinically used pentavalent antimonials - sodium stibogluconate (Pentostam^R) and meglumine antimoniate (Glucantime^R) (Croft, 1988). It is possible, however, that this *in vitro* model can provide useful information on the drug sensitivity of the parasite and may prove useful in studies on the mechanisms of drug action. For this reason, promastigotes of *L. donovani* were used in this study, in testing the antileishmanial activity of a number of potential metabolic inhibitors of trypanothione metabolism as well as a limited number of other potentially antitrypanosomal compounds.

Amastigote infections of macrophages respond to the clinically used antileishmanial compounds (the pentavalent antimonials, amphotericin B and pentamidine) and so this model resembles more the situation of the parasite *in vivo* than do promastigotes *in vitro*. Thus this test has good predictive value for antileishmanial activity and it is also possible that it can provide some information on the role of the host cell as well as the importance of parasite form in determining the drug sensitivity of the parasite. Also, since the aim of future drug development for leishmaniasis is to find less toxic drugs, any toxicity signs to the macrophage *per se* will be a useful indication that the drug under consideration may have potentially toxic side effects. In this study, two methods of assessing antileishmanial activity using this model have been used: determining the proportion of macrophages which become infected with parasites and the mean number of amastigotes per infected macrophage.

The culture technique used to support growth of intracellular *L. donovani* amastigotes in mouse PECs was a modification of that described by Neal and Croft (1984). Using this method, infected macrophages were 76-98% infected with amastigotes and each infected macrophage contained a mean number of 8.1 ± 1.0 amastigotes at the end of the experiment. These numbers are higher than those reported by Neal and Croft (1984), although the ratio of infecting amastigotes to macrophages was lower and the incubation period was shorter in my experiments. This difference in infectivity may be attributed to the different origin of peritoneal macrophages and the procedure which was used in order to obtain them. The different strain of parasite and the different culture conditions may also have contributed to observed differences (i.e. 20% HIFCS).

For convenience of handling, laboratory animal house space and economical use of drugs, the *in vivo* model used in this study was visceral leishmaniasis in inbred Balb/c mice and not the classical hamster model established by Stauber *et al*. (1958). Bradley and his colleagues have shown (1972; 1977) that a number of inbred mouse strains are highly susceptible to infection by the intravenous route and mouse models have been employed for recent chemotherapeutic studies by several workers (Black *et al*., 1977; New *et al*., 1978; Trotter *et al*., 1980a; Bray, 1987; Neal, 1987; Hunter *et al*., 1988). The mouse strains fall into two categories on the basis of liver parasite counts at the end of the 1st or 2nd week after infection which is referred to as the "acute phase" of the infection (Bradley and Kirkley, 1977). Among those mouse strains allowing rapid parasite proliferation during acute phase, the subsequent course also varies and some mice recover completely (i.e. NMRI) while others (i.e. B10.D2) support a continually increasing amastigote load during this second phase which is referred to as the "chronic phase" of infection. This distinction between acute and chronic phases is important for subsequent analysis and comparison of results from chemotherapeutic studies and it must be also take into account that the clinical and not the subclinical cases of visceral leishmaniasis are more difficult to treat with the standard drug

treatments (see section 1.2.). The Balb/c mouse strain is susceptible to infection with L. donovani and carries the $H\text{-}2^{d/d}$ haplotype which is associated with persisting heavy infection, with the prediction that the majority of infected mice will succumb by 130 days after infection (Blackwell et al., 1980; Bradley, 1987). The prime need in any in vivo chemotherapeutic study is an experimental model which reflects the disease, at least in certain essentials, in humans and dogs, and in this respect the chronic Balb/c mouse model was found suitable for this study. The pattern of infection in the L. donovani-Balb/c visceral leishmaniasis model observed in this study was similar to that described by Blackwell et al. (1980).

4.2. TRYPANOTHIONE METABOLISM AS A CHEMOTHERAPEUTIC TARGET FOR LEISHMANIASIS: THE EFFECT OF COMPOUNDS THOUGHT TO INHIBIT TRYPANOTHIONE METABOLISM IN VIVO AND IN VITRO

Drugs designed against those parasitic enzymes which are essential for the survival of the parasite and absent from the host, are widely regarded as a rational approach to chemotherapy. Recent work in trypanosomatids has shown that trypanothione metabolism meets these criteria and its potential role as a chemotherapeutic target is highlighted by the fact that a number of trypanocidal drugs such as DFMO (Bacchi et al., 1983; Bacchi and McCann, 1987; Fairlamb et al., 1987; Bellofatto et al., 1987) and arsenicals (Fairlamb et al., 1989) interfere with the metabolism and functions of this important metabolite. Moreover, current research in the chemotherapy of experimental trypanosomiasis (Jennings, 1988a, b; Jennings, 1990) supports the hypothesis that a number of compounds thought to interfere with polyamine/trypanothione metabolism are likely to act synergistically. These findings are consistent with the concept that the trypanothione oxidation-reduction system of the trypanosomes is the main target of these drug combinations (Henderson and Fairlamb, 1987; Jennings, 1990). DFMO blocks the polyamine biosynthetic pathway at ornithine decarboxylase and so gradually the pool of

trypanothione is reduced. Trypanothione can react irreversibly with the arsenicals to give the Mel-T adduct (Fairlamb et al., 1989) and therefore, the efficiency of arsenical treatment should depend upon the pool of trypanothione present at the time of arsenical chemotherapy and on how quickly it can be replaced by biosynthesis. Since arsenicals combine with trypanothione and effectively remove it from the system, a stage should be reached when it requires only minimal quantities of the arsenical drug to complete the destruction of the parasite. For this reason it has been suggested (Jennings, 1990) that a period of DFMO treatment is required before the arsenical is given, so that the depletion of trypanothione pool can take place. For the same reason it has been suggested to give the arsenical towards the end of the DFMO therapy (Jennings, 1990).

As arsenic and antimony are similar in some ways, another possibility is that antimony could replace the arsenic in the trypanocide (Jennings, 1990). This was initially recognised by Friedheim (Friedheim, 1953) who prepared analogues of melarsen (MSb) and melarsoprol (MSbB). These compounds were very effective against T. gambiense trypanosomiasis (Friedheim, 1953), however, were never exploited. In view of this, the possible potentiation of the existing available antimonial compounds with DFMO was investigated (Jennings, 1988a, b; Jennings, 1991a, b) but the potentiation observed against T. brucei, although 100% cures could be obtained, was not as obvious as that found with the arsenical compounds. However, no cures with antimonial monotherapy were obtained in the CNS-trypanosomiasis mouse model (Jennings, 1990; 1991a, b).

The synthesis of trypanothione can also be affected by blocking the polyamine pathway at S-adenosylmethionine decarboxylase (SAM-decarboxylase) by the guanylhydrazones (Ulrich et al., 1982; Ulrich and Cerami, 1984) and also by diminazene diaceturate (Berenil^{R}) and pentamidine (Lomidine^{R}). Jennings et al. (1987), obtained cures of CNS-trypanosomiasis with a combination of DFMO and 1,3,5, triacetylbenzene tris (guanylhydrazone) trihydrochloride (i.e. TBG) and it has been also shown (Jennings, 1988a, b) that it was possible to reduce the DFMO administration period by using a

combination of DFMO, TBG and the arsenical melarsoprol and still obtain satisfactory cure rates.

Diminazene diaceturate, as well as binding to DNA, also affects SAM-decarboxylase (Bitonti et al., 1986a, b) and has been reported to be potentiated by DFMO (Bacchi and McCann, 1987; Jennings, 1990).

Another way of embarrassing the trypanothione oxidation-reduction pathway of trypanosomes could be by increasing the amount of peroxide which has to be detoxified by this system. A number of nitro-compounds such as nitrofurans and nitroimidazoles have been reported to undergo "futile redox cycling" producing oxygen radicals and hydrogen peroxide (Henderson et al., 1988). If this is so, then the combination of such nitroimidazoles with any of the potential other inhibitors of trypanothione metabolism such as for instance the arsenical drugs, should be synergistic. A combination of the 5-nitroimidazoles, MK 436 (12.5 mg/kg), L634,549 (25 mg/kg) and fexinidazole (30 mg/kg) all were curative when used with a dose of 2.5 mg/kg Trimelarsan (Jennings, 1990).

The above mentioned compounds including DFMO, a number of 2-nitroimidazoles and 5-nitroimidazoles, guanylhydrazones, the diamidine Berenil$^{(R)}$, the arsenical Trimelarsan$^{(R)}$ and also the pentavalent antimonial sodium stibogluconate (Pentostam^{R}) and buthionine sulfoximine (BSO), have been used in this study in order to test if they are similarly synergistic against Leishmania species and in particular L. donovani, as they have been shown to be against T. brucei. Suramin has also been included as it has been shown to act synergistically with the 5-nitroimidazoles against CNS-trypanosomiasis (Jennings et al., 1983).

4.2.1. THE NITROIMIDAZOLES

4.2.1.1. The nitroimidazoles in monotherapy experiments in vitro and in vivo

Nitroheterocyclic compounds are known for their selective toxicity towards anaerobic and micro-aerophilic organisms although several are active against supposed aerobes (Hamilton-Miller and Brumfitt,

1976; Coombs, 1977; Muller, 1981; Hof et al., 1986).

Susceptible anaerobes such as trichomonads, are assumed to reduce the nitro-group of these drugs in ferredoxin-linked reactions leading to the formation of cytotoxic products (Moreno et al., 1984). Against African trypanosomes, nitro-compounds are thought to exert their cidal action by "futile redox-cycling" (Docampo and Moreno, 1984). Several of these compounds, such as the 5-nitroimidazoles and 2-nitroimidazoles, have been reported to have useful antitrypanosomal activity when used alone (Jennings and Urquhart, 1983; Jennings et al., 1984) or in combination with other compounds (Jennings and Urquhart, 1983; Jennings et al., 1984; Zweygarth and Rottcher, 1987a, b; Jennings, 1988a, b; Jennings, 1990). The results of chemotherapeutic studies (Jennings and Urquhart, 1983) with three of the 5-nitroimidazoles, L611,744, L634,549 and MK 436, against early (3-day) mouse infections of T. brucei showed them to have varying trypanocidal activities and confirmed the initial observations of Cuckler et al. (1970) on the anti-trypanosome activity of nitroimidazoles.

The 5-nitroimidazole MK 436 was found to be fully effective at less than 250 mg/kg/day in treating the chronic and acute stage of Chagas disease in mice and in dogs (Miller et al., 1979; Wyvratt, 1990). At therapeutic doses, the compound appeared superficially to be free from toxic effects (Malanga et al., 1981). It was effective against T. cruzi-infections unresponsive to nifurtimox and benznidazole (Wyvratt, 1990). In three months oral toxicity studies in rats at the level of 3000 mg/kg/day, a number of drug-related toxic effects were observed. MK 436 was found to be mutagenic in the Ames test and in some in vivo cytogenic tests (Wyvratt, 1990).

MK 436 was also tested against L. donovani in mice in this study and showed interesting but not curative antileishmanial activity (Table 5, Figure 6).

Among the nitroheterocyclic compounds tested against leishmanias were the nitroimidazoles metronidazole and benznidazole. Metronidazole, a 5-nitroimidazole, has been reported to have good activity against L. m. mexicana in hamster peritoneal exudate cells

when the compound was at very high concentrations (Mattock and Peters, 1975b) but it was found to be inactive on *L. infantum* in NMRI mice (Peters *et al.*, 1980a). Moreover, Keithly and Langreth (1983) tested the drug against *L. donovani* and *L. mexicana* in hamsters and found no activity against either. Neal and Croft (1984) also tested the activity of metronidazole against *L. donovani* in mouse peritoneal exudate cells and found it totally inactive. Metronidazole has been tried in the treatment of patients with cutaneous leishmaniasis (Wahba and Cohen, 1977) and Ethiopian mucocutaneous leishmaniasis (Belehu *et al.*, 1978) but without any success. In this study metronidazole was found to be ineffective against growing promastigotes, in agreement with the earlier results (Table 3, Figure 4).

There have been differing reports concerning the antileishmanial activity of the 2-nitroimidazole, benznidazole. Peters *et al.* (1980a,b) found it to be inactive against *L. infantum* in mice, but active against *L. major* and *L. m. amazonensis* in mice and tissue culture and it has been suggested that this compound may be useful in treating the cutaneous disease. In this study, benznidazole was found to be inhibitory to growing promastigotes, but only at the highest level tested (100 μg/ml) (Table 12, Figure 13).

Other nitroimidazoles tested in this study were the 5-nitroimidazoles, L611,744, fexinidazole, L634,549, Go 10,213 and dimetridazole and the 2-nitroimidazoles Ro 15-0216 and Ro 15-6547. There were not any previous reports on the activity of these nitroimidazoles against *Leishmania* species. The majority of the nitroimidazoles tested in this study, showed better activity *in vitro*, giving up to 100% reduction in parasite numbers, than *in vivo*. It may be possible to attribute this to differences of their catabolism by the host (i.e. pharmacokinetic profiles) and/or to pharmacokinetic problems limiting exposure of the parasite to the drug. With the exception of benznidazole, the 2-nitroimidazoles showed relatively better activity (Table 12, Figure 13) than the 5-nitroimidazoles (Table 3, Figure 4). This correlates well with the fact that the 2-nitroimidazoles can be reduced more easily than the

5-nitroimidazoles by the cells. With the exception of dimetridazole, all the nitroimidazoles showed interesting antileishmanial activity *in vivo* (60-80% reduction in liver parasite loads).

The *in vivo* activity of the 2-nitroimidazole, Ro 15-0216 was less than that of the 5-nitroimidazoles and was apparently dose independent (Table 13, Figure 6). This was almost certainly due to its more rapid metabolism by the host (see also section 4.2.1.2.e). Experiments in dogs and sheep revealed a short half-life for Ro 15-0216 (unpublished observations quoted by Zweygarth and Rottcher, 1987a).

The trypanocidal activity of Ro 15-0216, has been previously tested in stocks of *T. b. brucei*, *T. b. evansi*, *T. vivax* and *T. congolense* and *T. simiae* (Zweygarth and Rottcher, 1987a, b), revealing different sensitivities. Due to the short half life, administration of the compound continuously in the drinking water was superior to a single intraperitoneal injection (Zweygarth and Rottcher, 1987a). Ro 15-0216 has also shown promising activity against acute infection of rats with *T. rhodesiense* and *T. gambiense*, remarkable activity against chronic *T. rhodesiense* in rabbits, but inconsistent results were obtained with *T. rhodesiense* in vervet monkeys (Stohler, 1990). Acute infections of sheep with *T. brucei*, *T. evansi* and *T. congolense*, also responded well to treatment (Stohler, 1990). Acute and chronic infections of sheep with *T. brucei* as well as chronic infections showing CNS involvement were cured by treatment for six days. *In vitro*, only low concentrations were required to inhibit growth of *T. rhodesiense*, *T. gambiense*, *T. congolense* or *T. vivax* (Stohler, 1990).

Ro 15-0216 has not been reported, so far, to show any pharmacological properties which might preclude its use in man. It had low acute oral toxicity in mice, rats and dogs after oral administration, and was well tolerated in rats at 300 mg/kg/day and dogs at 50 mg/kg/day in 13-week oral tolerance studies (Stohler, 1990). In pharmacokinetic studies of Ro 15-0216 in mice, rats, dogs and sheep, important species differences were observed (Stohler, 1990). A major drawback of the compound is that it was mutagenic in

the Ames test (S. typhimurium) and induced genetic damage in S. cerevisiae in the range 0.3-3 μg/ml (Stohler, 1990).

The 5-nitroimidazole, fexinidazole differs from other standard compounds of the same type by its high activity against T. cruzi, lower toxicity and comparative ease of chemical synthesis (Raether and Deutschlander, 1979; Raether and Seidenath, 1983). Like all 5-nitroimidazoles, it exhibits a strong in vitro and in vivo action on trichomonads and Entamoeba histolytica and was also found to be moderately active against several gastrointestinal nematodes (Raether and Seidenath, 1983). In this study, it has shown interesting antileishmanial activity against L. donovani, being superior to the other nitroimidazoles tested in vivo (Table 5, Figure 6). This, however, was not unexpected since it has been reported (Raether and Seidenath, 1983) to possess perfect requirements concerning stability and metabolisability and its main metabolites proved to be as active as the parent compound.

Various toxicological studies have been carried out with fexinidazole. The compound was well tolerated in mice, rats, guinea-pigs, rabbits and dogs and there is a wide range between the effective and the maximum tolerated doses (maximum oral dose: 10,000 mg/kg body weight) (Raether and Seidenath, 1983). In this study it was shown to be well tolerated in mice (4 x 200 mg/kg) but it proved toxic to macrophages at concentrations of 50 μg/ml and above. These concentrations, however, are far higher than those that can be attained in serum (1.3 ± 0.1 μg/ml). Fexinidazole, like other nitroimidazoles, was shown to be mutagenic in various strains of bacteria at concentrations ranging from 2 to 500 μg/ml (Gericke, referred to in: Raether and Seidenath, 1983).

The in vitro and in vivo results for the nitroimidazoles showed that many of these compounds have an interesting antileishmanial activity when used as monotherapy. Used in the experimental mouse model, they have shown no toxic effects and can be given at dose levels as high as 1 g/day. A number of similar drugs such as metronidazole and dimetridazole, used in the treatment of trichomoniasis and other protozoal and anaerobic bacterial

infections, are also mutagenic to bacteria in other tests under anaerobic conditions, although it is considered by some that these tests have no predictive value as regards the mutagenic risk for man (Boisseau, 1987, personal communication). Metronidazole (Flagyl^{R}) for instance, which is widely used in human medicine, is Ames test positive and yet has been used at relatively high dose rates and for prolonged treatment periods without any major side effects. Nevertheless, such mutagenic compounds are unlikely to be selected for treatment of human disease. It is possible, however, that they could be used for treating the disease in dogs. My results suggest that some of the nitroimidazoles are worth pursuing as possible drugs against canine leishmaniasis. The 2-nitroimidazole, Ro 15-0216 which cured *T. b. brucei* infection in sheep, is under further development as antitrypanosomal agent (WHO, 1989).

Unfortunately, the other nitroimidazoles which have been used in this study are not generally available, as the pharmaceutical companies which produced them have suspended their development. Thus, it now seems probable that there is little prospect that they will be developed for clinical use either as antitrypanosomal drugs or as antileishmanials.

4.2.1.2. The nitroimidazoles in combination experiments *in vitro* and *in vivo*

The main approach of this project was to evaluate combination therapy using compounds thought to interfere with trypanothione metabolism. Large effects were observed although in no case were all the parasites eradicated. The following combinations were tested in this work.

4.2.1.2.(a) Effect of the 5-nitroimidazoles against *L. donovani* in mice in combination with DFMO

There are no previous reports on the combination of DFMO with 5-nitroimidazoles against *Leishmania* species. These combinations have been shown to cure experimental murine *T. brucei* CNS-infections (Jennings, unpublished results).

In this study, however, no potentiation was observed (Table 6, Figure 7). In some experiments this could have been due to the fact that the percentage suppression obtained with the 5-nitroimidazoles as monotherapy was very high and therefore DFMO addition could not show any potentiation. Only the combination involving the nitroimidazole MK 436, which gave the lowest suppression value as monotherapy, and DFMO resulted in a significant increase in efficacy. Probably, lower dose levels of the 5-nitroimidazoles and DFMO were required in order to determine whether or not these compounds are additive or synergistic.

The 5-nitroimidazole L611,744, which was available in sufficient quantity, was studied in a greater detail in combination therapy with DFMO. The results produced gave conflicting evidence of potentiation. This could have been caused by the lack of DFMO intake control (see also section 4.2.4.1.), which in this experiment resulted in a wide variation in the % suppression caused by DFMO. It may also have been influenced by the 5-nitroimidazole and the dosage regimen used. Four doses of L611,744 at 200 mg/kg gave a significant increase when used with DFMO (Table 8, Figure 9), while 12 daily doses of 100 mg/kg gave only a marginal increase (Table 7, Figure 8). Thus, giving the nitroimidazole at higher dose levels and for a shorter period of time, at the end of DFMO therapy, appeared to be more beneficial. This suggests that an initial reduction of the trypanothione pool by DFMO is necessary and therefore giving the nitroimidazole at the end of DFMO therapy when the trypanothione pool has been reduced, makes the combination more effective.

4.2.1.2.(b) Effect of the 5-nitroimidazole L634,549, diminazene diaceturate (Berenil^R) and melarsonyl potassium (Trimelarsan^R), alone and in combination, against *L. donovani* in mice

The results of chemotherapeutic studies against chronic mouse infections of *T. brucei* with two 5-nitroimidazoles, MK 436 (80 mg/kg) and fexinidazole (30 mg/kg), showed them to be effective at four daily doses, when administered in combination with diminazene diaceturate (Berenil^R) (Jennings *et al.*, 1980). Also MK 436 (12.5

mg/kg), L634,549 (25 mg/kg) and fexinidazole (30 mg/kg) all were curative when combined with doses of melarsonyl potassium (Trimelarsan[R]) (2.5 mg/kg) (Jennings, 1990; Jennings, 1991a). None of these drugs administered singly at these levels produced any permanent cures although a high percentage of the mice could be cured with four doses of fexinidazole at 250 mg/kg.

L634,546 and Trimelarsan have not been tested for activity against *Leishmania* species before. In this study, the activity of Berenil (Table 9, Figure 10) was similar to that reported previously (Keithly and Fairlamb, 1988). The 5-nitroimidazole, L634,549 markedly suppressed the number of parasites present in the liver but in no instance there was a complete suppression of the infection (Table 9, Figure 10). Combination of these compounds did not improve their ability to suppress infection. It seems likely therefore, that if these drugs act against similar targets in trypanosomatids as has been hypothesised, they are either not effective inhibitors of these targets in leishmanias, or they do not readily get to the targets. It is also possible that the importance of these metabolic reactors is not so crucial to survival and growth in leishmanias as it is in trypanosomes.

4.2.1.2.(c) The effect of the 5-nitroimidazoles MK 436, L634,549 and fexinidazole and suramin, alone and in combination, against *L. donovani* in mice

These combinations did not improve the ability of the single compounds to suppress experimental infections of mice with *L. donovani* (Table 10, Figure 11). Permanent cures, however, were obtained in a mouse model of *T. brucei* CNS-trypanosomiasis when treatment was carried out with suramin (20 mg/kg) followed by 4 consecutive daily doses of 250 mg/kg MK 436 or 70 mg/kg L634,549 or 80 mg/kg L611,744 (Jennings *et al.*, 1983), or 30 mg/kg fexinidazole (Jennings and Urquhart, 1983), even though, none of these drugs administered singly elicited 100% permanent cures.

More recently Arroz and Djedje (1988) experimented with a combination of suramin and metronidazole, which had shown promise in

mice (Raseroka and Ormerod, 1985; 1986), in a moribund patient with multidrug-resistant T. b. rhodesiense. The initial apparent cure, however, was followed by a relapse 3 weeks after the end of treatment.

Based on previous work in trypanosomes (see section 4.2.), a plausible hypothesis can be formulated for synergism between DFMO, arsenicals, diamidines and nitroheterocyclic compounds. However, this is not the case for suramin. Suramin may not have a clearly defined single target site of action since it inhibits a large number of enzymes (Fairlamb and Bowman, 1980). An early event noted previously is the progressive inhibition of glycolysis and respiration (Fairlamb and Bowman, 1977; Fairlamb and Bowman, 1980). More recently, suramin has been also shown to displace low density lipoproteins (LDL) from surface receptors of mammalian cells as well as that of trypanosomes (Furlong, 1989; Coopens et al., 1989). None of these observations, however, offer a clear biochemical link with the effects of the other classes of trypanocidal compounds. Despite this, suramin was screened in this study because of its known trypanocidal action, for any effects on polyamine and thiol metabolism in leishmanias. There is only one previous report on suramin as monotherapy and in combination against Leishmania species (Keithly and Fairlamb, 1988). Keithly and Fairlamb (1988) reported a 70% reduction in parasite load by suramin alone when the compound was used against L. donovani in mice and this is considerably higher than that obtained in this study (34%). However, the standard deviation observed and the shorter length of treatment used in this study, make a direct comparison difficult. The combination of suramin with DFMO (3%) has been reported (Keithly and Fairlamb, 1988) to improve marginally their ability alone to suppress L. donovani infections in mice, however, the two compounds were in effect less than additive. It seems likely therefore that suramin does not affect polyamine and thiol metabolism in leishmanias.

4.2.1.2.(d) The effect of the 5-nitroimidazole MK 436 given at various dose levels alone and in combination with verapamil against L. donovani in mice

Verapamil, a cardiac-active calcium antagonist used mainly as a coronary vasodilator, has recently been shown to affect drug efflux in a multiple drug resistant carcinoma cell line, resulting in drug accumulation within the cells (Slater et al., 1982; Rogan et al., 1984; Fojo et al. 1985). A similar effect was subsequently shown with chloroquine-resistant Plasmodium falciparum (Martin et al., 1987). It has also been found that in vitro verapamil can reverse nifurtimox-resistance in T. cruzi and, although to a lesser extent, antimony-resistance in L. donovani (Neal et al., 1989).

Verapamil alone appears to have mild antimalarial properties in vitro (Jacobs et al., 1988), but only at toxic levels, and was found to be inactive against T. cruzi and L. donovani (Neal et al., 1989). This was also confirmed for L. donovani in the present study.

One attempt was made to examine if it was possible to increase the effectiveness of the nitroimidazoles by concurrent verapamil administration. Verapamil was used in combination with the 5-nitroimidazole MK 436 (Table 11, Figure 12). The results showed no increased effect even when the nitroimidazole was used at the highest dose level (4 x 200 mg/kg). At all other levels there was a slight increase in percentage suppression which was statistically significant only at the dose level of 4 x 50 mg/kg. This suggests that verapamil does not significantly affect the concentration of the nitroimidazole in the drug-sensitive parasite. This is perhaps similar to the finding that verapamil did not exert potentiation of the drug effect when used with chloroquine against the drug-sensitive strain of P. falciparum (Jacobs et al., 1988).

4.2.1.2.(e) In vivo activity of Ro 15-0216 in combination with diminazene diaceturate (Berenil^R) and melarsonyl potassium (Trimelarsan^R, Mel W^R)

Synergism of Ro 15-0216 (40 mg/100 ml of drinking water x 5 days)

with diminazene diaceturate (Berenil^{R}) (2.5-4 mg/kg) was demonstrated in _T. congolense_-infected mice resulting in complete cure (Zweygarth and Rottcher, 1987a). The combination of Ro 15-0216 with Berenil was also found to be successful in swine _T. simiae_ infections (Zweygarth and Rottcher, 1987b). Ten mg/kg of Berenil and 50 mg/kg of Ro 15-0216 or 20 mg/kg Berenil and 25 mg/kg of Ro 15-0216 were equally effective, while either drug alone was ineffective. Multiple administration of compound Ro 15-0216 (4 x 20 mg/kg; 3 x 50 mg/kg) at 2 hourly intervals did not cure the animals. Synergism of Ro 15-0216 with suramin was also demonstrated in _T. b. evansi_-infected mice, (Zweygarth and Rottcher, 1987a).

In this study, in order to overcome the putative problem of rapid metabolism and increase its effectiveness, Ro 15-0216 was administered in the drinking water. Observations made by Jennings _et al_. (1984) in _T. brucei_ infections showed that divided doses of 5-nitroimidazoles were more effective than the same quantity given on a single occasion. Combination of Ro 15-0216 with Trimelarsan (4 x 30 mg/kg) did not improve the ability of the compounds to suppress the experimental infection in mice. When Ro 15-0216 (20 mg/100 ml of drinking water) was combined with Berenil, (4 x 40 mg/kg), a small potentiation was observed (Table 14, Figure 14).

The lack of efficacy of nitroimidazoles in the experiments discussed in section 4.2.1.2. above, suggests that such combinations have little potential for development for antileishmanial treatment.

4.2.3. THE GUANYLHYDRAZONES

Guanylhydrazones have received considerable attention as antineoplastic agents (Dave _et al_., 1977). Their antineoplastic activity was thought to be due, at least in part, to interference with polyamine biosynthesis via inhibition of mammalian S-adenosyl-L-methionine decarboxylase (SAM decarboxylase) (Pegg and Hibasami, 1979). Of particular significance was the finding that homogenates of _T. brucei_ contained a high specific activity of

methylglyoxal bis-guanylhydrazone (MGBG)-sensitive SAM decarboxylase (Dave et al., 1978). The aminopropylation of putrescine to form spermidine in T. brucei (Bacchi et al., 1979) requires decarboxylated SAM, as is the case in other organisms. In several trypanosomatids including T. brucei, the important respiratory enzyme α-glycerophosphate dehydrogenase is selectively activated by spermidine and spermine in vitro (Bacchi et al., 1978). Thus, the guanylhydrazones appeared to be an interesting class of substances warranting more extensive screening against African trypanosomes. Several aromatic guanylhydrazones have been tested (Ulrich et al., 1982) for antitrypanosomal activity and significant differences in activity demonstrated that the nature of aromatic bridge linking the guanylhydrazone units was of considerable importance.

One of these guanylhydrazones, TBG tested against early T. brucei infections in mice (Ulrich and Cerami, 1984; Jennings et al., 1987) proved to be the most active of all. This was also found to be the case in this study for L. donovani promastigotes in vitro (Table 15, Figure 16). However, in contrast to its in vitro activity, TBG was found to be less active than the guanylhydrazone DBG, when tested in vivo (Table 16, Figures 17 & 18). This could be due to differences in conformational flexibility between these two compounds (Ulrich and Cerami, 1984), with the conformation preferred for the uptake system being different from that preferred at the active site.

Jennings et al. (1987) reported that treatment of late T. brucei infections in mice with TBG (4 x 15 mg/kg or 4 x 10 mg/kg, twice daily) in combination with DFMO (2% soln. for 14 days) or suramin (1 x 20 mg/kg) resulted in permanent cures. It has been also shown that if DFMO and TBG are combined with arsenical treatment, then the period of DFMO administration necessary to cure chronic murine trypanosomiasis can be reduced from 14 to 8 days (Jennings, 1988a, b). In this study, however, combinations of TBG and DBG with Pentostam(R) and DFMO did not show any promising potentiation in activity against L. donovani in mice (Table 16, Figures 17 & 18).

There is only one previous report on the activity of

guanylhydrazones against *Leishmania* species and this referred to the activity of MGBG alone and in combination with DFMO against *L. donovani* amastigotes *in vitro* (Neal and Croft, 1984). In both cases MGBG was inactive and in addition, when alone, was toxic to macrophages.

The lack of efficacy of the guanylhydrazones tested *in vivo* in this study suggests that these compounds have little to offer as antileishmanials. The antileishmanial activity of the compounds against promastigotes, however, provides some encouragement that other compounds of this series, with different pharmacokinetic properties, may have more promise.

4.2.4. TESTING OF OTHER POTENTIAL INHIBITORS OF TRYPANOTHIONE METABOLISM

4.2.4.1. DL-α-DIFLUOROMETHYLORNITHINE (DFMO), (EFLORNITHINE[R])

DL-α-difluoromethylornithine (DFMO), a selective enzyme-activated irreversible inhibitor of ornithine decarboxylase, is a key enzyme for the synthesis of polyamines (Metcalf *et al*., 1978). It is effective against *T. b. brucei* (Bacchi *et al*., 1980), *T. b. gambiense* (McCann *et al*., 1981a), *T. b. rhodesiense* (McCann *et al*., 1981b) and *T. congolense* (Karbe *et al*., 1982) in mice.

DFMO has been extensively used against the Gambian form of human sleeping sickness with promising results. It was effective against the early and late stages of the disease and also against arsenical-refractory cases (Van Nieuwenhove *et al*., 1985).

DFMO acts synergistically with a variety of commercial trypanocides against early stages of murine *T. b. brucei* infections (McCann *et al*., 1983). Combination of DFMO with suramin (Clarkson *et al*., 1984), melarsoprol (Jennings, 1988a, b) and diminazene diaceturate (Jennings, 1990; Zweygarth and Kaminsky, 1991) are effective even in cases with central nervous (CNS) involvement.

Against *Leishmania* species, DFMO was found to be able to block amastigote to promastigote transformation *in vitro* (Coombs *et al*.,

1983). DFMO, 1 mM, in the culture medium completely prevented transformation of L. mexicana amastigotes. Promastigotes of this species, however, were far less sensitive to DFMO since 50 mM failed to block division. The high intracellular and extracellular putrescine levels observed in L. mexicana late log phase promastigotes (Coombs and Sanderson, 1985) may be responsible for the insensitivity of promastigotes of this species to DFMO (Coombs et al., 1983). More recently Kaur et al. (1986) examined the susceptibility of L. donovani promastigotes growth to DFMO. At DFMO concentrations as low as 50 mM, growth was completely blocked. Cells incubated with DFMO for 70 hrs were completely depleted of putrescine and had approximately 50% less spermidine than the controls. Keithly et al. (referred to in: Bacchi and McCann, 1987) using promastigotes of L. donovani and L. braziliensis guyanensis grown in RE III medium, obtained >80% inhibition of growth with 5 mM DFMO.

In this study the high susceptibility of L. donovani promastigotes to DFMO was also confirmed (Table 17, Figure 19). However, amastigotes growing in macrophages in vitro exhibited lower susceptibility to the drug than did promastigotes in vitro (Table 18, Figure 20). There was an apparent small reduction in amastigote numbers which, however, was not statistically significant. The difference in susceptibility of L. donovani amastigotes as compared to that of promastigotes could be due to possible stage differences in drug uptake, ODC turnover rates, the enzyme's affinity for DFMO, or the regulation of expression of ODC. Keithly and Fairlamb (referred to in: Keithly and Fairlamb, 1988) found no evidence for differential uptake or concentration of DFMO, nor utilisation of alternate enzymatic pathways for polyamine biosynthesis in their in vitro testings with L. donovani, L. mexicana and L. b. guyanensis.

Neal and Croft (1984) found 13% reduction of infected macrophages at 27 μg/ml of DFMO. This result is different to that found in this study where, at this concentration, no reduction of infected macrophages was observed. This could be due to the longer

treatment period and different origin of macrophages and method used to be elicited by Neal and Croft.

Keithly et al. (referred to in: Bacchi and McCann, 1987) have also used DFMO singly and in combination with Pentostam(R), pentamidine, suramin and bleomycin against L. b. guyanensis and L. donovani infections in mice (BALB/c ByJ). Depending upon the species DFMO can be synergistic, additive, or have no effect when combined with other drugs (Keithly and Fairlamb, 1988). DFMO, given at 3% in the drinking water for 2 weeks beginning 24 hrs preinfection, suppressed development of L. donovani in spleen cells by 51% (Keithly et al., referred to in: Bacchi and McCann, 1987) . DFMO was synergistic with the antitumor antibiotic bleomycin and showed an additive effect when in combination with suramin, allopurinol riboside, Berenil(R) and Pentostam(R), in the L. donovani mouse model (Keithly and Fairlamb, 1988). DFMO, given at 3-5% in the drinking water for 3 weeks starting 24 hrs postinfection to mice infected with L. b. guyanensis, was 50-74% suppressive to amastigote formation. DFMO was not synergistic with Pentostam(R) and bleomycin in this model (Keithly et al., referred to in: Bacchi and McCann, 1987). These results coincided with the finding (Keithly and Fairlamb, 1988) that DFMO significantly reduces putrescine and trypanothione levels in leishmania and these DFMO-induced changes in metabolite levels were similar to those reported for T. b. brucei (Fairlamb et al., 1987). The effect of DFMO alone appeared to be cytostatic rather than cytocidal because non-dividing promastigotes could survive in culture for at least 11 days (Keithly and Fairlamb, 1988). This effect must be due to a depletion of polyamine levels and trypanothione, because its effect can be completely reversed by addition of putrescine (Keithly and Fairlamb, 1988). Thus it seems necessary to combine DFMO with a cytocidal drug to effect a complete cure.

With DFMO, the continuous presence of relatively high concentrations is necessary, since the target, ODC, is a rapidly inducible enzyme. The standard DFMO treatment regimen in vivo, used throughout this study, consisted of giving a 2% solution of DFMO in

the drinking water continuously for a period of 14 days, starting approximately 4-5 days after infection. This regimen has been used since the first successful experiments against trypanosomes using combinations of DFMO and bleomycin (Bacchi _et al_., 1980) and suramin and DFMO (Clarkson _et al_., 1984). Pharmacokinetic studies on DFMO in mice (Romijn _et al_., 1987) showed that oral administration of DFMO during the day-time resulted in 10- to 15-fold lower levels than administration during night and discontinuation of treatment resulted in 50% decrease of DFMO levels in serum and tissues in approximately 6 hrs. For this reason the mice in this study were placed under a 2 hour light plus 4 hour dark routine, which was reported (Gillet _et al_., 1986; Jennings, 1988a, b) to have the effect of spreading the intake of DFMO more evenly throughout the 24 hour period and also substantially increase its intake. However, a major problem with oral DFMO to mice via the drinking fluid is the lack of drug intake control, which may result in wide variations in drug concentrations between individual animals and thus making it difficult to quantitate the _in vivo_ effect of the drug combinations. Romijn _et al_. (1987) found that the individual uptake of DFMO in the drinking water during a 14 hrs treatment period ranged between 350-2800 mg/kg. They found, however, a significant correlation between the dose of DFMO which was taken up by each mouse and the concentration of DFMO in the serum and other tissues. This variation in drug uptake could explain the relatively large variation in parasite suppression by DFMO that was observed in some experiments of this study.

DFMO was also used in this study not only as a 2% but also as a 4% solution and its effect on liver, spleen and bone marrow parasite load was assessed after 7, 14 and 30 days of administration (Table 19, Figure 21). DFMO suppressed significantly parasites in the liver and to a lesser extent in the spleen and bone marrow. This was expected since pharmacokinetic studies in mice dosed with oral DFMO (2%) have shown that the highest concentrations of DFMO are present in organs which are part of the uptake and elimination pathway and these are the intestine, liver and kidney, whereas there

was not an accumulation of DFMO in other tissues (Romijn _et al_., 1987).

In this study, it was observed that the consumption of the 4% solution was reduced in comparison to the 2%, this finding suggesting that the 4% solution was less palatable to mice resulting in a reduced intake. This could explain why there was not a significantly different effect on liver and bone marrow parasite load between these two DFMO concentrations. However, it does not explain the finding that the 4% solution was more suppressive than the 2% solution, on spleen parasite load. More careful monitoring of fluid and so drug intake is required to help reduce these findings.

DFMO given continuously for 30 days had a very beneficial effect and all organs screened had very low parasite burdens. This indicates that it may be possible to clear all tissues with a treatment regimen involving a prolonged period of DFMO administration in combination with a cytocidal drug. The major disadvantage of this is the difficulty of DFMO administration and the quantities of the drug required for such a long treatment period. None of the combinations and day regimens I tested, however, had this effect (Table 29, Figures 32, 33, & 34).

4.2.4.2. DIAMIDINES

A number of diamidine compounds have been tested against _Leishmania_ species showing moderate to low activity _in vivo_ (Hanson _et al_., 1963; Mattock and Peters, 1975b; Hanson _et al_., 1977). The differences observed in the activity of these compounds are thought to be related to the degree of their affinity to the transport system. Previous studies with _T. brucei_ have shown (Damper and Patton, 1976) that the diamidine pentamidine, is accumulated in blood forms via a substrate-specific, carrier-mediated, concentrative, energy-coupled process and that other diamidines are competitive inhibitors of pentamidine uptake. Studies with structural analogues of pentamidine, used to characterise the pentamidine transport system in terms of structural specificity,

have shown that diamidines containing a methylene chain as the bridge between the aromatic amidine moieties were the most effective inhibitors and compounds with the amidines in the *meta* position to the linkage bridge have a higher affinity for the transport site (Damper and Patton, 1976).

The diamidine, diminazene diaceturate (Berenil[R]) is an antiprotozoal drug reported to be very effective against trypanosomes and babesia of cattle, sheep and several other domestic animals (Dalvi, 1988). Its good tolerance in cattle, sheep, dog and man is well documented, but camels and horses can show severe toxic reactions (Tacher, 1982). Chemically it is pp'-diamidinodi-azoaminobenzene diaceturate trihydrate, thus it is chemically related to diamidine compounds such as pentamidine and propamidine (Hawking, 1958). It binds non-specifically with serum and other proteins and from toxicological standpoint is neurotoxic and accumulates mainly in the liver, kidney and brain (Dalvi, 1988). Although the exact mechanism of its action is not known, the drug has been shown to inhibit trypanosomal SAM-decarboxylase (Bitonti *et al*., 1986a, b) and has been reported to be potentiated by DFMO (Bacchi and McCann, 1987). Moreover, Jennings (1990) reported that DFMO (2% soln. in the drinking water x 15 days) in combination with diminazene diaceturate (1 x 40 mg/kg) 4 days after the start of DFMO treatment, cured 47 of a total of 49 mice infected with *T. brucei*. It was also possible to reduce the period of DFMO administration to 6 days and still obtain reasonable cure rates (5/6 mice) (Jennings, 1990). Certainly Berenil does not work solely through polyamine metabolism, since it has been shown to block selectively kinetoplast DNA synthesis (Newton, 1972), but the irreversible inhibition of SAM-decarboxylase might contribute to the compound's overall efficacy as a trypanocide. At the very least, we can say that polyamines are somehow involved in the cytotoxicity of Berenil, pentamidine and similar compounds, since spermidine or spermine can block the curative effects of these compounds *in vivo* (Bacchi *et al*., 1983; Bitonti *et al*., 1986a). Further studies are needed to elucidate more fully the role of inhibition of SAM-decarboxylase by

Berenil in the trypanocidal effect of the drug and the mechanism by which the inhibition occurs. Berenil has also been tested against *Leishmania* species *in vivo* and was found to be highly active against *L. major* and *L. mexicana amazonensis* in mice (Peters *et al.*, 1980b) but with only moderate activity against *L. infantum* in mice (Trotter *et al.*, 1980a). In the present study, Berenil showed marked inhibition of promastigote growth (Table 17, Figure 19) and good antileishmanial activity against amastigotes of *L. donovani* growing in PECs, (67% amastigote reduction at 5 μg/ml) (Table 18, Figure 20). However, it proved to be toxic to macrophages at concentrations of 10 μg/ml or above. This could be expected since it is known to be clinically toxic. Similar observations were made by Neal and Croft (1984) who found toxicity of Berenil to macrophages at concentrations of 9 μg/ml and above. In this study, Berenil was also tested *in vivo* at various concentrations (Table 20, Figure 22). It showed good activity, however, it did not eradicate all the parasites even when used at high dose levels. Concentrations above 40 mg/kg did not increase significantly the percentage parasite suppression as determined on day 23 post infection. Trotter *et al.* (1980a) reported a delayed action of Berenil against "*L. infantum* LV9" which suggests that an extended duration of the post-treatment observation period may be necessary in order to evaluate more accurately the results of high drug dose regimens. This could be due to the fact that the compound is not rapidly and completely metabolised in the body of certain species (cattle, rabbits, rats, mice) and the appreciable liver concentrations and the course of the blood/plasma levels may be responsible for a certain prophylactic effect of Berenil observed in cattle (Klatt and Hadju, 1976; Gilbert and Newton, 1982; Gilbert, 1983; Kellner *et al.*, 1985). Contrary to these findings, a rapid fall in blood levels of the drug following intramuscular dosage to dogs was reported (Bauer, 1958; Anika and Onyeyili, 1989) and for this reason it seems rather unlikely that Berenil could be effective as antileishmanial for treating the disease in dogs. Also, since its activity is compromised by the associated toxicity, it is not an

ideal drug for treating the disease in humans.

The diamidine, pentamidine (Lomidine^{R}) can also affect the synthesis of trypanothione by blocking the polyamine pathway at SAM-decarboxylase (Bacchi and McCann, 1987). It has not been fully investigated in the CNS-trypanosomiasis mouse model, but it has been reported that with a dose rate of 100 mg/kg cures can be obtained with one or more consecutive daily doses given 4 days after the start of DFMO administration (Jennings, 1990). Against Leishmania species, pentamidine has shown different activities in the hands of different workers and in different test systems. In vitro the drug was active at high concentrations against promastigotes of L. pifanoi, L. braziliensis, L. tropica and L. enriettii (Jimenez and Ercoli, 1965). In this study, however, pentamidine methanesulfonate was found to be very active against L. donovani promastigotes even at very low concentrations (Table 17, Figure 19). Pentamidine diisethionate showed (Mattock and Peters, 1975b) only a moderate level of action against amastigotes of L. major (in dog sarcoma cell line) and L. mexicana (in hamster) and none against L. donovani (in dog sarcoma cell line). Contrary to that, Berman and Wyler (1980) found it very active (ED_{50}=0.02 µg/ml) against L. donovani growing in human macrophages. The differences in sensitivity reported, could be due to a combination of stage and species differences, different host cells employed or to the different chemical structure of the diamidine used, which could determine the drug sensitivity of the parasite. Pentamidine was also tested in vivo in this study (Tables 26 and 27, Figures 28 and 29) and showed no appreciable activity. In addition it caused toxic side effects and mortality of a few mice (see also section 4.3.).

4.2.4.3. DL-BUTHIONINE-(S,R)-SULFOXIMINE (BSO)

The formation of peroxides by cells appears to be an unavoidable consequence of aerobic metabolism and glutathione (GSH) plays a major protective role against the endogenous oxidant stress that results from high intracellular levels of H_2O_2. Reports that

trypanosomes lack catalase (Fulton and Spooner, 1956) focused attention on H_2O_2 and the regulation of its intracellular concentration. Buthionine sulfoximine (BSO) is a specific inhibitor of γ-glutamylcysteine synthetase, the first enzyme of glutathione synthesis (Griffith and Meister, 1979). Administration of BSO has been reported to lead to prolonged survival times or cures of *T. brucei* infections in mice (Arrick *et al.*, 1981).

BSO has not been tested previously against *Leishmania* species. In the present study, in which it was tested *in vitro* against both promastigotes (Table 17, Figure 19) and amastigotes (Table 18, Figure 20) of *L. donovani*, it showed some activity but only against promastigotes. This could be due to the fact that the medium (RPMI-1640) used for growing macrophages contained GSH which could have replenished the intracellular GSH levels of amastigotes depleted by BSO.

BSO tested at various concentrations in mice infected with *L. donovani* did not eradicate all the parasites even when used at high levels, although it caused marked reduction in parasite numbers (Table 19, Figure 23). This finding supports the suggestion of interference of the host or the host cell in some other way. Preliminary experiments indicated that BSO is metabolised very rapidly by the animal and it is necessary to administer the drug very frequently (every 1.5 hrs) to maintain chemotherapeutic levels in the circulation (Arrick *et al.*, 1981).

4.2.4.4. NITROFURANS

As well as the nitroimidazole compounds used in this study, I also tested a nitrofuran, nitrofurazone, which has been used in patients with sleeping sickness resistant to arsenicals (Lowenthal *et al.*, 1977). In this context the drug has acquired a reputation for toxicity of almost unacceptable degree, although salvaging a number of patients with otherwise a hopeless prognosis (Lowenthal *et al.*, 1977). A shorter hospital stay than that required for melarsoprol treatment and oral instead of intravenous therapy were the potential

advantages of nitrofurazone. With the exception of nifurtimox, no other nitrofuran appears to have been tested against *Leishmania* species previously. Nifurtimox, which was not tested in this work, is an anti-*T. cruzi* compound. It has been reported to have moderate activity *in vitro* against *L. mexicana* and *L. donovani* amastigotes (Mattock and Peters, 1975b) and has been used to treat human cutaneous and mucocutaneous leishmaniasis (Guerra *et al*., 1981; Werner and Barretto, 1981), although it shows toxic side effects.

In the present study, nitrofurazone showed interesting antileishmanial activity against promastigotes, giving up to 100% reduction (Table 17, Figure 19). In contrast, it had no activity *in vivo* and indeed it showed an antagonistic effect when it was combined with Berenil and Trimelarsan (Table 21, Figure 24). This difference in the susceptibility between promastigotes and amastigotes may be due to permeability differences between these two stages of the parasite. The inactivity of nitrofurazone *in vivo* observed in this study together with its reported toxicity, suggested that further experimentation using this compound as a potential antileishmanial was not worthwhile.

4.2.4.5. ANTIMONIAL COMPOUNDS

The antimonial compound used in this study was sodium stibogluconate (Pentostam^R). Sodium stibogluconate has been assessed as an antileishmanial in a multitude of systems, both *in vitro* and *in vivo* and the extent of the information available on the clinical use of this compound is too large to allow a comprehensive coverage in this thesis. The compound has little or no activity against promastigotes in culture (Al-Khateeb *et al*., 1977; Berman and Wyler, 1980) and this was also confirmed in this study against *L. donovani* (Table 17, Figure 19). Experimental *in vivo* infections, however, do respond to treatment with the drug (Beveridge, 1963). Trotter *et al*. (1980a) reported an ED_{50} of 22 mg Sb^V/kg/day x 5, whereas Neal (1987) found an ED_{50} of 11.2 mg Sb^V/kg/day x 5, against *L. donovani* in mice. Similar results were obtained in the present study where a

dose level of 10-25 mg Sb^V/kg/day x 4, resulted in 48-66% parasite suppression (Table 22, Figure 6). Methodological differences such as the size of inoculum and hence challenge to the host, route of administration, timing of treatment, strain of parasite and host animal used, may serve to explain differences in results reported by different workers.

The compound has been also incorporated in liposomes in attempts to potentiate its activity, and some hopeful results have emerged from this. Sodium stibogluconate in liposomes was shown to be 700-fold more efficacious against experimental kala-azar in rodents than the free drug (Alving et al., 1978). New et al. (1978), however, found only 10-fold enhancement in mice infected with L. donovani and more recently (New et al., 1983) it was also reported that free sodium stibogluconate did not clear L. donovani from the liver, but in the encapsulated form, the compound did clear the parasites. Experimental cutaneous leishmaniasis with L. major and L. m. amazonensis was demonstrated to be more susceptible to sodium stibogluconate entrapped inside liposomes than the drug free in solution (New et al., 1981). More recently, nonionic surfactant vesicles (niosomes) have also been investigated for their potential as antileishmanial drug carriers (Baillie et al., 1986; Hunter et al., 1988). Niosomes appear to be more stable and possess significant cost effective advantages over liposomes. Unfortunately, despite having a similar biodistribution, niosome encapsulated drugs are somewhat less effective than liposome preparations against animal models of leishmaniasis.

In earlier studies using free sodium stibogluconate, it was found that pre-dosing the host cells (dog sarcoma cells) prior their infection with amastigotes of L. donovani seemed to increase significantly the antileishmanial activity of this drug and it has been suggested that antimonials may exert an effect on amastigotes during their brief extacellular transit from one host cell to another in vivo (Mattock and Peters, 1975b). Similar results were found using L. m. mexicana in hamster peritoneal macrophages. Using cultured human monocytes infected with L. donovani and L. tropica,

Berman and Wyler (1980) were able to demonstrate the high sensitivity of the intracellular amastigotes to this antimonial with an ED_{50} of 5 μg Sb^{V}/ml against L. donovani. I obtained similar results in my study using L. donovani in mouse peritoneal exudate cells (Table 18, Figure 20). Neal and Matthews (1982) reported total elimination of L. donovani from mouse peritoneal macrophages by this compound at 81 μg/ml. I was unable to confirm this in my study, and there was not total elimination of parasites even at a higher drug level (100 μg/ml). This could be attributed to experimental variables such as length of incubation time and infecting ratio of amastigotes to macrophages. Coombs et al. (1983) demonstrated that transformation of L. m. mexicana amastigotes to promastigotes was sensitive to the drug, suggesting that the amastigote itself is affected by sodium stibogluconate and that prior metabolism by the host cell is not essential for activity. The difference in the sensitivity of amastigotes and promastigotes observed could be explained by the differential permeability of this two distinct forms of the parasite. In spite of this drug having been used clinically for many years, there seems to be no conclusive evidence on how it exerts its effect and the mode of action of the antimonial compounds remains to be elucidated.

4.2.5. DETECTION OF ADDITIVE OR SYNERGISTIC INTERACTIONS IN CHEMOTHERAPY BETWEEN COMPOUNDS THOUGHT TO INTERFERE WITH TRYPANOTHIONE METABOLISM IN VIVO AND IN VITRO

The results of the monotherapy experiments showed that a number of compounds used in the present study have some antileishmanial activity when used alone. These compounds were investigated in more detail in combination experiments in vitro and in vivo by using relatively low drug concentrations so that the detection of synergistic and additive effects would be possible (Tables 23, 24 and 25, Figures 25, 26, 27a and 27b). The results of these studies have shown that these combinations were less effective than had been hypothesised. There appeared to be no more than an approximately

additive effect with any combination. Trimelarsan appeared to reduce the effect produced by all the other drugs in most cases. Combinations involving five or more drugs proved toxic to macrophages. It should be noted, however, that combinations involving so many drugs are impractical and have only a theoretical value. The overall conclusion from these experiments was that although combinations involving DFMO, fexinidazole, Berenil and Pentostam have interesting activity against L. donovani in culture and in mice, they were not apparently synergistic. There are several possible explanations for this. One possibility is that some of the drugs used in the combinations are not as effective inhibitors of the same target sites in L. donovani as they are in trypanosomes or even that these target sites differ between trypanosomes and leishmanias. It is also possible that some of these compounds such as the diamidines (see also section 4.2.4.2.1.) or nitroimidazoles may not act solely through polyamine and trypanothione metabolism and that inhibition of these pathways plays only a part in the overall efficacy of the compounds as antileishmanials. For instance, it has been demonstrated (LaRusso et al., 1977) that reductively activated metronidazole binds to bacterial and phage DNA and yeast tRNA. More recently it has been demonstrated (Sorensen et al., 1990) that the 2-nitroimidazole Ro 15-0216 targets mammalian DNA topoisomerase II and enhances enzyme-mediated DNA cleavage uniquely at specific DNA sequence elements.

Possible carrier-mediated processes or transport loci participating in the uptake and transport of more than one of the compounds may explain the antagonistic and less than additive effects observed in some combinations.

There is only one previous report, by Keithly and Fairlamb (1988), on the use of combination chemotherapy targeting trypanothione metabolism in Leishmania species. Combinations involving Berenil(R) or Pentostam(R) and DFMO had an additive effect against experimental visceral leishmaniasis and this is in agreement with the results obtained in this study.

In the final analysis, chemotherapy is only an adjunct to the process of host immunity and the immune response is an important element in the activity of most antileishmanials. The disease patterns produced by inoculation of *Leishmania* parasites in mouse strains of various genetic backgrounds vary considerably, depending on genetically determined factors contributed by both the host and the parasite. The immune response-linked genetic mechanisms underlying susceptibility or resistance to leishmania infections determine the capacity of the host to overcome, or not, an established infection through the development of immune effector mechanisms and thus critically decide the outcome of infection in its later stages. Thus the success of treatment should vary with the inherent ability of different hosts to mount an antibody response. This suggests that further studies involving testing of these compounds in different host species such as, in the case of *L. donovani*, the dog, may be needed to elucidate more fully the role of inhibition of trypanothione metabolism in *Leishmania* species by these compounds and the mechanism by which the inhibition occurs. In trypanosome infections it is well established that antitrypanosomal antibodies are adequate to clear the bloodstream of parasites during DFMO therapy and that an intact immune response is necessary for cures of the disease to be obtained (de Gee *et al.*, 1983; Bitonti *et al.*, 1985; Jennings, 1988a, b; Zweygarth and Kaminsky, 1991). An additive effect *in vitro* may be found to be potentiating *in vivo* due to additional influence of the immune response and this has been demonstrated in trypanosomes (Zweygarth and Kaminsky, 1991). Such potentiation, however, was not observed in this study. Therefore, a more adequate immune response in trypanosome infections as compared to that in leishmania infections may also serve to help explain the potentiation of drug combinations observed against the former parasites.

4.3. *IN VIVO* EXPERIMENTS INVOLVING THE DRUGS OF CURRENT USE, SODIUM STIBOGLUCONATE (PENTOSTAM^R) AND PENTAMIDINE (LOMIDINE^R), IN COMBINATION WITH DFMO AND BSO

In one attempt to maximise the effectiveness of the drugs currently being used in the treatment of leishmaniasis, sodium stibogluconate (Pentostam^R) and pentamidine, these drugs were tested *in vivo* in some detail in the Balb/c mouse model. They were used both alone, with different lengths of treatment time (Table 26, Figure 28) and in combination, by using different treatment regimens (Table 27, Figures 29 & 30). The results revealed that a more favourable response to treatment was achieved when an inhibitory concentration of the antimonial was maintained for most of the day, by using multiple low daily doses, than when a high single dose was employed. In addition, the total dose of Pentostam required to produce a particular level of suppression was lower when the drug was administered twice (rather than once) daily and for longer period of time. These findings are consistent with the known pharmacokinetics of antimony in man (Chulay *et al*., 1988) after parenteral administration of pentavalent antimonials. These pharmacokinetic data have been interpreted by Chulay *et al*., (1988) in terms of a three phase pharmacokinetic model representing an initial absorption phase (mean $t_{1/2}$=0.85 hrs), a very rapid elimination phase (mean $t_{1/2}$=2.02 hrs) which is responsible for the removal of most of the drug from the body, and a slow elimination phase (mean $t_{1/2}$= 38-76 hrs) which provides evidence for some accumulation of the drug. These results are also in accord with the findings of Chulay and his associates (1983) who reported that treatment of human visceral leishmaniasis in Kenya with sodium stibogluconate at a dose of 10 mg Sb^V/kg every 8 hrs for 10 days appeared to be a safe alternative to conventional treatment.

Pentamidine by itself showed no appreciable activity in this mouse model and in addition it caused some toxic side effects and mortality of a few mice. The poor efficacy of pentamidine against leishmania in mice, however, has been noted previously by Trotter

and his associates (1980a, b), who found it to be inactive at 50 mg/kg/day x 5, against L. major, L. mexicana amazonensis and L. donovani.

Another possibility investigated in this study, was the combination of DFMO with Pentostam and pentamidine (Table 27, Figure 29). The potential advantage of this combination is that all of these drugs have been passed for use in humans. These combinations were carried out with the hope that DFMO might lower the Pentostam or pentamidine dose necessary to suppress infection. Keithly and Fairlamb (1988) have previously reported the use of DFMO singly and in combination with Pentostam against L. b. guyanensis, L. m. mexicana and L. donovani infections in mice (BALB/c ByJ). The effect was additive at best, and in L. mexicana infections suppression was only marginally better than treatment with DFMO or Pentostam alone. Similar results were obtained in this study. Moreover, addition of pentamidine resulted in an antagonistic effect. The combination of Pentostam, pentamidine and BSO was also tested but showed little promise (Table 27, Figure 30). Thus, these combinations are also rather disappointing and not worth pursuing.

4.4. A STUDY ON THE PREVENTION OF THE RELAPSE OF VISCERAL LEISHMANIASIS IN THE MOUSE MODEL, USING COMPOUNDS THOUGHT TO INTERFERE WITH TRYPANOTHIONE METABOLISM

A problem in the chemotherapy of visceral leishmaniasis is the relapse of the disease after an apparently successful treatment. Thus it was of interest to develop an experimental relapse model of leishmaniasis in the Balb/c mouse and test the efficacy of the new compounds and most promising drug combinations, in clearing the parasites not only from liver but also from spleen and bone marrow. It is already known that the Balb/c mice maintain chronic heavy infections involving mononuclear phagocytes throughout the body (Blackwell et al., 1980) and this was also observed in this study where parasite load maintained at high levels until the end of the experiment (DAI 80). The results clearly demonstrated (Table 28,

Figure 31) that treatment with both iv and ip administration of sodium stibogluconate solution (Pentostam^{R}) greatly reduced the number of parasites found in liver but to a lesser extend the parasite load in the spleen and bone marrow, as determined on day 23 after infection (DAI 23) and this is in agreement with previous reports (Carter et al., 1988; Carter et al., 1989). The low parasite loads found on DAI 23 persisted and no increase in numbers occurred over the next 15 days. Eventually, this apparent resistance does wane and liver parasite burdens in sodium stibogluconate-treated animals are known (Carter et al., 1988; Carter et al., 1989) to return to control levels by day 50 post drug treatment. In this study, however, this was confirmed only for spleen and bone marrow parasite loads, whereas liver parasite loads, although showing an increase, did not reach the control levels. This could be a consequence of the higher dose level of the antimonial used in this study and its known (Chulay et al., 1988) slow terminal elimination phase suggesting that the drug persists in the liver for longer period of time. Although the total organ content of antimony might be unmeasurable, there could be sufficient accumulation of drug in a subcellular region such as the lysosomal compartment which would be sufficient to suppress parasite growth. There is also some evidence from other investigations on the phagocytic activity of macrophages, that liver macrophages might have been left in an activated state following chemotherapy using sodium stibogluconate (Carter et al., 1989). Treatment did not have a similar effect on spleen and bone marrow parasite load. This could reflect heterogeneity in macrophage populations as it has been shown that macrophages from different tissues vary in their susceptibility to L. donovani infection and that Lsh gene is expressed variably in different tissues (Olivier and Tanner, 1987). Thus it is not inconceivable that infection with L. donovani has different effects on different macrophage populations and that cells from these populations respond differently to drug treatment. It is also possible that the drug shows inability to reach the infected macrophages at spleen or bone marrow sites. The low rate of blood perfusion through spleen and

bone marrow compared with liver and the high initial clearance rates of drug through the urine (Chulay et al., 1988) tends to support this latter proposition. However, it should be noted that neutron activated analyses showed that antimony reached the spleen of mice and 24 hrs post-dosing antimony concentrations in the liver and spleen were comparable (Hunter et al., 1988).

The results from use of the drug combinations (Table 29, Figures 32, 33, 34 & 35) showed them to reduce significantly the number of parasites not only in liver but also in spleen and bone marrow. They did not give a total clearance of the parasites but they proved to be more effective than Pentostam itself. The percentage suppression in liver as determined on DAI 39 was higher than that of DAI 23 in combinations other than the combination of Pentostam with DFMO. This could be due to the presence of residual drug with slow action such as has been reported with Berenil (see section 4.2.4.2.1).

In some groups of mice the presence of bacteria in the organs which were screened gave evidence of acquired infection with other organisms and a competitive relationship could have resulted in an apparent increase of the percentage suppression of the combination such as in the case of the combination involving Pentostam and DFMO. Eperythrozoon are known to affect immune responses to protozoa and the course of infection (Peters, 1965; Ott and Stauber, 1967).

Particular attention was given to DFMO to assess the potential of the drug for preventing the relapse of visceral leishmaniasis. It showed good activity but did not give a total clearance from all tissues (see section 4.2.4.1.).

Thus, the results obtained, presented in section 3.2. and discussed in section 4.2. of this thesis, showed that many of the compounds thought to inhibit the synthesis or interfere with the activity of trypanothione have antileishmanial activity. However, none of the combinations appeared to be synergistic under the conditions used although at high concentration they were better than sodium stibogluconate itself. At the very least, we can say that there are indications that the polyamine and trypanothione pathways of leishmanias are somehow involved in the cytotoxicity of most of

the compounds tested.

Trypanothione metabolism has potential as drug target that has yet to be fully realised. Although studies on the metabolism and functions of trypanothione in trypanosomatids are still in their infancy, present work is helping to optimise combination therapy of existing drugs and to provide a rational approach for development of new and better drugs. This study was simply a step towards this direction. Where do we go from here? With the modern techniques now available for drug development and drug delivery, perhaps we should be updating our approach to the problem of chemotherapy. Many more metabolic studies are needed to unravel at the molecular level the biochemical pathways that occur in trypanosomatids. This information should enable an increasingly more rational approach to be taken in the design of molecules to be synthesised and tested. In trypanosomatids trypanothione reductase (TR) is essential for maintaining trypanothione as $T[SH]_2$ and therefore represents a target for the design of new trypanocidal drugs. The gene encoding TR from the cattle pathogen _T. congolense_ has been cloned, sequenced (Shames _et al_., 1988) and expressed in _Escherichia coli_ (Sullivan _et al_. 1989) and shows more than 50% homology with human glutathione reductase (GR) (Shames _et al_., 1988). Sequence data from _L. donovani_ (Taylor _et al_., 1989) support these findings, suggesting similar reaction mechanisms for GR and TR. However, there are also important differences. TR will not reduce GSSG, neither human GR will reduce $T[S]_2$ to any significant extent (Shames _et al_., 1986). In addition, the mixed disulfide of N^1-glutathionylspermidine and glutathione and the N^1-glutathionylspermidine disulfide are substrates for TR but not GR (Henderson _et al_., 1988). Thus these findings suggest that TR targeting in trypanosomatids is a promising approach for drug design.

Molecular modelling of both enzyme and substrate and the accurate elucidation of the three dimensional structure of TR is necessary in order to assist in the design of its specific inhibitors. One such attempt is the recent modelling study of _T. congolense_ TR (Murgolo _et al_., 1989).

4.5. THE EFFICACY OF A DRUG-CARRIER SYSTEM, OCTYL DEGROL OIL, IN THE CHEMOTHERAPY OF VISCERAL LEISHMANIASIS

One alternative to finding new drugs involves improving delivery of existing compounds to the target cell, thus decreasing the toxicity to the host cell and improving the therapeutic index. During recent years several attempts have been made to improve the efficacy of existing anti-protozoal drugs by increasing their bio-availability to the parasite. For example, daunorubicin, a potent trypanocide _in vitro_ but inactive _in vivo_, was conjugated to bovine serum albumin or ferritin and regained its _in vivo_ activity (Williamson and Scott-Finnigan, 1978). The most dramatic results of targeted chemotherapy, however, have been those obtained using liposome- or niosome-entrapped drugs against macrophage-inhibiting protozoa. Preferential uptake of drug-loaded vesicles by cells of the reticulo-enthothelial system target the drug passively to the infected phagocytes of this tissue. The potential of drug-loaded vesicles for the delivery of pharmacological agents to macrophage dwelling parasites was quickly recognised. Liposomes (Alving, 1986) and niosomes (Baillie _et al_., 1986) containing sodium stibogluconate were shown to be more effective than free drug in the treatment of experimental visceral and cutaneous leishmaniasis (see also section 4.2.4.5.).

One of the drawbacks in using sodium stibogluconate in the chemotherapy of leishmaniasis, is its rapid metabolism and very short half life in the mammals (Otto _et al_., 1947; Pamplin _et al_., 1981; Chulay _et al_., 1988). In one attempt to overcome this and achieve effective drug levels for longer, a new experimental compound, octyl degrol oil, which was effective in allowing slow release of drugs, was investigated. The preliminary results I obtained (Table 30, Figures 36 & 37) showed the drug in suspension with the octyl degrol oil to be more efficacious than the free drug, suggesting that further experiments for evaluating octyl degrol oil as a slow-release drug carrying system for leishmaniasis, may prove to be worth pursuing. Unfortunately there is as yet no

other information available on this new carrier system and lack of material prevented me from carrying out more experiments with it.

4.6. THE ANTILEISHMANIAL ACTIVITY OF DNA-INTERCALATORS, PLATINUM AND RHODIUM DRUG COMPLEXES AND 8-HYDROXYQUINOLINES

The results from testing these experimental compounds, showed the 8-hydroxyquinoline compounds and the majority of DNA intercalators to have interesting antileishmanial activity against promastigotes of L. donovani, whilst the platinum and rhodium drug complexes were relatively inactive with the exception of Rh (III)-T which showed some activity at the highest level tested (100 µg/ml) (Table 31, Figures 38, 39 and 40). There was insufficient material available to allow in vivo testing. A number of related platinum and rhodium drug complexes, tested against trypanosomes in vivo (Osuna et al., 1987; Jennings, unpublished results) and against L. donovani amastigotes in vitro and in vivo (Croft et al., 1990) were reported to have some activity and it has been suggested (Croft et al., 1990) that the amastigote-mitochondrial complex is the primary site of their action. These compounds possibly warrant further study.

There have been no previous reports on the activity of the 8-hyroxyquinolines against Leishmania species, although several related quinoline compounds tested showed very interesting antileishmanial activity in vitro and in vivo, being in some cases several hundred times more potent than the standard pentavalent antimonial meglumine antimoniate (Hanson et al., 1977; Kinnamon et al., 1978; Peters et al., 1980a). The highest activity was shown by the quinoline compound, WR 6026 which was 474 times as active as meglumine antimoniate when given im and 708 times as active as meglumine antimoniate when given orally. WR 6026 was also tested against visceral leishmaniasis in dog giving a 94% reduction in liver parasite load at 3.25 mg/kg/day for 5 days (Chapman et al., 1979). Clearly these compounds have great potential.

There have been several reports on antiprotozoal drugs such as the diamidine compounds and some nitroimidazoles which themselves

or their metabolic products interfere with DNA synthesis by binding and degrading intact DNA. There are no previous reports on these newly synthesised DNA intercalators tested in this study. The promising results obtained in this study with the DNA-intercalators, suggest that further evaluation of these compounds would be worthwhile.

Overall, the present study has provided information on the susceptibility of L. donovani to a number of drugs and drug combinations. Many were relatively ineffective but some deserve further study and possibly could lead to better ways of treating canine leishmaniasis.

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