ISSN 0325-951X

PROBLEMATICA DE LA ENFERMEDAD DE CHAGAS
Simposio internacional. Academia Nacional de Medicina
Buenos Aires, 19-20 abril 1999

MEDICINA (Buenos Aires) 1999; 59 (Supl. II): 179-187

FUTURE PROSPECTS FOR THE CHEMOTHERAPY OF CHAGAS' DISEASE

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Abstract Over the last two decades, progress towards new drugs for the treatment of Chagas' disease has been disappointing. However, as a result of the parasite genome sequencing projects, the possibility of identifying novel drug targets through genomics, proteomics and bioinformatics has never been better. Progress towards the development of novel therapeutics, from target identification and validation by chemical and genetic means through to rational drug design, is illustrated with reference to the metabolism and functions of trypanothione, with particular emphasis on trypanothione reductase, one current drug target of choice.

Resumen ***Perspectivas futuras de la quimioterapia de la enfermedad de Chagas.*** Durante las dos últimas décadas no se han realizado progresos satisfactorios sobre drogas eficaces para el tratamiento de la enfermedad de Chagas. Sin embargo, como resultado de estudios sobre la estructura del genoma del *Trypanosoma cruzi,* de la estructura molecular de las proteínas y de la aplicación de la bioinformática, se ha generado la posibilidad de identificar en el *Trypanosoma cruzi* nuevos blancos para medicamentos específicos. El progreso hacia una nueva quimioterapia de la enfermedad de Chagas se ha facilitado por el diseño racional de moléculas activas. El descubrimiento de la tripanotiona, una molécula característica de los tripanosomatideos y el conocimiento de su metabolismo y funciones, lo mismo que el de la enzima tripanotiona reductasa, ha permitido identificar nuevos blancos para fármacos antichagásicos mas eficaces que los utilizados hasta ahora.

Key Words: drug discovery, trypanothione reductase, target validation, trypanosomiasis, leishmaniasis

There is an indisputable and urgent need for new, effective and safe drugs for the treatment of Chagas' disease, particularly for the late-stage of the disease[1-4]. Although public health measures such as vector control have greatly decreased the incidence of new infections, that is of little consolation for the 18 million or so people currently infected with *Trypanosoma cruzi*. Moreover, if any lessons are to be learned from previous malaria eradication programmes one ignores the risk of the emergence of insecticide resistance at one's peril. Alternative modes of transmission of the disease by blood transfusion, by transplantation or transplacentally continue to be of clinical concern. Thus, new drugs are also required for the effective treatment of the acute phase of infection in order to replace the currently available drugs, nifurtimox and benznidazole, which are often poorly tolerated and frequently ineffective.

Interest within the pharmaceutical industry in drug discovery against tropical diseases and other 'orphan diseases' has been in decline for several decades. The major reason for this is economic. In an increasingly global and competitive market, one only has to compare the high development costs (currently estimated at US $ 200-400 million per successful product) with the poor prospects of a reasonable economic return to appreciate the nature of the problem. Given this depressing situation, scientists have been stimulated to find alternative and less costly approaches towards drug discovery by 'rational drug design[5-9] and also to develop improved therapeutic strategies using existing drugs through combination chemotherapy or optimisation of drug administration[10].

Strictly speaking, it is not yet possible to 'rationally' design a new drug and the term 'rational inhibitor design' is to be preferred. Converting an effective inhibitor into a useful drug depends on a number of important biological factors that fall under the broad disciplines of pharmacology and toxicology. While some of these undesirable properties of a molecule (e.g. poor uptake, rapid metabolic inactivation, inappropriate tissue distribution and rapid excretion) can sometimes be predicted and avoided or overcome through chemical modification of the lead compound, the discipline of 'rational pharmacology' is still in its infancy. Thus 'rational drug design' from bench to bedside is still not realisable in practice.

The key features of current thinking in drug discovery involve identification of a novel drug target, its isolation and detailed characterisation of its molecular and kinetic

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properties, target validation by chemical or genetic means, the identification of lead inhibitors and their subsequent optimisation to improve their pharmacological and toxicological properties. Each of these steps is considered in more detail below. This is followed by an overview of trypanothione metabolism with special reference to *T. cruzi* and an analysis of how far trypanothione reductase has progressed as a chemotherapeutic target.

Target identification

To date, chemotherapeutic targets have been identified either through comparative biochemistry (e.g. ergosterol), comparative biology (e.g. the kinetoplast), or through studies on the mode of action of experimental compounds or drugs (e.g. trypanothione). The principal broad areas for chemotherapeutic intervention are listed in Table 1 and the reader is referred to various reviews for detailed information on the current status of these potential targets. As pointed out in a recent review, many of these remain to be unambiguously demonstrated to be *bone fide* drug targets[11].

For the future, the advent of parasite genome projects means that the prospects for the identification of entirely novel therapeutic targets are excellent (Figure 1). In theory, once the genome of *T. cruzi* is completed, all of the potential drug and vaccine candidates in the parasite will be known. In practice, interpreting the full significance of this wealth of information will undoubtedly be a major challenge, particularly for those working in the emerging fields of functional genomics, proteomics and bioinformatics. In my view, these new disciplines should be regarded as complementary tools to be added to the classical approaches of drug discovery rather than as replacements.

The ideal drug target

There are several criteria that need consideration in selecting a potential drug target. First, it should be essential for the survival of the parasite in the appropriate life cycle stage (i.e. the amastigote). An enzyme or pathway that converts a prodrug into a toxic compound would be an exception to this caveat (see below). Second, an ideal target should be uniquely found in the parasite and absent from the human host. The biosynthesis of ergosterol and trypanothione are two excellent examples in *T. cruzi*. In reality, enzymes or metabolic pathways are rarely unique and therefore the chosen target must be sufficiently different from the host homologue to permit selective inhibition. (Although it is true that a single amino acid difference in the active site of an enzyme can result in selective inhibition with the appropriate compound, it should be noted that a single point mutation in the target gene of the parasite can lead to complete resistance!). A second alternative is that the chosen target may be essential for parasite survival, but due to by-pass pathways, non-essential for the host. Finally, for rational drug design, the target should be amenable to study at the mechanistic and structural level, ideally a small soluble enzyme and/or simple metabolite.

Target validation

Having identified a potential target that meets these criteria it is necessary to demonstrate the essential nature of the molecule for the growth and survival of the parasite. This is called 'target validation' and can be achieved by either chemical or genetic methods, preferably both (Figure 1).

The classical method involves the use of selective inhibitors or existing drugs that specifically inhibit the target within the intact parasite and have a cytostatic or cytotoxic effect. 'Chemical validation' with novel compounds that are structurally unrelated to any existing drugs has the added advantage of providing a useful novel chemical perspective for future inhibitor design. Ideally a series of analogues with a range of potencies should be tested to show that there is a correlation between target sensitivity and cytocidal (or cytostatic) activity. Unfortunately, variable uptake, metabolism or excretion of ex-

TABLE. *Potential Chemotherapeutic Targets in* T. cruzi

Trypanothione (thiol, polyamine and AdoMet metabolism)	5, 22, 87-89
Purine salvage (transport and interconverting enzymes)	87, 90
Folate and pteridine metabolism (e.g. DHFR-TS & PTR1)	91
mRNA biosynthesis (*trans*-splicing and cap biosynthesis)	92, 93
Kinetoplast (RNA editing and kDNA replication)	94, 95
Ergosterol biosynthesis (e.g. sterol C-14 demethylase)	2, 96-98
Transialidase, GPI-anchor and ether lipid biosynthesis	99-101
Proteinases (e.g. cysteine proteinases)	102-104
Glycosome (energy metabolism)	105-108

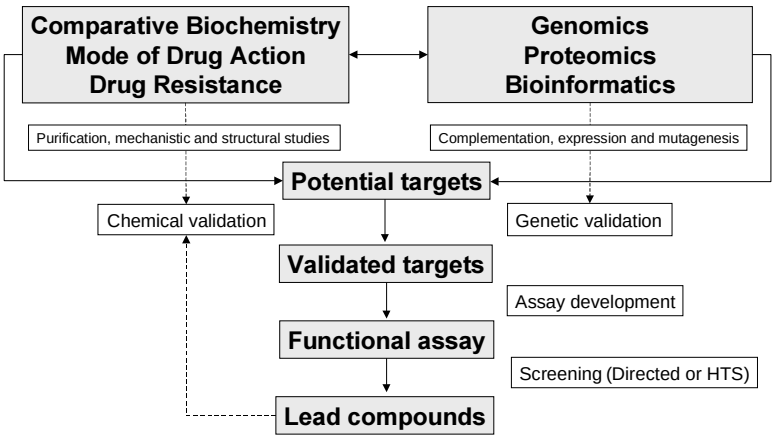

Fig. 1.– Chemical and genetic approaches to target discovery and validation.

perimental compounds by the parasite may obscure such a correlation.

In the pharmaceutical industry, with its access to vast numbers of chemical compounds (collections of synthetic chemicals, natural products and combinatorial chemical libraries), chemical validation is the preferred method. Potential targets are evaluated by initially identifying lead inhibitors by high throughput screening (HTS) against the isolated target and subsequently demonstrating that leads are toxic to the intact parasite.

In an academic environment, with limited access to such chemical diversity, "genetic validation" is often the preferred approach. This involves elimination or alteration of a target's function by various forms of genetic manipulation. The strength of this approach is that deletion of all copies of a gene encoding a target results in complete elimination of the product. It is therefore equivalent to achieving 100% inhibition by a chemical compound, something that may be difficult to achieve in the absence of specific and potent enzyme inhibitors. If a null mutant can be produced then the target is not essential for survival of the parasite. However, this sweeping statement needs some qualification. First, it should be born in mind that gene 'knockouts' are usually carried out in the insect vector stage of the life cycle where the gene may not be essential. Second, phenotypic studies in rich culture medium may not adequately reflect the nutritional and environmental conditions encountered in the human host. Third, the non-essential nature of a gene does not always mean that the enzyme or pathway is not a drug target. The salvage of purines, which are essential for RNA and DNA synthesis in all trypanosomatids, provides an interesting example. Although lines of *L.donovani* lacking hypoxanthine-guanine phosphoribosyltransferase (HGPRT) can grow normally due to multiple by-pass pathways[12], they are >1,000-fold resistance to allopurinol[13], as a consequence of their inability to convert the pro-drug allopurinol into its toxic nucleoside triphosphate analogue[14]. Thus, in this example, genetic manipulation validates the mode of action of allopurinol and alerts us to a possible mechanism by which resistance might arise. Clearly, a thorough knowledge of the cell biology and biochemistry of the particular life-cycle stage of an organism is important for correct interpretation of this type of functional genomic approach.

Another current limitation of gene disruption or gene deletion in *T.cruzi* and *Leishmania spp.* is that null mutants cannot be produced for genes that are essential for survival. In *Leishmania spp.* attempts to eliminate essential genes by sequential rounds of gene replacement with drug resistance markers leads to changes in allelic copy number through alterations in ploidy or changes in chromosome structure[15, 16]. Such changes are often considered to be diagnostic that the product of a particular gene is essential. Methodological difficulties associated with gene targeting such as inducing an adverse effect on the expression of an adjacent gene need to be ruled out. Therefore, it is advisable to demonstrate that chromosomal null mutants can be achieved in the presence of an episomal copy of the gene[17].

In the case of the African trypanosome, inducible expression systems are available which allow conditional expression of a gene in the presence of a chemical inducer such as tetracycline[18]. One can therefore produce a transgenic organism in which the chromosomal allelic copies of the gene have been deleted by means of homologous replacement with a drug-resistance gene, but contain a supplementary copy of the target gene in another locus whose expression is under control of an inducing agent. By altering the amount of inducer in the medium one can therefore study the phenotypic effects

of different levels of gene expression. This powerful and sophisticated tool has yet to be developed for *T. cruzi.*

The Drug Discovery Process

Limitations of space do not allow a full treatment of this subject and the reader is referred elsewhere for further details[19, 20]. However, some of the salient features are discussed below.

Once a target has been validated, a functional assay must be developed for high-throughput screening against diverse molecular libraries generated by combinatorial chemistry or other large compound collections. Depending on the nature of the target, colourimetric, fluorescence, luminescence or radioisotopic methods are possible. The automated assay has to be specific, cheap, reproducible and capable of miniaturisation to fit a 96-, 384- or even 1536 microtitre-plate-assay format[21]. A typical HTS programme may test 100,000 compounds per day and screen about one million samples in total, so the functional target usually has to be produced by expression in a recombinant system. Parallel screening or secondary screening of potential leads against the human homologue may be required to maximise selectivity.

Combinatorial chemistry is undoubtedly successful in identifying large numbers of lead inhibitors. However, many leads are eliminated during screening against the intact parasite *in vitro* or *in vivo* because a compound does not enter the parasite or lacks selectivity. Those that do show activity need to be prioritised and optimised not only in terms of potency against the target, but also for enhanced activity against the parasite *in vitro* and *in vivo* and for ease and diversity of chemical synthesis. Where possible, structure-based drug design[5-9] is used to guide analogue synthesis for evaluating structure-activity relationships within a series of compounds. It should be noted that selection of the best drug candidate for pre-clinical development will also depend on the pharmaceutical features of the compounds and not simply on the potency against the isolated target. Safety is as important as efficacy of a drug candidate. Preliminary toxicology and pharmacology studies in animals will give an indication of likely undesirable side effects that may arise in pre-clinical development.

Even with the application of rational inhibitor design and rational pharmacology, the rate of attrition of drug candidates is still depressingly high: less than 20% of research projects reach clinical trial and only 10% of compounds in development achieve registration. For this reason, several drug targets and several candidate drugs should be at various stages of the development pipeline to guard against the high price of failure.

Metabolism and functions of trypanothione

Before considering the prospects of the development of a new drug against trypanothione reductase, it is relevant to consider what is known about the general metabolism and functions of trypanothione.

Trypanothione (N^1,N^8-*bis*(glutathionyl)spermidine, $T[SH]_2$) has only been found in parasitic trypanosomes and leishmania and constitutes the major acid-soluble thiol in these cells [22]. The biosynthetic pathway to $T[SH]_2$ and the central role of trypanothione reductase (TryR) is illustrated in Figure 2. *T. cruzi* differs from *T. brucei, C.fasciculata* and *Leishmania spp.* in that it is unable to synthesise putrescine (a diamine) *de novo* and is entirely dependent on exogenous diamines or polyamines (e.g. spermidine) for growth[23]. Consequently, it possesses discrete transport systems for polyamines and diamines that can be regulated in response to intracellular requirements for polyamines[24]. *T. cruzi* is less restrictive in the polyamines it will take up from the medium and conjugate with glutathione than other trypanosomatids. For example, it can use cadaverine instead of putrescine to synthesise aminopropylcadaverine to form the homotrypanothione analogue of trypanothione[25]. This lack of discrimination for putrescine and spermidine may be another target for therapeutic attack.

A number of important protective, regulatory and metabolic functions have been ascribed to this metabolite and its associated enzymes. One of the principal protective functions of trypanothione involves defence against oxidant stress by the detoxification of free radicals (R·), the removal of peroxides (ROOH) and the reduction of disulphides (RSSR) (Fig. 2)[22].

Free radicals that are formed during normal aerobic metabolism or as a consequence of reductive metabolism of the nitrofuran drugs, nifurtimox and benznidazole, can be rendered harmless via non-enzymatic interaction with $T[SH]_2$ or other thiols such as glutathione (GSH) forming trypanothione disulphide ($T[S]_2$) and glutathione disulphide (GSSG), respectively[26-28].

Like other organisms, accumulation of disulphides would adversely affect thiol-redox balance and general metabolic activity of the trypanosome. Thus, $T[S]_2$ is constantly regenerated to $T[SH]_2$ by means of TryR, an NADPH-dependent flavoenzyme[29-31]. Since trypanosomatids lack glutathione reductase, reduction of GSSG and other disulphides (RSSR) is a two-step process. First, GSSG undergoes thiol-disulphide exchange with $T[SH]_2$ to form 2 GSH and $T[S]_2$. Second, $T[S]_2$ is reduced by TryR as above. Although exchanges between thiols and disulphides occur readily non-enzymatically in other trypanosomatids, *T. cruzi* possesses a trypano-thione-glutathione thiol transferase (TGTT) to catalyse this process[32, 33]. The reason for this is not known, but it is pos-

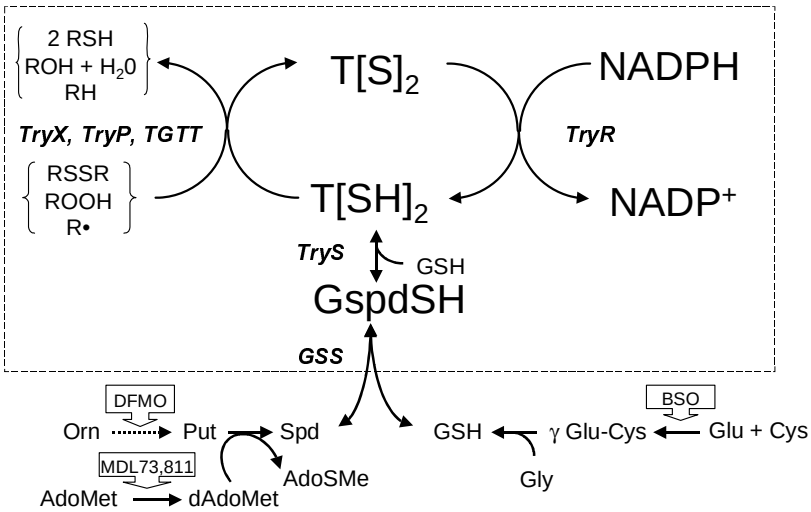

Fig 2.– Biosynthetic pathway to trypanothione and its antioxidant functions in *T.cruzi*. The boxed area is unique to the parasite whereas the pathway for the synthesis to the precursors glutathione (GSH) and spermidine (Spd) are also present in the host. Unlike mammalian cells and other trypanosomatids *T.cruzi* lacks ornithine decarboxylase and is unable to synthesise putrescine (Put) *de novo* (dotted arrow). The enzymes that are unique to the parasite are glutathionylspermidine synthetase (GSS), trypanothione synthetase (TryS), tryparedoxin (TryX), tryparedoxin peroxidase (TryP), trypanothione:glutathione thiol transferase (TGTT) and trypanothione reductase (TryR).

sible that trypanothione levels could become rate limiting in *T. cruzi* when the cells are starved of polya-mines[23]. However, the amastigote stage resides in the cytosol of the host cell and consequently should be bathed in an abundance of polyamines. Gene knockout experiments should shed further light on the role of this unusual enzyme.

Another inevitable consequence of aerobic metabolism in all organisms is the formation of peroxides. Removal of hydrogen peroxide and organic peroxides is achieved by means of TryR, $T[SH]_2$, tryparedoxin (TryX)[34] and tryparedoxin peroxidase (TryP)[35], which together constitute the 'trypanothione peroxidase system' originally discovered in *T.brucei* and *C. fasciculata*[36]. Recen-tly, the genes for TryP and TryX have been cloned, sequenced, expressed and functionally reconstituted with TryR[37-39]. It has been claimed that *T. cruzi* does not have a specific trypanothione peroxidase system[40]. However, as a result of the *Leishmania* genome project a functional trypanothione peroxidase system has been discovered in *Leishmania spp.* [41], and, since similar genes are present in the *T.cruzi* genome project database, this assertion needs careful re-evaluation.

Another possible protective function of $T[SH]_2$ involves the metabolic inactivation of foreign chemicals (xenobiotics) and other toxic compounds such as heavy metals. A glutathione *S*-transferase has been purified from *T. cruzi* that could play such a role[42, 43]. However, the isolation of this enzyme predates the discovery of trypanothione and it is not known whether this enzyme will accept $T[SH]_2$ rather than GSH. In leishmania, resistance to trivalent antimonial and arsenical compounds is mediated via a pump that excretes conjugates of trypanothione and heavy metals[44, 45]. It has been proposed that a 'trypanothione *S*-transferase' could be involved, however, such an enzyme has not been identified in leishmania so far[46].

Regulation of intracellular thiol-redox balance has been proposed as a means of regulating intracellular metabolic processes in many organisms. However, definitive evidence for such a regulatory role is still lacking[47]. In mammalian cells, regulation of the levels of intracellular polyamines is critical for growth and differentiation[48-51]. However, in *T.brucei* the rate limiting step catalysed by ornithine decarboxylase does not appear to be controlled in the same manner as in other cells[52-55] and polyamine transport is absent[56]. In *C. fasciculata* the levels of trypanothione and its precursor, glutathionylspermidine, are regulated in response to growth conditions[57]. It is possible that accumulation of glutathionylspermidine at the expense of trypanothione acts as a store for spermidine that can be rapidly released once the cell encounters

favourable growth conditions as found for *Escherichia coli* [58]. *E. coli* is the only non-trypanosomatid found so far that is able to synthesise glutathionylspermidine (but not trypanothione)[59]. Significantly, glutathionylspermidine synthetase (GSS) from both *E. coli* [60] and *C.fasciculata* [61] are actually bifunctional enzymes that possess a distinct amidase domain capable of hydrolysing glutathionylspermidine to glutathione and spermidine in addition to the domain catalysing ATP-dependent synthesis of glutathionylspermidine from glutathione and spermidine. How these two opposing reactions are controlled to prevent futile hydrolysis of ATP is not known. It will be interesting to see whether the *T. cruzi* enzyme possesses a similar capability. Since the levels of trypanothione and glutathionylspermidine in *T.cruzi* epimastigotes reflect the availability of polyamines and diamines in the medium[23], intracellular levels of free polyamines may be regulated in a similar manner to *C. fasciculata*.

The amino acid sequence[37, 38] and three-dimensional structure[62] of tryparedoxin shows some similarity to thioredoxin from other organisms. Like thioredoxin is other organisms, it has been suggested that reduction of ribonucleotides may involve transfer of reducing equivalents from NADPH via TryR, $T[SH]_2$ and TryX to ribonucleotide reductase[63]. Further work is required to establish whether this is the sole route for the formation of deoxyribonucleotides since this may represent yet another 'Achilles heel' for chemotherapeutic attack.

Trypanothione reductase as a chemotherapeutic target

Given the central role of TryR in the anti-oxidant functions of trypanothione and the fact that glutathione reductase (GR) is absent from trypanosomatids, TryR has received most attention as a potential therapeutic target. Human GR and parasite TryR show a pronounced ability to discriminate between their disulphide substrates[29, 64, 65], so that design of selective inhibitors of TryR is a strong possibility. The enzyme from *T. cruzi* has been purified and extensively characterised for its enzymatic properties[31, 66] and catalytic mechanism[67] and its three-dimensional structure determined to high resolution with substrates and inhibitors[68-71].

A number of existing therapeutic agents and experimental compounds with trypanocidal activity *in vivo* and *in vitro* are inhibitors of TryR[28, 30, 72-82]. However, these observations can not be interpreted as chemical validation of the essential nature of this drug target, since the compounds are not sufficiently selective or potent. We have therefore attempted reverse genetic approaches to demonstrate the essential requirement for TryR by expression of anti-sense RNA[83], overexpression of wild-type[84] or dominant-negative mutant TryR[85] and by gene disruption[86] or gene replacement[17]. In *T. cruzi*, we were unable to down-regulate TryR activity using plasmids expressing anti-sense RNA due to specific rearrangements in a proportion of the plasmids that flipped over the *TRYR* gene to the sense orientation[83]. In *Leishmania spp.,* it proved impossible to disrupt or delete all *TRYR* alleles, indicating that leishmania are unable to survive in the complete absence of TryR activity. Partial reduction (50-85%) of TryR activity does not affect growth in culture as promastigotes or amastigotes. However, these organisms are more sensitive to oxidant stress in activated macrophages, but not to exogenously added hydrogen peroxide[85, 86]. Similar unpublished studies in collaboration with Professor Clayton's laboratory using a tetracycline-inducible system indicate that African trypanosomes show increased sensitivity to oxidant stress once the level of TryR activity falls to 10% of normal. Moreover, infectivity and virulence in mice is dependent on tetracycline-regulated TryR activity: in the absence of tetracycline an infection can not be established.

Thus, all of the currently available evidence indicates that TryR is a valid target for drug design in *Trypanosoma* and *Leishmania spp.* However, greater than 90% inhibition may be required to kill the parasites in the absence of oxidant stress. The essential requirement for TryR also implies that its substrate is also essential and therefore biosynthesis of trypanothione (and/or homotrypanothione in *T. cruzi*) is also likely to be a chemotherapeutic target in these parasites.

Based on the data obtained thus far for chemical and genetic validation of TryR as a drug target, Glaxo-Wellcome has commenced a collaborative project for the development of an assay for HTS. Once leads have been identified for optimisation, we should be in a strong position to test the feasibility of chemotherapeutic intervention in animal models. Although it is hoped that a single drug might be developed against three major parasitic diseases, the requirement for a drug to reach therapeutic levels in the heart, central nervous system and the liver, spleen and bone marrow may prove too exacting: separate products may be required for each disease. Although the costs of drug development are high, with the financial help of WHO/TDR and other charitable organisations, they are not insurmountable.

Acknowledgements: I am most grateful to the Wellcome Trust for their continuing support. I thank Dr. WN Hunter for his useful comments.

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