Validating targets for antiparasite chemotherapy

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SUMMARY

The enzymes and receptors in parasites that can be qualified as targets for antiparasite chemotherapy should perform essential functions in the parasites and demonstrate some feasibility for selective inhibition. They can be tentatively identified through detailed analysis of various aspects of metabolisms in the parasites or elucidation of the mechanisms of action among proven antiparasitic agents. Preliminary verifications of these putative targets can be indicated by *in vitro* antiparasite activity of an inhibitor of the target. However, before a major long-term effort to pursue in-depth structure-activity analysis of the target is to be committed for specific inhibitor design, further validations of the target are essential to insure that future studies are not misguided. One old-fashioned approach to validate a target in the pharmaceutical industry is by correlating target inhibitions with antiparasitic activities among large numbers of drug derivatives. The results are often indicative but hardly ever conclusive. Another method is by comparing the putative drug targets between the drug-sensitive and the drug-resistant parasites for potential discrepancies. Unfortunately, the latter often result from indirect causes, such as reduced drug transport, instead of an alteration of the drug target itself. The third experimental approach is by disrupting the gene encoding the putative target in parasite, which can provide the most conclusive evidence on whether the target plays an indispensible role in the parasite. But special conditions are needed for the gene knockout mutants to survive to exhibit their phenotypes and to allow genetic complementation studies for further verifications. Furthermore, gene knockout experiments are often difficult to perform on cells of multiple ploidy or genes of multiple copies, and are currently applicable only to a limited number of protozoan parasites. In the current article I have tried to take a cursory look at some eleven putative drug targets among various parasites, each supported by well-established antiparasitic agents identified as its inhibitors. I have also considered the evidence for validity of each of them and the potential means of further verifying their validity.

Key words: Dihydropteroate synthetase, Pyruvate: ferredoxin oxidoreductase, Trypanothione reductase, Nucleoside phosphotransferase, Glycosome, Lanosterol C-14α demethylase, Dihydrofolate reductase-thymidylate synthetase, Purine phosphoribosyltransferase, Mitochondrial electron transport, Chloride ion channel, Ornithine decarboxylase.

INTRODUCTION

Parasitic infections should be relatively easy to treat, when comparing with cardiovascular disorders or cancer, because the aetiologic agents causing these diseases are clearly identified in almost all the cases. Recent successes in the *in vitro* cultivations of many of the important parasites have enabled us to study these organisms by methods similar to those employed in investigating bacteria, fungi and nematodes, including biochemistry, molecular biology, cell biology, structural biology and developmental biology. These new approaches to understand the parasites are becoming increasingly important for discovering targets for antiparasite chemotherapy. The classical methods of screening natural products and synthetic chemicals in parasite-infected animal models or tissue cultures are yielding diminishing returns nowadays, because easy discoveries have already been made in the past. Assays with much enhanced sensitivities become necessary for detecting active ingredients of minute quantities among natural products. Recent development of new technologies in making various combinatorial libraries of novel chemicals further demands the availability of assays capable of monitoring the inhibitory activities from an extremely low quantity of drugs. Thus, identification of an enzyme or a receptor in a parasite with an essential role in sustaining the survival or growth of the latter is becoming an increasingly important task. Assays for such an enzyme or receptor should be capable of detecting inhibitors with a very high sensitivity.

Some parasites inhabit enriched living environments when compared with free-living organisms. This has led to specific metabolic adaptations, including deficiencies in the metabolism of the parasites (Wang, 1984). The simplified metabolic pathways usually contain a rate-limiting enzyme whose normal function is indispensable to the parasite. The same enzyme in the mammalian host may not be essential, due to the more complicated metabolic activities. Also, due to the distant evolutionary relationship between a parasite and its host, there may be sufficient distinctions between the equivalent enzyme of the two origins to allow the design of specific inhibitors of the parasite protein. This is a situation when the parasite molecule can be recognized as a *bona fide* target for antiparasite chemotherapy. Another way to identify a target in a

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parasite is by understanding the mechanism of action of a well-established antiparasite agent originally discovered by old-fashioned screening methods (Wang, 1982). The study of mechanism of resistance to the drug can be most useful in discovering biological aspects of a parasite previously unknown or unsuspected. But thorough studies must be pursued in order to identify the genuine target for drug action so that it can be useful for new drug discoveries. A chemical compound usually has multiple biological effects, but most of them are likely to be irrelevant to the antiparasite activity and must be weeded out at the very beginning of attempts to elucidate the compound's mode of action.

Occasionally, however, a drug may act on numerous essential enzymes in a parasite with similar potencies, which makes discussion of a specific drug target meaningless. A good example could be the antitrypanosomal drug suramin, which is an inhibitor of a variety of dehydrogenases and kinases with little discrimination (Wang, 1995). There are many apparently essential glycolytic enzymes in African trypanosomes that are similarly susceptible to suramin. Theoretically, such an enzyme inhibitor should exhibit wide ranging toxicities to mammalian enzymes as well, and thus have no therapeutic value. But suramin is relatively non-toxic to mammals because it cannot enter mammalian cells, whereas it can be bound to serum proteins and transported into the trypanosomes via endocytosis. Thus, in reality, the real target for suramin in African trypanosome is its endocytotic machinery.

Once a therapeutic target is identified by metabolic or drug action studies, a major research effort is initiated to isolate and express the gene encoding the target protein and to characterize the function and structure of this protein for specific inhibitor design. Such studies are time consuming and expensive. It is thus essential that the target be fully validated by a variety of means during and immediately following its identification. This task has become increasingly important in recent years, because classical metabolic and mode of action studies are receiving diminishing emphasis. Many researchers are now trying to cut this part of the research short (if it is too difficult and time consuming) in order to get into gene clonings. Consequently, many enzymes identified in parasites have been accepted as targets for antiparasite chemotherapy without much scrutiny. The proteases in parasites, for example, are a family of enzymes generally accepted as targets, but few have been fully validated (see Coombs & Mottram, this volume). This current trend may be partly attributed to the tremendous successes in developing protease inhibitors for antiviral and antihypertensive uses, partly due to the availability of numerous protease inhibitors in pharmaceutical industry, and partly because of the fact that some parasites may require protease activities for penetrating the mammalian host tissue and digesting proteins for amino acids. However, the profile of proteases in an eukaryotic microorganism or a helminth can be exceedingly complicated. There is not yet any convincing evidence from any parasite indicating that one single protease is essential for its survival. A frequent practice is to show inhibition of a parasite protease with an inhibitor such as a peptidyl halogenated methyl ketone and then demonstrate the toxicity of this compound toward the parasite, but this does not necessarily validate the protease as an antiparasite chemotherapeutic target. The halogenated methyl ketones are alkylating agents known to alkylate proteins in a non-specific manner. This is a precarious situation and it is important that it alerts the scientific community to place more emphasis on vigorous target validation prior to making any major research commitment to long-term studies aiming at discovering new drugs.

There can be two types of aiming at discovery targets for antiparasite chemotherapy. The first type is one of which has not yet had a specific inhibitor that demonstrates antiparasite activity in patients, animal models or even *in vitro* cultures of the parasite. These targets, though not necessarily invalid, are at too early a stage for meaningful discussions. The second type includes those where inhibitors are known to possess antiparasite activities. This fact alone, however, does not validate the enzyme or receptor as a genuine therapeutic target either. Supporting evidence from other studies should be provided for true validations. These include (1) comparisons of profiles between enzyme (receptor) inhibition and antiparasite action among a large number of chemical derivatives of the drug; (2) characterizations of the putative drug target from drug-resistant mutant parasites; and (3) knockouts of the gene encoding the putative target accompanied by subsequent complementations by the missing gene. The first kind of study has frequently been carried out among pharmaceutical houses due to the availability of large numbers of drug derivatives and their profiles of biological activities. One successful example was in the mechanism of action studies on ivermectin in the early years of its development (Pong & Wang, 1982). The problem with this approach is in comparing the *in vitro* activities of a large group of chemical compounds with their *in vivo* activities without in-depth knowledge on the absorption, distribution and metabolism of each compound. Thus, whenever an exceptional case arises, there is a strong tendency to either ignore it or explain it away based on assumptions. The conclusion is, therefore, often not as solid as one would like to see from a strict scientific standard. As for the second type of investigation, it is generally a good idea to compare the putative drug targets between the drug-sensitive and the drug-resistant parasites for verification. Unfortunately, the latter often turn out to be drug-transport mutants and so do not

Table 1. Validated targets for chemotheraputic attack of parasites

Targets	Parasites	Inhibitors
Dihydropteroate synthetase	Apicomplexa	Sulfadoxine
Pyruvate: ferredoxin oxidoreductase	Anaerobic protozoa	Metronidazole
Trypanothione reductase	Kinetoplastida	Nifurtimox
Nucleoside phosphotransferase	Protozoa	Allopurinol riboside
Glycosome	African trypanosomes	SHAM + glycerol
Lanosterol C-14α demethylase	*Leishmania* and *T. cruzi*	D0870
Dihydrofolate reductase-thymidylate synthetase	Protozoa	Pyrimethamine
Purine phosphoribosyltransferase	Certain protozoa and trematodes	Allopurinol
Mitochondrial electron transport	Apicomplexa	Atovaquone
Chloride ion channel	Nematodes and arthropods	Ivermectin
Ornithine decarboxylase	African trypanosomes	DFMO

provide useful data. It is only in rare occasions that one finds in pyrimethamine-resistant *Plasmodium falciparum* a specific mutation in its dihydrofolate reductase that makes the enzyme less susceptible to pyrimethamine inhibition (Peterson, Milhous & Wellems, 1990). The third approach, to knock out the gene encoding the putative target followed by complementations with the missing gene, may prove to be the most conclusive way of validating a target. One of the most recent successful cases was the double knockout of the ornithine decarboxylase-encoding gene from *Trypanosoma brucei* (Li *et al*. 1996) and the subsequent complementation of the knock-out mutant with a cloned ornithine decarboxylase gene (Sommer *et al*. 1996). The results provided conclusive evidence that ornithine decarboxylase in *T. brucei* performs an essential function – that of producing polyamines which are at very low concentration in mammalian blood (see below). A loss of this enzyme activity brings *T. brucei* into the dormant G1 phase. These results qualify the enzyme as a *bona fide* target for anti-*T. brucei* chemotherapy.

The usefulness of the gene knockout approach is limited. The knockout of a gene encoding an essential enzyme means that the gene knockout will be lethal to the parasite, unless there is a specific way to enable the knockout mutant to survive and grow so that the subsequent complementation could be performed. In the case of *T. brucei* ornithine decarboxylase gene-knockout, the mutants can grow in culture medium supplemented with putrescine and the complemented revertants can be selected in medium without added putrescine. It was also fortunate that the ornithine decarboxylase in *T. brucei* is encoded by a single copy gene and so it was technically simple to disrupt. For a putative target encoded by multi-copy genes scattered among different chromosomes, the standard gene knockout technique may not apply well. Gene knockout works also best on haploids, for obvious reasons. Diploids, such as trypanosomes, are still easy to work on, but higher ploidy such as *Giardia* will be extremely difficult to handle. As for the helminths, homologous DNA integration has not been possible as yet, but the free-living *Caenorhabditis elegans* could be developed as a model using the tc1 transposon insertion technique to knockout the genes encoding putative targets identified in parasitic nematodes.

So far, gene knockouts and complementations can be carried out only with trypanosomes, leishmania and *Toxoplasma gondii*. With the rapid progression being made in DNA transfection techniques applicable to the protozoan parasites (Table 1), however, there will be more of them amenable for this type of investigation in the near future.

In this review article, I take a cursory look at a few of the putative targets that have already *bona fide* antiparasite drugs identified as their inhibitors, and consider the evidence for validity and potential as a means of further verifying their validity.

ENZYMES OR RECEPTORS FOUND ONLY IN PARASITES

These molecules would appear to be the most suitable candidates for therapeutic targets. But as discussed previously, uniqueness alone is not sufficient. The function of the enzyme must be proven essential for the survival and for development of the parasite.

Enzymes for dihydropteroate synthesis in apicomplexan parasites

Plasmodium, *Toxoplasma* and *Eimeria* have long been known to respond to sulphonamides and sulphones. Sulphadoxine is one of the most commonly used sulpha drugs on *P. falciparum*, and has been found to inhibit 7,8-dihydropteroate synthetase (DHPS) in this parasite (Zhang & Meshnick, 1991). It was found that resistance to sulphadoxine in *P. falciparum* could occur by three different mechanisms (Dieckmann & Jung, 1986). Uptake of sulphadoxine

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could be markedly reduced, which, unfortunately rendered no help in validating the drug target, but the reduced conversion of sulphadoxine to the toxic analogue of dihydropteroate and the enhanced syntheses of *p*-aminobenzoic acid (PABA) in the drug-resistant *P. falciparum* did support DHPS as the target for the antimalarial action of sulphadoxine. *P. falciparum* was shown to synthesize pteroyl-polyglutamate from GTP, PABA and L-glutamate (Krungkrai, Webster & Yuthavong, 1989) but lack dihydrofolate synthetase (Ferone, 1977). Since the DHPS in various plasmodia have been shown to catalyse the synthesis of dihydrofolate from dihydropterin-6-CH_2OH and PABA-glutamate without going through 7,8-dihydropteroate formation (Ferone, 1973), DHPS in *P. falciparum* could be subject to inhibition by either sulphadoxine or the antifolate pyrimethamine. Data pointing to such a likelihood have been available for some time (Krungkrai *et al*. 1989).

Thus DHPS from *P. falciparum* can be considered to be a validated target for antimalarial chemotherapy. Recently, the gene encoding this enzyme was cloned from *P. falciparum* and found to be a gene for a bifunctional enzyme that includes dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK) at the amino terminus (Triglia & Cowman, 1994). Discrepancies in the sequences of the DHPS portion of the genes from sulphadoxine-sensitive and -resistant *P. falciparum* were observed. Correlations between sulphadoxine resistance and point mutations in the bifunctional protein have been established (Brooks *et al*. 1994). Further analysis of this enzyme should allow better understandings of sulpha drug resistance in *P. falciparum* and provide opportunities for designing more novel and potent inhibitors.

Pyruvate : ferredoxin oxidoreductases in anaerobic protozoan parasites

Anaerobic protozoan parasites depend primarily on ferredoxin-like or flavodoxin-like low-redox-potential electron transport proteins for electron disposition. In trichomonad flagellates and rumen ciliates, ATP production takes place primarily in a membrane-bound organelle known as the hydrogenosome (Müller, 1980). By the actions of pyruvate:ferredoxin oxidoreductase (PFOR), ferredoxin and hydrogenase in the organelle, pyruvate derived from glycolysis is converted to acetyl-CoA to generate ATP with electrons channelled to protons to produce H_2. Although *Entamoeba histolytica* (Reeves *et al*. 1977) and *Giardia lamblia* (Schofield, Edwards & Krautz, 1991) do not possess hydrogenosomes, similar electron transport via PFOR and ferredoxin also plays a major role in their energy metabolism. PFOR has no counterpart in mammals. It is incapable of reducing pyridine nucleotides because of its low redox potential (approximately -400 mV), but the low potential allows transfer of electrons from pyruvate to the nitro groups of a large family of 5-nitroimidazoles to form cytotoxic products (Muller, 1986). Thus, although there has not yet been a specific inhibitor of PFOR that has been shown to act also against anaerobic protozoa, the 5-nitroimidazoles are known to act against these protozoa as a consequence of the catalytic action of PFOR. This fact to some extent validates PFOR as a therapeutic target, at least in the activation of pro-drugs, but some reservations remain because the real consequence of inhibiting PFOR has not yet been recorded for any of the anaerobic parasites. Townson, Upcroft & Upcroft (1996) observed recently that PFOR activity was decreased in all metronidazole-resistant lines of *Giardia duodenalis*, by as much as 5-fold. Some anaerobically metronidazole-resistant *T. vaginalis* have no detectable PFOR activity but still manage to grow, albeit poorly (Johnson, 1993). This suggests that inhibiting PFOR may not be toxic to the parasite. It would be interesting to perform gene knockout on PFOR gene in these anaerobes and to monitor if the knockout is lethal or simply makes the parasites less able to survive in their natural environment.

Unfortunately, that approach is not yet available for these parasites. The genes encoding PFOR have been isolated recently from *E. histolytica* (Rodrigues *et al*. 1996), *T. vaginalis* (Hrdy & Muller, 1995) and *Giardia* (GenBank accession number L27221). They share 35–45% sequence identities. PFORs are dimeric or tetrameric proteins of 240 kDa subunits. Considerable research efforts will be required before their detailed structures can be resolved for specific inhibitor design. Meanwhile, major progress has been made recently in stable genetic transfections of *E. histolytica* (Hamann, Nickel & Tannich, 1995; Vines *et al*. 1995) and *G. lamblia* (Yee & Nash, 1995; Yu *et al*. 1995). It will not be long before gene knockout can be used to verify the validity of PFOR as a therapeutic target in these parasites.

Trypanothione reductase in Kinetoplastida

Trypanothione, a conjugate between glutathione and spermidine, is one of the truly unique features of kinetoplastida (Fairlamb *et al*. 1992). The presence of trypanothione metabolism in these organisms defies obvious rationalization because the organisms must first synthesize or scavenge enough glutathione in order to begin synthesizing trypanothione. The two compounds have such similar redox potentials that thiol-disulphide exchange reactions between the two can occur non-enzymatically (Fairlamb & Cerami, 1992). Although the reduced trypanothione remains in one molecule whereas reduced glutathione is separated into two, the former is not

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an ordinary dithiol due to the long distance between the two cysteine residues. Its association with trivalent arsenicals is much weaker than that of 2,3-dimercaptopropanol or lipoic acid (Fairlamb *et al*. 1987). Thus the selective advantage of the presence of trypanothione in kinetoplastids remains a mystery. It is no doubt a unique feature, but there must be sufficient evidence to indicate that it is also essential for the parasites before trypanothione metabolism can be validated as therapeutic target.

The existence of trypanothione reductase instead of glutathione reductase in kinetoplastids suggests an essential role for trypanothione to mediate the redox balance, and thus, trypanothione reductase could be a potential target for chemotherapy. Considerable in-depth studies on the structure of this enzyme have been carried out by X-ray crystallographies (Kuriyan *et al*. 1991; Hunter, this volume). Much effort has also been focused on synthesizing and screening inhibitors of trypanothione reductase as potential antitrypanosomal drugs (Krauth-Siegel & Schonect, 1995) under the assumption that the enzyme performs a crucial function in maintaining the redox balance in the parasites. But recent over-expressions of trypanothione reductase in *Trypanosoma cruzi* and *Leishmania donavani* by DNA transfection did not enhance resistance of the transfectants towards drugs thought to induce oxidative stress (Kelly *et al*. 1993). A 40% to 60% decrease in the content of trypanothione and monoglutathionyl spermidine caused by a depletion of spermidine in *T. brucei* (Fairlamb *et al*. 1987) did not increase susceptibility of the cells towards oxidative stress, but instead, pushed the cells into dormancy (Giffin *et al*. 1986). Two well-known antitrypanosomal agents, melarsen oxide and nifurtimox, were found inhibitory to trypanothione reductase (Fairlamb, Henderson & Cerami, 1989). But in the absence of corroborating evidence connecting drug-resistance of a mutant parasite with loss of drug sensitivity of the enzyme (or the equivalent), it is too early to conclude on the mechanisms of action of these drugs. The validity of trypanothione metabolism as a therapeutic target thus still remains to be verified.

Nucleoside phosphotransferases in protozoan parasites

All the protozoan parasites studied to date except for *Acanthamoeba* have been found deficient in *de novo* synthesis of purine nucleotides (Wang, 1984; Berens, Krug & Marr, 1995). This finding has focused much research attention on the purine salvage pathways in the parasites because these pathways have obviously become the only supply-lines for the purine nucleotide pool in these protozoa. There is a unique salvage enzyme, purine nucleoside phosphotransferase, identified among the *Leishmania* species (Nelson *et al*. 1979). It can transfer a phosphate group from a variety of monophosphate esters to the 5′-position of purine nucleosides as well as some of the analogues such as allopurinol riboside, formycin B (Carson & Chang, 1981), 9-deazainosine and thiopurinol riboside (Marr & Berens, 1983). These nucleoside analogues can be either converted to the triphosphates and incorporated into nucleic acids of *Leishmania* or, in the case of allopurinol riboside, turned into inhibitors of other essential purine salvage enzymes and so exert antiparasite activity (Looker, Marr & Berens, 1986). One major problem associated with the purine nucleoside phosphotransferase in *Leishmania* is, however, that it is apparently performing a non-essential function for the parasite. There are several other major purine phosphoribosyltransferases in *Leishmania* that apparently constitute main pathways of purine salvage (Tuttle & Krenitsky, 1980). Thus a mere inhibition of purine nucleoside phosphotransferase is unlikely to deplete the purine nucleotide pool in *Leishmania*. Allopurinol riboside acts not as an inhibitor but a 'false substrate' of the enzyme and it is the products which happen to exert inhibitory actions on several other targets in the parasite (Looker *et al*. 1986). This 'subversive substrate' approach has raised several concerns: (1) the antileishmanial activity of allopurinol riboside is unrelated to the lack of *de novo* synthesis of purine nucleotides in *Leishmania*; (2) the activity depends on an inability, shared by a number of leishmanial enzymes, to distinguish between the hypoxanthine and the allopurinol moiety of their substrates in order to convert allopurinol riboside all the way to the toxic products through a chain of reactions. Although a successful case as such could occur in a fortuitous way, it will be difficult to design a much improved 'false substrate' better recognized by all the enzymes in the chain reactions resulting in a more toxic product(s); (3) consequently, it will be difficult to identify a suitable 'false substrate', with a relatively high efficiency of conversion at each step of the chain reactions to produce a sufficient level of the toxic product(s). Thus, the 'false substrate' is most unlikely to acquire a highly potent antiparasite activity exemplified by the rather weak antileishmanial activity of allopurinol riboside in clinical trials (Saenz *et al*. 1989). Furthermore, there is not an easy and rational way of identifying a chain of enzymes that will all accept the same structural 'falsehood' in their substrates. Neither metabolic studies nor gene knockouts will help. It must rely on random screenings, which should be something to be avoided at present.

Metabolic studies indicated that many anaerobic protozoan parasites are incapable of *de novo* synthesis of both purine and pyrimidine nucleotides. In *Tritrichomonas foetus* (Wang *et al*. 1983*b*) and *G. lamblia* (Aldritt, Tien & Wang, 1985) TMP is provided by a single, isolated salvage pathway that converts exogenous thymidine to TMP by the action

of a thymidine phosphotransferase. The enzyme activity is not affected by thymidine kinase inhibitors such as acyclovir, but is inhibited by guanosine or 5-flurodeoxyuridine; both compounds also inhibit the *in vitro* growth of the parasites. Although further investigations will be required for validation, this enzyme appears to be an attractive putative target for chemotherapeutic treatment of trichomoniasis and giardiasis.

Glycosomes in African trypanosomes

The bloodstream forms of African trypanosomes, such as *T. brucei*, depend entirely on glycolysis for ATP production (Brohn & Clarkson, 1980). The glycolysis proceeds at a rate 50 times that in mammalian cells, obviously a means of compensating for the poor energy yield from glycolysis. This high rate is made possible in *T. brucei* by enclosing the first 7 glycolytic enzymes and two glycerol-metabolizing enzymes in single-membrane-bound organelles, glycosomes (Opperdoes & Borst, 1977). The glycosomes generate 3-phosphoglycerate from glucose and this is released into the cytoplasm for pyruvate and ATP production. Glycosomes are thus self-contained in ATP-ADP balance, but must depend on a dihydroxyacetone phosphate (DHAP): glycerol-3-phosphate (G3P) shuttle catalysed by a glycosomal G3P dehydrogenase and a mitochondrial G3P oxidase for regeneration of NAD from NADH (Visser & Opperdoes, 1980; Visser, Opperdoes & Borst, 1981). Under anaerobic conditions, G3P cannot be oxidized back to DHAP and thus accumulates inside the glycosome. This, together with the rising level of intraglycosomal ADP due to depletion of NAD, eventually drives a reversed glycerol kinase-catalysed reaction to generate ATP and glycerol in the glycosome (see Clayton & Michels, 1996, for fuller details).

This intricate compartmentalization of glycolysis by glycosomes, which has no parallel in mammals, attracted researchers to inhibit the mitochondrial G3P oxidase with salicylhydroxamic acid (SHAM) to bring *T. brucei* into an anaerobic condition, and then try to stop glycolysis with added glycerol (van der Meer, Vershijs-Broers & Opperdoes, 1979). Bloodstream *T. brucei* treated with the SHAM–glycerol combination lysed within minutes. The same combination is also effective in suppressing *T. brucei* parasitaemia in infected animals. Recently, ascofuranone, a prenylphenol antibiotic, was found to inhibit non-competitively the ubiquinol-dependent mitochondrial O_2 uptake in bloodform *T. brucei* with an estimated K_i of 2·34 nM (Minagawa *et al*. 1966). In combination with 4 mM glycerol, ascofuranone demonstrated a minimum inhibitory concentration of 30 nM on the *in vitro* growth of *T. brucei*. The *T. brucei* mitochondrial G3P oxidase has been recently identified and partially purified (Chaudhuri *et al*. 1995). It is anticipated that more structural information on this enzyme will become available following cloning of the encoding gene (Chaudhuri & Hill, 1996) for designing more specific inhibitors with less non-specific toxicities than SHAM.

The qualification of the glycosome as a therapeutic target lies in its vital function in African trypanosomes as well as its unique compartmentalization of glycolytic enzymes. The individual glycolytic enzymes inside the glycosome are, without a doubt, each playing an essential role in trypanosomal energy metabolism. Genes encoding most of these enzymes have been cloned, sequenced and expressed (Opperdoes & Michels, 1993). Three dimensional structures of some of the recombinant enzymes have been analysed in X-ray chrystallography and efforts are in progress for designing and synthesizing specific inhibitors based upon the structural information obtained (Verlinde *et al*. 1994). However, other than the considerably higher isoelectric points that the trypanosomal enzymes have demonstrated in their primary sequences, there have not been any major structural distinctions discovered between the parasite and host enzymes that could be related to distinctive catalytic characteristics. There has not yet been any potent specific inhibitor found. In view of the scarcity of potent inhibitors of glycolytic enzymes in general, the individual glycolytic enzymes of trypanosomes may not be as attractive a therapeutic target as the intact glycosome in its entirety.

An interesting aspect of glycosomes concerns the mechanisms underlying the import of proteins which are synthesized by free polysomes in the cytoplasm (Sommer & Wang, 1994). Inhibition of the import of glycosomal protein either partially or totally is expected to disrupt glycolysis in African trypanosomes. Recent development of expression vectors for transfection of *T. brucei* has made it possible to express reporter proteins in the organism and monitor their import into the glycosome. The results (Sommer *et al*. 1992; Blattner *et al*. 1992) showed that the import depends on the very last three amino acids at the C-terminus of the protein as a targeting signal. However, unlike the peroxisomal protein import in mammalian cells, which depends on the C-terminal tripeptide serine–lysine–leucine (SKL) and its very close homologues (Gould *et al*. 1989), the required tripeptide sequences for targeting glycosomal import is much more relaxed. With the SKL sequence as a starting point, S can be substituted by any other small polar amino acid, K can be replaced by those amino acids capable of forming H bonds and L is replaceable by other hydrophobic amino acids (Wang, 1995). This discrepancy in targeting signals between peroxisomal and glycosomal import could constitute an opportunity for selective inhibition of the latter and

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provide antitrypanosomal chemotherapy. Further studies will be necessary to indicate if a specific blocking of the glycosomal targeting signal by an inhibitor will demonstrate anti-*T. brucei* activity.

INDISPENSABLE ENZYMES OR RECEPTORS IN PARASITES

These enzymes/receptors can be designated targets for antiparasitic chemotherapy without other considerations, because their inhibition should bring the parasites under control. However, it is also important to compare each with its counterpart in the mammalian host for an assessment of the feasibility of designing specific inhibitors against the parasite enzyme. This assessment may begin with a comparison of protein sequences; any enzyme/receptor with a sequence identity above a certain percentage should not be pursued any further. A real comparison will not be possible until well resolved 3-dimensional structures become available, which will take time. It has been generally believed that if the mammalian counterpart does not play an indispensable role in the mammalian host, there may not be need for inhibitors that are highly specific for the parasite enzyme, but the reality is often more complicated than simple theories. For instance, the presence of *de novo* synthesis of purine nucleotides in mammals appears to suggest that mammalian purine phosphoribosyltransferases may not play a crucial role in mammals, but loss of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity in humans causes gout and Lesch-Nyhan Syndrome (Seegmiller *et al*. 1967). Thus, it may well be that for indispensable enzymes in parasites with counterparts also present in the host, in general it will be necessary to design inhibitors specific towards the parasite enzyme.

Lanosterol C-14α demethylase in Leishmania *and* Trypanosoma cruzi

The azole antifungal agents ketoconazole, miconazole and itraconazole are known to act by inhibiting the cytochrome P-450-dependent C-14α demethylation of lanosterol in the ergosterol biosynthetic pathway (Yoshida & Aoyama, 1987). *Leishmania* and *T. cruzi* also contain ergosterol as the principal sterol in the plasma membranes (Haughan & Goad, 1991). Furthermore, the antifungal azoles inhibit the biosynthesis of ergosterol in *Leishmania* by blocking C-14α demethylation of lanosterol and they inhibit *in vitro* growth of *Leishmania* (Beach, Goad & Holz, 1988). Similar observations were also made on *T. cruzi* in subsequent studies (Goad *et al*. 1989). More recently, another antifungal agent, the bis-triazole, D0870, was tested in the mouse models with short-term and long-term *T. cruzi* infections, and demonstrated a most encouraging *in vivo* anti-*T. cruzi* activity (Urbina *et al*. 1996; Urbina, this volume). Based on our knowledge from fungi, the cytochrome P-450 which demethylates lanosterol at the C-14α position is likely to be present and play an essential role in both *Leishmania* and *T. cruzi* (but see Urbina, this volume).

By transforming a *Saccharomyces cerevisiae* host with an *S. cerevisiae* genomic library and screening the resultant transformants for resistance to ketoconazole, Kalb *et al*. (1986) were able to isolate the structural gene encoding lanosterol 14 α-demethylase. It has an open reading frame of 1590 nucleotides encoding a 60·7 kDa protein (Kalb *et al*. 1987). When the gene was disrupted by integrative transformation, the resulting *S. cerevisiae* required ergosterol for growth only in the absence of oxygen, but other studies have indicated that this enzyme requirement can be by-passed as a result of additional mutations in yeast (King *et al*. 1985). The 14 α-demethylation of lanosterol is also required for the biosynthesis of cholesterol in mammals (Trzaskos, Kawata & Gaylor, 1986). Rat liver cDNA encoding this enzyme was identified and shown to bear significant sequence homology with yeast lanosterol 14 α-demethylase; 49·9 % nucleotide identity and 37·7 % amino acid identity (Aoyama *et al*. 1994). The human CYP51 cDNA coding for the same enyzme was also recently isolated and expressed and found to have a deduced amino acid sequence 93 % and 38–42 % identical to rat and fungal lanosterol 14 α-demethylases, respectively (Stromstedt, Rozman & Waterman, 1996). In a previous investigation, the selective antifungal action of azole antimycotics was found not to be due to inherent sensitivity differences between fungal and mammalian lanosterol 14 α-demethylases *per se*, but rather to the presence of other azole-susceptible isozymes only in mammalian systems (Trzaskos & Henry, 1989). Thus, as a rather non-selective inhibitor of adrenal and gonadal steroid synthesis, ketoconazole may exert a variety of endocrine effects, but remains an orally acceptable drug against certain systemic mycoses.

It is not yet known how much distinction there is between the lanosterol 14 α-demethylase of *Leishmania* and *T. cruzi* and that of humans. But if the usefulness of azole antimycotics can be cited as a precedent, a case can be argued for searching for potent but not necessarily specific inhibitors of the parasite enzymes.

Dihydrofolate reductase – thymidylate synthetase bifunctional proteins in protozoan parasites

Dihydrofolate reductase (DFHR), a classical target in antimicrobial and anticancer chemotherapies has been shown also to be a *bona fide* therapeutic target in the *Plasmodium*, *Toxoplasma* and *Eimeria* species with pyrimethamine being the typical enzyme inhibitor exerting antiparasite actions. Development

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of pyrimethamine-resistance in *P. falciparum* has become widespread (Wernsdorfer & Payne, 1991). Most of the cases can be attributed to specific point mutations in *P. falciparum* DHFR rendering the enzyme less susceptible to pyrimethamine inhibition (Peterson *et al*. 1990).

A highly unusual feature of DHFR in apicomplexan parasites as well as Kinetoplastida is that it is always associated with thymidylate synthetase (TS) in a bifunctional protein. The genes encoding these bifunctional proteins in most of the protozoan parasites have been isolated, sequenced and analysed (Ivanetich & Santi, 1990). DHFR is always at the N-terminal portion whereas TS occupies the C-terminus. Though sharing the same protein, the two enzymatic functions do not appear to be interdependent. The gene fragment encoding the DHFR portion of the *P. falciparum* bifunctional protein was expressed (Sirawaraporn *et al*. 1993) and found to function normally. It is not known why the DHFR of apicomplexan and kinetoplastids exists in a bifunctional protein with TS. One likely explanation would be that since these organisms do not perform *de novo* synthesis of purine nucleotides, the primary function of the tetrahydrofolate formed from the catalytic function of DHFR is to provide 5,10-methylene-tetrahydrofolate as a cofactor of the TS-catalysed reaction. Thus there is no need to separate the two enzymatic activities into two different proteins. The anaerobic protozoan parasites *Trichomonas* and *Giardia* do not perform *de novo* synthesis of either purine or pyrimidine nucleotides and no DHFR activity has yet been detected in these organisms.

The DHFR-TS bifunctional proteins in apicomplexa and kinetoplastids are well validated targets for chemotherapy. There is an urgent need at present to understand the detailed three-dimensional structures of these proteins, including that of the pyrimethamine-resistant DHFR from *P. falciparum*, for the design of specific inhibitors in the future.

Purine phosphoribosyltransferases

The absence of *de novo* synthesis of purine nucleotides among the protozoan parasites and certain trematodes results in their primary dependence on one or two purine salvage enzymes for replenishing the purine nucleotide pool. Detailed metabolic analysis has indicated that *G. lamblia* depends primarily on a guanine phosphoribosyltransferase (GPRT) and an adenine phosphoribosyltransferase (APRT) (Wang & Aldritt, 1983), *T. foetus* relies on just the one salvage enzyme, hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT) (Wang *et al*. 1983*a*), and *Schistosoma mansoni* requires an HGPRT and an APRT (Dovey, McKerrow & Wang, 1984). The lack of interconversions between the adenine and guanine nucleotides in these organisms further emphasises the essentiality of each of the purine phosphoribosyltransferases for survival and growth of these parasites. *G. lamblia* GPRT (Sommer *et al*. 1996), *T. foetus* HGXPRT (Chin & Wang, 1994) and *S. mansoni* HGPRT (Yuan *et al*. 1990) have each been cloned, over-expressed and characterized. The detailed three-dimensional structure of *T. foetus* HGXPRT has been resolved in X-ray crystallography (Somoza *et al*. 1996) and compared with that of human HGPRT (Eads *et al*. 1994). The aim is to discover an inhibitor that can deplete the purine nucleotide pool in the parasite rather than, as described above, finding a 'false substrate' that may be turned into a cytotoxic product by the enzyme.

Purine phosphoribosyltransferases have been identified in all the other protozoan parasites investigated except for *Trichomonas vaginalis* (Heyworth, Gutteridge & Ginger, 1982) and *E. histolytica* (Lo & Wang, 1985), which contain only purine nucleoside kinases. However, experimental evidence to suggest that any of the purine phosphoribosyltransferases provides the primary means of salvaging purines for those protozoan parasites is lacking. In fact, there is evidence pointing to the contrary in some cases. The gene for HGXPRT in *Toxoplasma gondii* was knocked out in recent studies (Donald *et al*. 1996). The knockout mutant, lacking all detectable HGXPRT activities, grows normally as the wild-type. This is apparently attributed to the presence of another major purine salvage enzyme, adenosine kinase, in *T. gondii* (Schwartzman & Pfefferkorn, 1982) capable of providing 75% of the purine nucleotide pool. Thus, the best one could expect from *T. gondii* HGXPRT as a therapeutic target is to identify a 'subversive substrate' for it. But then, as discussed previously, the choice of HGXPRT as such a target has no relation to the lack of *de novo* synthesis of purine nucleotides in the parasite. Any enzyme could be capable of generating a cytotoxic product.

Allopurinol is apparently recognized as a substrate by the HGPRT in *Leishmania* (Tuttle & Krenitsky, 1980) and converted to cytotoxic products as in the case of allopurinol riboside (Marr & Berens, 1983 and the above). It was tested on visceral leishmaniasis and demonstrated moderate therapeutic activities (Kager *et al*. 1981). Subsequent clinical trials have yielded equivocal results.

Mitochondrial electron transport in apicomplexan parasites

Many 2-hydroxynaphthoquinones have demonstrated therapeutic activities against apicomplexan parasites. Parvaquone and buparvaquone have been developed for the treatment of theileriosis in cattle and other domestic animals (McHardy, 1992). Atovaquone is now on clinical trials as an

antimalarial drug (see Gutteridge, this volume). The 2-hydroxy-naphthoquinones are analogues of ubiquinone. The primary site of action of atovaquone in *P. falciparum* is the cytochrome bc 1 complex where an apparent drug binding site is present in cytochrome b (Fry & Pudney, 1992; Vaidya *et al*. 1993). In *P. falciparum*, ubiquinone plays also an important role as an electron acceptor for dihydroorotate dehydrogenase. Consequently, pyrimidine biosynthesis in *Plasmodium* is also inhibited by atovaquone (Ellis, 1994). Atovaquone has also been found active against the *T. gondii* cysts in the brains of mice (Araujo *et al*. 1992).

A group of 4-hydroxyquinoline derivatives, including buquinolate, decoquinate and methyl benzoquate, have been shown to be relatively non-toxic but highly effective anticoccidial agents. They act on the parasites by inhibiting their mitochondrial respiration with a point of block between the ubiquinone and cytochrome b (Wang, 1975, 1976). Strains of *Eimeria tenella* resistant to the 4-hydroxyquinolines had their mitochondria isolated, examined and found to be insensitive to drug inhibition (Wang, 1976). Thus, the ubiquinone-cytochrome b portion of the mitochondrial electron transport chains among apicomplexan parasites also shares the common feature of being a therapeutic target. They also share the same trait of high frequencies in developing drug resistance (Wang, 1976). Further investigations will be necessary to gain a greater understanding of this therapeutic target and ways of coping with the high frequencies of drug-resistance development.

The membrane chloride ion channels in nematode and arthropod parasites

A family of natural compounds, the avermectins and milbemycins, were discovered as highly potent anthelmintics with also powerful insecticidal activities (Campbell *et al*. 1983). They act at the junction of ventral cord interneuron and motorneuron to immobilize the nematode (Kass *et al*. 1980) and at the neuromuscular junction of arthropods to cause paralysis (Fritz, Wang & Gorio, 1979). Their mechanism of action is to open the chloride ion channels in the neuronal membranes of the nematode and the muscle membranes of the arthropod to hyperpolarize the cells so that they can no longer respond to incoming stimuli. Picrotoxin, a specific blocker of the chloride ion channels, can reverse all the physiologic effects of avermectins and milbemycins. The chloride ion channels in the mammalian central nervous system and the arthropod neuromuscular junctions are associated and regulated by the γ-aminobutyric acid (GABA) receptor. But the regulation and the structure of chloride ion channels in the nematode have remained unclear until it was recently identified and isolated from the free-living nematode *Caenorhabditis elegans* (Cully *et al*. 1994). It turns out to be a glutamate-gated chloride ion channel composed of α- and β-protein subunits. cDNAs encoding the two subunit proteins were cloned, sequenced and found to share considerable identities with the α- and β-subunits of the mammalian GABA and glycine receptors (Cully *et al*. 1994; Martin *et al*. this volume).

This glutamate-gated chloride ion channel is undoubtedly a validated target for anti-nematode action. However, it is not totally clear how distinct a pharmacological property it has when compared with the GABA receptor-chloride ion channel complex in the mammalian central nervous system. The avermectins act strongly on the complex from rat brain through a potentiation of GABA- and benzodiazepine-binding to open the channel (Pong & Wang, 1982). It is apparently due to the failure of avermectins to cross the blood brain barriers into the central nervous system of the mammals that has made them relatively safe to use (Campbell *et al*. 1983). It is a good example of differential drug efficacy dictated by the accessibility of the target.

Ornithine decarboxylase in African trypanosomes

Polyamines found in almost all living organisms, are universally required for cellular proliferation (Tabor & Tabor, 1984). Ornithine decarboxylase (ODC), the enzyme that controls the formation of the polyamine putrescine, is an enzyme characterized by its striking inducibility and short half-life (20 min) in mammalian cells. The polyamines are generally present at relatively high levels in most mammalian tissues. An exception is the bloodstream where the presence of high levels of polyamine oxidase reduces the polyamines to a barely detectable level (Gahl & Pitot, 1979). The primary habitat for African trypanosomes is the bloodstream and so this provides the parasite with little chance to take up any polyamine. Instead it needs to rely on its own polyamine biosynthetic machineries. Thus, an effective inhibition of the key enzyme ODC in the synthetic pathway should deplete the trypanosomes of polyamines and inhibit proliferation.

Eflornithine, DL-α-difluoromethylornithine (DFMO), is a suicide inhibitor of mouse ODC (Poulin *et al*. 1992). The enzymic catalysis forms a covalent adduct between the decarboxylated and defluorinated DFMO and the residue cysteine 360 in the enzyme. DFMO is a new antitrypanosomal drug applied to humans with good therapeutic activity against *T. brucei gambiense* infections, but less activity against *T. brucei rhodesiense* infections (Milford *et al*. 1992). The drug depletes the polyamines from *T. brucei* and retards its growth (Fairlamb *et al*. 1987), but the inhibitory effects can be reversed by adding exogenous putrescine (Phillips, Coffino & Wang, 1987). There is thus little

doubt that DFMO acts on the trypanosomes by inhibiting their ODC activity. In our subsequent investigations, the single copy ODC gene was deleted from *T. brucei* by double gene knockouts (Li *et al.* 1996). The cloned knockout cells showed no detectable ODC activity and cannot grow in regular culture medium without adding putrescine and replacing the serum with heat-inactivated bovine serum in order to inhibit the serum polyamine oxidase. These dormant ODC-knockout cells could be, however, complemented to grow in the regular culture medium by transfection with a wild-type *T. brucei* genomic library (Sommer *et al.* 1996). All the cloned complemented cells turned out to contain a plasmid with the full-length *T. brucei* ODC gene. *T. brucei* ODC has been thus fully validated as a therapeutic target.

It was, however, unclear why, by acting as a suicide inhibitor of ODC, the drug can effectively block the growth of *T. brucei* but remain remarkably non-toxic to the mammalian host (Schechter & Sjoerdsma, 1986). A comparison between the trypanosome and the mouse ODC indicated that DFMO is indeed a suicide inhibitor of both enzymes (Bass *et al.* 1992) with a 3-fold lower K_i value against the mouse enyzme (Phillips *et al.* 1987). The amino acid sequence of *T. brucei* ODC derived from the encoding gene has a 62% identity with that of the mouse ODC. The only major difference between the two enzymes is that the mouse ODC has an extra 36 amino acid peptide at the C-terminus which happened to be postulated by Rogers, Wells & Rechsteiner (1986) as the PEST sequence involved in triggering the rapid *in vivo* degradations of mammalian ODC. This hypothesis led to tests of the *in vivo* half-life of *T. brucei* ODC, which was found to be greater than a day. More recently, R. Kaminsky of the Swiss Tropical Institute demonstrated that the ODC in DFMO-susceptible *T. gambiense* has an extraordinarily long half-life, whereas ODC in the DFMO-refractory *T. rhodesiense* has a rather short half-life (personal communication). There is apparently a close relationship between the intracellular half-lives of ODC and the susceptibilities of the cells toward DFMO. A simple explanation for this phenomenon would be that for ODC species with rapid *in vivo* turnovers, the newly synthesized active ODC molecules are constantly and rapidly replacing the DFMO-inhibited ODC molecules. The cells with rapid ODC turnover are thus less affected by the inhibition of ODC.

It is rather amusing to learn that the circumstances can turn a common enzyme such as ODC into a well validated target for chemotherapy: (1) the ODC function becomes only indispensable in African trypanosomes when there are few polyamines in their living environment; (2) the *T. brucei* ODC differs very little from mouse ODC in their overall amino acid sequences. It will be most difficult to design an inhibitor that only inhibits *T. brucei* ODC. However, due to the very slow turnover of the trypanosome enzyme, any general suicide inhibitor of ODC would be expected to have a more intensified toxicity toward the trypanosomes as has indeed turned out to be the case (Schecter & Sjoerdsma, 1986). Therefore, the essential function of trypanosomal ODC is not caused by an unusual metabolism in the parasite and the uniqueness of trypanosomal ODC is not due to any unusual geometries of its active site. This is a peculiar case where further understanding of the structure of a therapeutic target may not help the design of a better antiparasite drug.

CONCLUSION

There are two families of apparently attractive, putative targets for antiparasite chemotherapy; those found only in parasites and those found indispensable for parasites. The unique ones must be proven to perform an essential function as well, whereas the indispensable ones must demonstrate some feasibility for specific inhibition. These different approaches are but two sides of the same coin, which argues for a single, unified standard for validating therapeutic targets in all cases.

There are probably many more well-publicized putative targets for antiparasite chemotherapy nowadays that have not been covered by this review. Some of them may be missed due to the ignorance of this author. But many of them are left out because they have not yet fulfilled the single unified criterion proposed by this author, even though some of them have already received certain attractive generic names. In a time when 'rational' approaches to antiparasite chemotherapy are receiving much attention from the scientific community, it makes sense for the researchers involved in this line of work to be extremely cautious and conscientious in laying down solid foundations now in order to make our research efforts in the future credible, respectable as well as fruitful.

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