

Glycolysis as a target for the design of new anti-trypanosome drugs

Christophe L.M.J. Verlinde,¹ Véronique Hannaert,² Casimir Blonski,³ Michèle Willson,³ Jacques J. Périé,³ Linda A. Fothergill-Gilmore,⁴ Fred R. Opperdoes,² Michael H. Gelb,⁵ Wim G.J. Hol, I,⁶ Paul A.M. Michels²

¹Department of Biological Structure, Biomolecular Structure Center, University of Washington, Seattle, USA, ²Christian de Duve Institute of Cellular Pathology and Laboratory of Biochemistry, Université Catholique de Louvain, Brussels, Belgium, ³Groupe Chimie Organique Biologique, UMR-CNRS 5068, Université Paul Sabatier, Toulouse, France, ⁴Department of Biomedical Sciences, University of Edinburgh, Edinburgh, UK, ⁵Departments of Chemistry and Biochemistry, University of Washington, Seattle, USA, ⁶Howard Hughes Medical Institute, School of Medicine, University of Washington, Seattle, USA

Abstract Glycolysis is perceived as a promising target for new drugs against parasitic trypanosomatid protozoa, because this pathway plays an essential role in their ATP supply. Trypanosomatid glycolysis is unique in that it is compartmentalized, and many of its enzymes display unique structural and kinetic features. Structure- and catalytic mechanism-based approaches are applied to design compounds that inhibit the glycolytic enzymes of the parasites without affecting the corresponding proteins of the human host. For some trypanosomatid enzymes, potent and selective inhibitors have already been developed that affect only the growth of cultured trypanosomatids, and not mammalian cells. © 2001 Harcourt Publishers Ltd

INTRODUCTION

The parasitic protozoa *Trypanosoma* and *Leishmania* belong to the order of Kinetoplastida, and are the causative agents of several highly disabling and often fatal diseases, which include African sleeping sickness, Chagas' disease and leishmaniasis.

African sleeping sickness, caused by *Trypanosoma brucei*, is transmitted to humans through the bite of the tsetse fly of the genus *Glossina*. When introduced into the host, the trypanosomes proliferate in the blood and lymphatic systems, before invading the central nervous system. Sleeping sickness falls into two clinical categories depending on which trypanosome subspecies is responsible: *T. b. gambiense* causes a chronic disease that takes several years to progress to the second meningoencephalitic stage; *T. b. rhodesiense*, however, causes an acute form of the disease, taking just a few weeks to reach this second stage. Sleeping sickness is a daily threat to more than 60 million people in 36 countries of sub-Saharan Africa, 22 of which are among the least developed countries in the world. The estimated number of people thought to have the disease is between 300 000 and 500 000. If untreated, both forms of the disease are fatal at the second stage, and unfortunately the treatment

of African trypanosomiasis is still unsatisfactory.¹ Eflornithine, the sole drug developed in recent times, is effective only for late-stage gambiense disease and is very expensive. Two other drugs, pentamidine and suramin, are used for the treatment of early-stage gambiense and rhodesiense disease, respectively, but have serious side effects. Melarsoprol remains the first-line drug for late-stage disease of both forms of sleeping sickness, but is very toxic and even fatal. Moreover, none of the African trypanocides can be given orally.

Chagas' disease is caused by *Trypanosoma cruzi*, for which many kinds of wild and domestic mammals act as hosts and hence as reservoirs of the disease. This flagellated protozoan parasite is transmitted to humans in different ways, either by a blood-sucking reduviid bug which deposits its infective faeces on the skin at the time of biting, or directly by transfusion of infected blood or by congenital transmission. *T. cruzi* infection has a wide distribution in Central and South America, where it is endemic in 21 countries. The disease affects 16–18 million people, and about 5–6 million of these have developed chronic incurable complications (such as cardiac lesions, digestive disorders, peripheral neurological lesions) appearing 10–20 years after the initial acute phase of the disease. There have been significant improvements in the control of Chagas' disease by breaking the transmission of the disease through targeting the insect vectors. Treatment is available for acute stages of the disease only. New drugs are thus still needed, especially to overcome the chronic form of the disease.²

Over 20 different species of the genus *Leishmania* are known to be pathogenic for humans. They are all transmitted by the bite of an insect vector, the phlebotomine sandfly. The leishmaniasis are divided into three general clinical patterns according to the form of the disease: cutaneous, visceral and mucocutaneous. There are estimated to be over two million new cases of leishmaniasis each year in 88 countries, with 367 million people at risk. New drugs are needed for leishmaniasis because the standard treatments can only be given parenterally, and the treatment courses are long, expensive, and may elicit severe adverse reactions. Moreover, key products such as antimonials are being compromised by drug resistance.²

Chemotherapy, even if not satisfactory, remains the mainstay for the control of all these diseases: vaccine development has proved difficult because many of these parasites have developed intricate mechanisms for evading their host's immune system. The limited number of available drugs is simply a consequence of market economy principles: since people most at risk from tropical diseases are among the poorest in the world, there is currently little perceived financial incentive for pharmaceutical companies to invest in development of new drugs. Fortunately, the cell biology of trypanosomes is so extraordinary that they have been the subject of more fundamental research than most other protozoan parasites. Each of these unique features may become a target for new drugs, provided they prove to be essential for the survival of the parasites in the human host.³

An ideal target for intervention in the case of Trypanosomatidae appears to be the glycolytic pathway. The 10 enzymes of glycolysis are responsible for the break down

of glucose into pyruvate, with concomitant production of ATP. Extensive studies of metabolism in African trypanosomes leave no doubt that the bloodstream stage uses rapid death of these parasites. The same will possibly be true for *T. cruzi* and *Leishmania* spp. which, despite quantitative differences in the contribution of glycolysis to the overall free-energy supply compared to *T. brucei*, also utilize glucose as a major energy substrate.⁵⁻⁷ Moreover, the long evolutionary distance between trypanosomatids⁸ and their mammalian hosts, and the unusual organisation of the glycolytic pathway in the parasites⁹ have endowed the trypanosomatid enzymes with distinct properties. These provide opportunities for exploitation in structure- or catalytic mechanism-based

design of selective inhibitors which would not affect glycolysis in the host.

GLYCOLYSIS IN TRYPANOSOMATIDS

In Kinetoplastida, the seven glycolytic enzymes converting glucose to 3-phosphoglycerate are localized in specialized peroxisome-like organelles called glycosomes (Fig. 1),^{4,9} in contrast to the situation in other organisms where the glycolytic enzymes are cytosolic. The NADH produced by glyceraldehyde-3-phosphate dehydrogenase in glycolysis is reoxidized by molecular oxygen via a mitochondrial glycerol-3-phosphate oxidase to which the electrons are

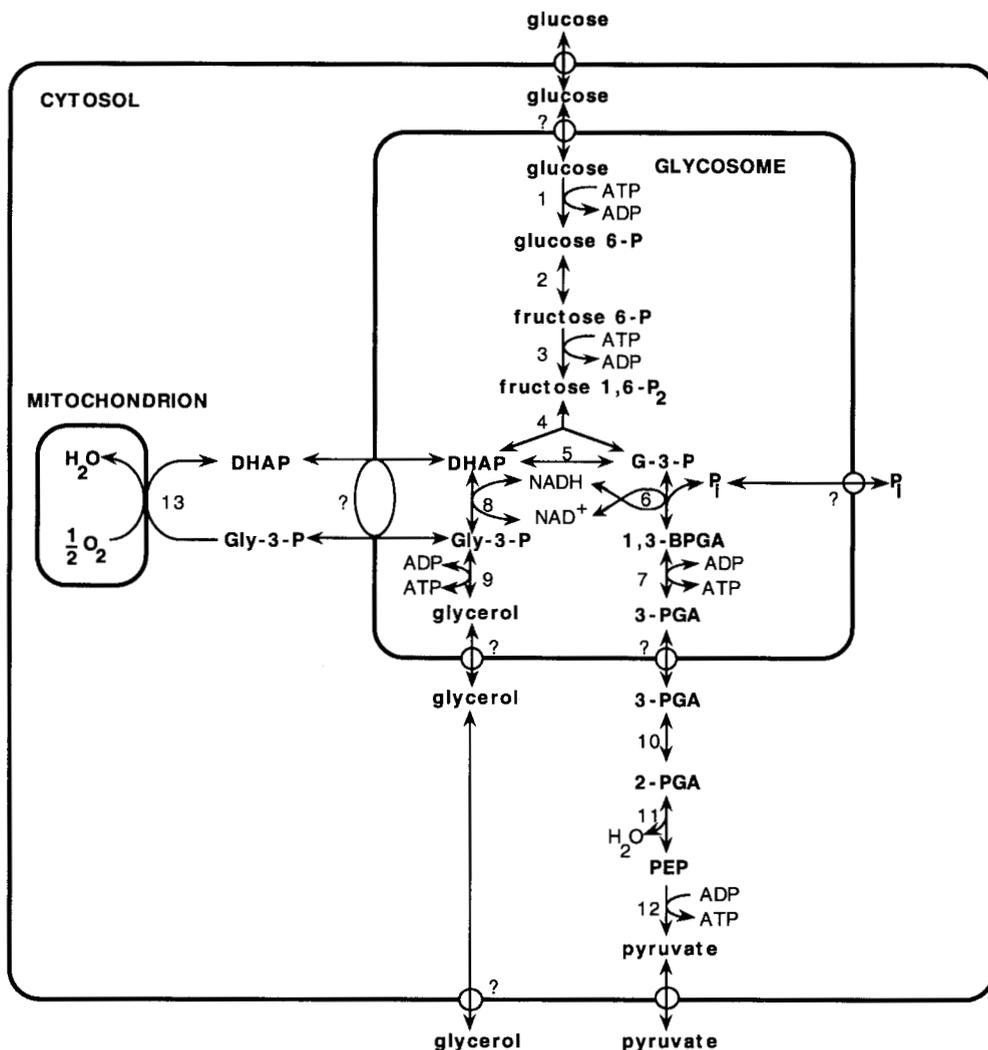


Fig. 1 The stoichiometric scheme of glycolysis in bloodstream-form *T. brucei*. (1) Hexokinase; (2) glucose-6-phosphate dehydrogenase; (3) phosphofructokinase; (4) aldolase; (5) triosephosphate isomerase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) glycerol-3-phosphate dehydrogenase; (9) glycerol kinase; (10) phosphoglycerate mutase; (11) enolase; (12) pyruvate kinase; (13) glycerol-3-phosphate oxidase. Substrate and metabolite transporters in membranes are represented by circles; the carrier molecules postulated for translocation of metabolites across the glycosomal membrane remain to be identified. Abbreviations: 1,3-PGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate; Gly-3-P, glycerol 3-phosphate; PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.

transferred through a glycosomal glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate: dihydroxyacetone phosphate shuttle. As a result of this organization, the intraglycosomal milieu is in redox and ATP balance. Net ATP synthesis occurs in the cytosol, in the reaction catalysed by pyruvate kinase. Under anaerobic conditions, the glycerol 3-phosphate is converted into glycerol with concomitant synthesis of ATP via the reverse action of a glycosomal glycerol kinase. The glycosomal ATP and NAD balance are thus maintained also under anaerobiosis, but one molecule of each glycerol and pyruvate are produced per molecule of glucose, instead of two molecules of pyruvate, and the net ATP production is decreased from two molecules to one. Due to this compartmentation, many regulatory mechanisms operating in other cell types cannot work in trypanosomes. This is reflected by the insensitivity of the glycosomal hexokinase and phosphofructokinase to compounds that act as activity regulators in other cell types.¹⁰⁻¹³

In principle, inhibition of any step of glycolysis in bloodstream-form *T. brucei* should lead to arrest of the glycolytic flux, and thus to death of the parasite, although the choice of triosephosphate isomerase as a target was controversial for a long time. It was reasoned that inhibition of this enzyme might not be effective in bloodstream-form parasites, because the parasites can convert dihydroxyacetone phosphate into glycerol with concomitant ATP production. However, it was recently shown, by a combination of genetics and computer modelling, that the enzyme is essential for trypanosome survival.¹⁴

Indeed, bloodstream-form *T. brucei* starved for glucose or incubated with the plasma-membrane glucose transporter inhibitor phloretin die within minutes.¹⁵ Also inhibitors of glyceraldehyde-3-phosphate dehydrogenase such as pentenolactone¹⁶ or bromopyruvate¹⁷ kill the trypanosomes, as do inhibitors of pyruvate efflux such as α -cyanocinnamic acid.¹⁸ However, all these inhibitors also block the equivalent human proteins. A method to block trypanosome glycolysis selectively and kill the parasites cultured *in vitro* is the inhibition of glycerol-3-phosphate oxidase by salicylhydroxamic acid (SHAM) in conjunction with preventing the reversal of the glycerol kinase by adding glycerol.¹⁹ However, this method has no direct therapeutic value, because impractically high concentrations of SHAM (1 mM) are required, and the concentrations of glycerol (5 mM) to be used are toxic for the host.²⁰ Recently, an alternative, highly specific, non-competitive oxidase inhibitor has been reported, the antibiotic ascofuranone,²¹ but no information is as yet available about its *in vivo* efficacy. Surprisingly, it was recently found that culturing bloodstream-form cells in the presence of SHAM or under anaerobiosis for 24 h resulted in cell death.¹⁴ Also, gene knockouts of glycerol-3-phosphate oxidase were unsuccessful.¹⁴ Previous experiments which suggested that the oxidase and glycerol kinase should be inhibited simultaneously to kill trypanosomes had always been performed for much shorter times.¹⁹ All data suggest that bloodstream-form trypanosomes may survive under anaerobic conditions for a short period, as a result of the glycerol-kinase dependent ATP production, but that prolonged growth and survival is not possible because of the 50% decrease in ATP yield. The glycerol-3-phosphate oxidase thus seems also to be a promising target for anti-trypanosome drugs.

Any selective inhibitor developed against a *T. brucei* enzyme may also be effective on the corresponding enzyme of other trypanosomatids, and vice versa. Most of the glycolytic enzymes are possibly also good drug targets in *T. cruzi* and *Leishmania* species, despite the larger contribution of mitochondrial processes in the mammalian stages of these parasites.⁵⁻⁷ For the mammalian amastigote stage of *T. cruzi* it has been shown that glycolysis is an essential process,²² and in *Leishmania* amastigotes glycolysis may still be important, despite the large dependence of these parasites on the oxidative breakdown of fatty acids for ATP synthesis.²³ However, the enzymes involved in glycerol metabolism seem to play a less important role in the reoxidation of glycosomal NADH and thus indirectly in the glycolytic flux of these two parasites. The presence of a glycerol-3-phosphate oxidase, or its gene, could not even be demonstrated in *Leishmania*.²⁴

GENERAL STRATEGIES FOR INHIBITOR DESIGN

The general strategy for enzyme inhibition can be illustrated by the scheme in Figure 2 where ES, EI and ESI are the Michaelis complex (enzyme E with bound substrate S), the enzyme-competitive inhibitor complex and the ternary complex resulting from binding of the inhibitor to the Michaelis complex, respectively. Blocking the flux of an essential metabolic pathway such as glycolysis in trypanosomes through inhibition of an enzyme by formation of complexes with competitive or uncompetitive inhibitors (EI and ESI, respectively) has two requirements. These are (1) that the targeted enzyme exerts sufficient control on the flux,^{25,26} and (2) particularly that the affinity of the inhibitor for the enzyme or for the Michaelis complex is high enough. High-affinity substrate analogues can fulfil this latter requirement and examples are given in this paper.

An alternative is to achieve transformation of the first EI complex into either a covalently bound complex (E-I) - in that case the inhibition becomes irreversible - or into a slowly isomerised complex EI* formed reversibly but having a very slow reverse-rate constant k_{-4} . The irreversible inhibition route k_4 implies that the substrate analogue I bears an electron acceptor center onto which a reactive group of the active site (such as the sulfur atom of a cysteine, or the nitrogen atom of a lysine) will react to make a covalent bond. The inhibition process is called suicide if the electron acceptor center on the inhibitor is unmasked by the enzyme in a preliminary step. Difluoromethylornithine (eflornithine), a drug currently used against gambiense sleeping sickness, is an example of a suicide inhibitor.²⁷

The formation of isomerised EI* complexes generally corresponds to situations where the inhibitor bears a structural similarity to the transition state of an enzymatic reaction or to a high-energy intermediate on the energy profile of the reaction. Such so-called transition-state analogues are often slow-binding inhibitors; the slow-binding process corresponds to the conformational change of the enzyme induced by the binding.²⁸ Transition-state inhibitors may be active in the femtomolar range.²⁹ They have found application in medicine and agrochemistry; an example is the recently

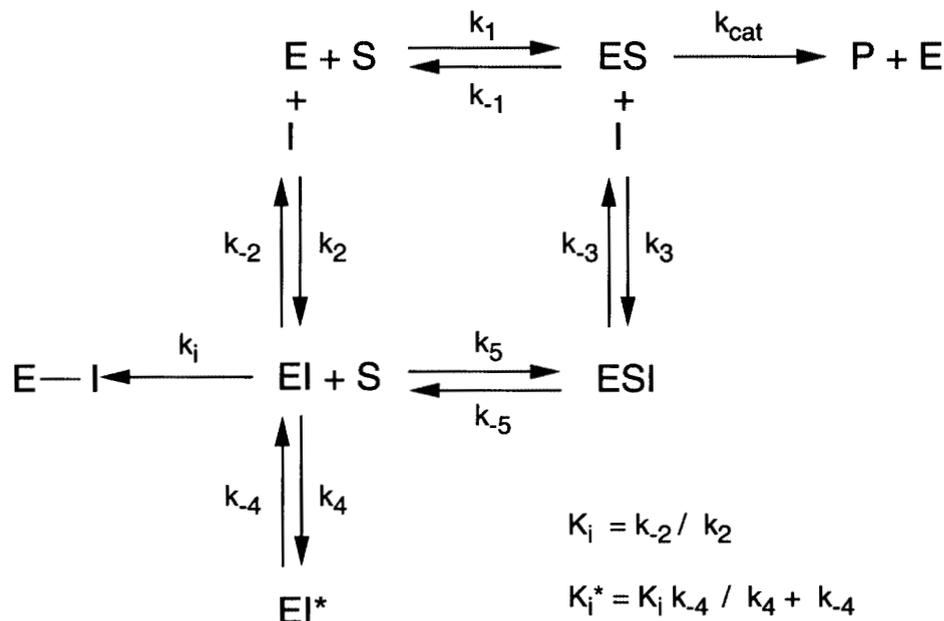


Fig. 2 Mechanisms of enzyme inhibition. The scheme shows both reversible and irreversible inhibition. There are three types of reversible inhibitors: I is a competitive inhibitor if it binds only to E and prevents S from binding. I is a non-competitive inhibitor if it binds to E and ES forms or I is an uncompetitive inhibitor if it binds to ES only.

developed anti-flu drug Relenza™, which is a transition-state analogue inhibitor of the viral neuraminidase.³⁰

Both irreversible and slow-binding inhibitors may be particularly suitable for inhibition of trypanosomal glycolysis. High intra-glycosomal concentrations of glycolytic intermediates have been estimated for bloodstream-form *T. brucei*.^{31,32} Enzyme inhibitors must thus possibly compete with substrates present at concentrations several times higher than the K_m values. For instance in the reaction catalysed by aldolase, the estimated concentration of fructose 1,6-bisphosphate is 210 times the K_m value, whereas in mammalian muscle the concentration is three times lower than the K_m .³¹ Inhibitors competitive with the aldolase substrate may thus not be very effective in trypanosomes, and irreversible inhibitors or transition-state analogues are therefore preferred. However, despite the high intra-glycosomal metabolite concentrations, several enzymes may work below saturation, because (1) in vivo the effective K_m 's are much higher than the values determined in vitro, as a result of the competition for active sites of the glycosomal enzymes and (2) there may be a significant sequestration of some metabolites to active sites because of the high enzyme concentrations in the organelles.^{31,33,34}

The sequestering of glycolytic enzymes inside glycosomes results not only in an increased concentration of glycolytic intermediates, restraining the efficacy of inhibitors competitive with these intermediates,³⁵ but it also results in three conservation relationships between glycosomal metabolites.³² These relationships can be derived from the stoichiometry of all reactions in Figure 1. The first relationship, although not immediately obvious, involves all phosphorylated glycolytic intermediates; their sum must remain

constant. The second and third relationships are that the sum of the glycosomal ATP, ADP and AMP, and the sum of NADH and NAD⁺ in the organelle are conserved. A result of these constraints is that the concentration of these metabolites cannot increase indefinitely. Therefore, compounds competitive with the nucleotide-substrates may be effective inhibitors of glycolysis if the concentration of these substrates in the glycosome is not too high. Calculations showed that potent inhibitors competitive with NAD⁺ and NADH or ATP and ADP inside the glycosome should be able to reduce the glycolytic flux considerably.^{26,36} Indeed, this prediction was experimentally confirmed; adenosine analogues which by in vitro assays were shown to be selective and potent inhibitors of glycosomal glyceraldehyde-3-phosphate dehydrogenase block the glycolytic flux in *T. brucei* and inhibit the growth of the parasites³⁷ (see below).

A last aspect about inhibitor design concerns selectivity: how to block the parasite enzymes without producing damage to glycolysis in the host. Several approaches can be considered.

Exploitation of metabolic differences

To kill a parasite, one might look for an enzyme or metabolic pathway that is essential for the parasite and absent in the host. Alternatively, one could exploit quantitative differences in metabolism, and select pathways and enzymes that exert a high level of metabolic control in the parasite and are much less effective in the host. This approach is one that has been taken into account in our design of selective glycolysis inhibitors as leads for new anti-trypanosome drugs, and this quantitative metabolic analysis has been discussed in detail in a recent review.²⁶

Exploitation of differences in three-dimensional enzyme structure

Superposition of the 3-D structures of parasite and host enzymes can reveal differences that can be exploited for the design of specific inhibitors.³⁸ For instance, comparison of the active sites of parasite and host glyceraldehyde-3-phosphate dehydrogenases has allowed the development of inhibitors with relatively high specificity and selectivity for the glycosomal enzyme of trypanosomatids,³⁷ (see below). Inhibitors may be designed to be analogues of substrates and other ligands, or may be 'mined' from the vast 3-D databases of small molecules in order to identify any that might have the appropriate shape and chemistry to fit the target site. Any molecules shown to affect the activity of the target enzyme can be optimized by rational design or by combinatorial chemistry and screening.

Exploitation of unique, reactive residues in or near the active site of the parasite enzyme

Although active-site residues of glycolytic enzymes are largely conserved, even when comparing trypanosomatid enzymes to their mammalian counterparts,³⁹ positions may be found where a nucleophilic residue is present only in the parasite enzyme. Such positions may therefore be exploited for irreversible binding of inhibitors, and an example (aldolase) is given in this review. Alternatively, a nucleophilic residue may become available specifically in the parasite enzyme for irreversible binding upon a conformational change induced by a first contact with the inhibitor. One possible approach to explore this possibility involves mapping the active site with substrate analogues. If needed, substrate analogues are first modified in order to obtain sufficient affinity, either empirically or by structure-based methods. Subsequently, electrophilic centers are introduced in the compounds to promote irreversible or slow-binding inhibition, if a reactive nucleophile is suitably located in the vicinity of the inhibitor binding site.

In addition to substrate- and structure-based drug design, high-affinity compounds can also be obtained by screening large libraries of synthesized compounds, and compounds of natural origin. A specific feature of this latter approach is that it may yield uncompetitive inhibitors with structures which could not be anticipated on the basis of the structure of the target protein.

TRYPANOSOMATID GLYCOLYTIC ENZYMES

This section will review the data currently available for the different enzymes of the glycolytic pathway of trypanosomatids and the development of selective inhibitors of these enzymes. Major focus will be on those trypanosomatid enzymes for which 3-D structural information is available: fructose-1,6-bisphosphate aldolase,⁴⁰ triosephosphate isomerase,⁴¹⁻⁴³ glyceraldehyde-3-phosphate dehydrogenase,⁴⁴⁻⁴⁶ phosphoglycerate kinase,⁴⁷ pyruvate kinase,⁴⁸ and glycerol-3-phosphate dehydrogenase.⁴⁹ While the latter enzyme is not glycolytic in the strict sense, it is essential in the glycosome to keep the metabolic pathway going. Because selectivity is a key issue for the development of therapeutics we will

point out relevant differences with the human counterpart enzymes, or if lacking, with another mammalian enzyme as a model for the human one.

Hexokinase

Hexokinase catalyzes the transfer of a phospho group from ATP to glucose. Humans possess four isoenzymes, designated as hexokinases I-IV, with different tissue distribution and kinetic properties.⁵⁰ Hexokinase IV, often called glucokinase, occurs in the liver and has a subunit molecular mass of approximately 50 kDa, very similar to the *T. brucei* enzyme.³⁴ The other human isoenzymes have about twice the subunit size, and have probably arisen by gene duplication followed by fusion. The *T. brucei* enzyme has only 36-37% identity with the human hexokinases (VH and PM, unpublished). Like other hexokinases, the trypanosome enzyme will accept as substrate a range of sugar molecules in addition to glucose. Moreover, it is not regulated by its product glucose 6-phosphate and has, in contrast to all human isoenzymes, a low specificity for ATP; it is able to use IPT, UTP and GTP as well. Crystal structures have been reported for the human brain hexokinase I,^{51,52} but no structure is available as yet for a trypanosomatid hexokinase. However, the low sequence identity, the different kinetic properties and structure modelling all suggest that the development of selective inhibitors of the trypanosomatid enzyme should be feasible. Therefore, the active site of the parasite hexokinase has been explored with N-acetylglucosamine derivatives bearing reactive alkyl or aryl groups. Indeed, various of these compounds inhibited the *T. brucei* enzyme. The best inhibitor was m-Br-phenylglucosamine, with a K_i value of 3 μM ; note that the K_m for glucose is 110 μM . In comparison, the activity of the inhibitor on yeast hexokinase is at least 50 times poorer; no data are as yet available about its effect on mammalian hexokinases.⁵³ Experiments to elucidate the mechanism of these N-acetylglucosamine derivative inhibitors have shown that they are not phosphorylated (i.e. they are not pseudo-substrates) despite a free 6-hydroxyl group. They cause conformational changes of the enzyme and promote ATP hydrolysis.^{54,55} Based on a 3-D model of the active site of *T. brucei* hexokinase, the synthesis of a new set of inhibitors is underway.

Glucose-6-phosphate isomerase

Glucose-6-phosphate isomerase catalyzes the aldose/ketose isomerisation of glucose 6-phosphate to fructose 6-phosphate. Amino-acid sequences of the enzyme from *T. brucei*, *T. cruzi* and *L. mexicana* have been determined.^{56,57} They show 72-76% identity amongst each other, and 57-59% identity with human glucose-6-phosphate isomerase. No crystal structures are available as yet for the enzyme from human or any of the trypanosomatids. Inspection of the sequences, and mapping them on the available crystal structures of the rabbit muscle, porcine and *Bacillus stearothermophilus* enzymes,^{58,59} do not provide obvious clues for the design of selective inhibitors. Conclusions about the feasibility of this enzyme as a drug target should possibly await the availability of a high-resolution crystal structure of a trypanosomatid enzyme.

Phosphofructokinase

Phosphofructokinase is a tetrameric enzyme catalysing the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate using ATP as phospho donor. Under physiological conditions the reaction is essentially irreversible. The human enzyme consists of identical or highly similar subunits of 85 kDa. The activity of this enzyme is regulated by a large number of metabolites and allosteric effectors. In contrast, the trypanosomatid enzyme has a subunit mass of only 53 kDa^{34,60} and its activity is only activated by its substrate fructose 6-phosphate and by AMP and ADP.^{10,11} The genes coding for phosphofructokinase of *T. brucei* and *L. donovani* have been cloned and sequenced,⁶⁰ and Lopez C and PM, unpublished). The percentage of amino-acid identity between the two trypanosomatid enzymes is 70%, but that with the human isoenzymes is very low (about 20%). Surprisingly, the trypanosomatid phosphofructokinases appear more similar to inorganic pyrophosphate (PP_i)-dependent phosphofructokinases,⁶⁰ despite their use of ATP as phospho donor. Although the ATP and PP_i-dependent enzymes are homologous, they are very different.³⁹ These differences offer great potential for the design of selective inhibitors. However, no crystal structure is available as yet for any PP_i-dependent phosphofructokinase, and for the ATP-dependent enzymes structural information is only available for bacterial enzymes.^{61,62} Therefore, current attempts to design selective inhibitors of the trypanosomatid enzyme are based on a very preliminary structure model of its active site. A first study has however been undertaken with fructose 6-phosphate analogues, namely 2,5-anhydro-D-mannitols bearing at position 1 various arylamino-groups.⁶³ These compounds appeared to bind at the ATP-binding site, instead of the fructose 6-phosphate site, with affinity constants of approximately 100 μM. A compound with an electrophilic isothiocyanate group displayed an irreversible inactivation pattern with K_i and k_i values of 130 μM and 0.26 min⁻¹, respectively. The residue involved in this specific inactivation of the parasite enzyme was identified by site-directed mutagenesis, Lys227. Based on this promising result, other compounds will be developed with a more reactive electrophilic center directed towards Lys227.

Fructose-1,6-bisphosphate aldolase

Fructose-1,6-bisphosphate aldolase catalyses the reversible aldol cleavage of fructose 1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Crystal structures have been determined for the enzymes of two trypanosomatids,⁴⁰ *T. brucei* and *L. mexicana*, both in the absence of substrate. Each monomer of the homotetrameric enzyme adopts a (β/α)₈ barrel fold, with the active site at the C-terminal portion of the barrel. The main characteristic of the site is the presence of a large number of charged residues which makes it less likely that high-affinity bioavailable competitive inhibitors can be found. The requirements for electrostatic complementarity of potential potent inhibitors are opposed by those promoting translocation across membranes.

In humans there are three counterpart aldolases: isoenzyme A in muscle tissue and erythrocytes, B in the liver, kidneys and the intestines, and C in the brain. The overall

sequence identity between each of the human enzymes and the parasite enzymes is about 47%, that between the two parasite enzymes 81%. Only crystal structures of the A enzyme have been determined, in the absence⁶⁴ and presence⁶⁵ of the substrate fructose 1,6-bisphosphate.

Efforts to arrive at suicide inhibitors of the *T. brucei* enzyme were not successful.⁶⁶ In contrast, and despite the conservation of most active-site residues, significant progress was made with two types of slow-binding inhibitors (EI^{*} complexes, Fig. 2): (1) β-dicarbonyl compounds which mimic the incoming (or leaving) groups in the carbon-carbon bond formation between dihydroxyacetone phosphate-enamine and glyceraldehyde 3-phosphate⁶⁷ and (2) bisphosphorylated aromatic derivatives bearing reactive groups, that mimic fructose 1,6-bisphosphate.⁶⁸ These compounds stabilize iminium ion reaction intermediates,⁶⁹ an effect that is highly dependent on the microenvironment provided by the enzyme. By taking advantage of slight sequence differences between the parasite and host enzyme, it was possible to arrive at inhibitors with high affinity for *T. brucei* aldolase and without any effect on the mammalian counterpart, even at 50 times higher concentration.

Triosephosphate isomerase

Triosephosphate isomerase catalyses the isomerization of dihydroxyacetone phosphate into D-glyceraldehyde 3-phosphate. Crystal structures have been determined for the *T. brucei*,⁴¹ *T. cruzi*,⁴² and *L. mexicana*⁴³ enzymes. The enzyme is a homodimer in which each monomer adopts a (β/α)₈ barrel fold, with the active site at the C-terminal portion of the barrel. In the presence of substrate the active site is only large enough to contain molecules the size of triosephosphates, but the site opens up in the absence of substrate. This plasticity is due to the movement of a loop that moves at its tip by as much as 7 Å.⁷⁰ The structure of human triosephosphate isomerase has also been determined.⁷¹ In overall sequence the human enzyme is between 51 and 53% identical to the trypanosomatid enzymes, which show 68–74% identity to each other. The active-site residues are fully conserved, hampering the development of selective inhibitors.

An early effort to design competitive inhibitors that would extend some 10 Å outside the closed active site, targeting a unique Ala 100-Tyr101 hydrophobic patch of the *T. brucei* enzyme, remains in an inconclusive state as the proposed complicated inhibitors were never synthesised.⁷² However, in the process, one inhibitor, N-hydroxy-4-phosphono-butanamide, was shown to bind to the enzyme in the open state.⁷³ Because the open state allows for the binding of bigger ligands, it might be a better starting point for drug design.

In another approach, modification of a cysteine in the homodimeric enzyme interface with methylmethanethiosulfonate led to the inactivation of *T. brucei* and *T. cruzi* triosephosphate isomerase.^{74,75} While the Cys residue is not part of the active site there is indirect evidence that the modification induces conformational changes that also involve the active site. Importantly, the human enzyme has a methionine at this position and is therefore not susceptible to the modification reagent. The importance of these studies

resides in the identification of a critical area in the enzyme interface as a target for drug design. Methylmethane thiosulfonate is a proof-of-principle reagent, not a drug as it will also covalently modify the hundreds of enzymes that rely on the presence of a Cys in their active site.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase catalyses the oxidative phosphorylation of D-glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate. Crystal structures have been determined for three parasite enzymes, in each case in complex with the cofactor NAD⁺: *T. brucei*,⁴⁴ *T. cruzi*,⁴⁵ and *L. mexicana*.⁴⁶ The three trypanosomatid enzymes are 80–90% identical to each other, and 45–48% to the human enzyme. Each monomer of the homotetrameric enzyme has two domains. The N-terminal domain binds NAD⁺ via its two nucleotide-binding Rossmann folds, and the cleft between the N-terminal and C-terminal domains harbours the active site of the enzyme.

From a comparison with the human enzyme structure,⁷⁶ (Read R, unpublished results) it appears that the active site of the enzyme and the neighbouring nicotinamide binding site for NAD⁺ are well conserved. However, the binding site for the adenosine portion of NAD⁺ exhibits significant differences between the parasite enzymes and the human

enzyme. Remarkably, in the parasite enzyme there is a narrow hydrophobic cleft adjacent to the 2'-hydroxyl of the adenosine ribose. This cleft is completely lacking in the human enzyme due to a different backbone conformation, warranting the name 'selectivity cleft' for the parasite enzymes.⁷⁷ Hence, it should be possible to design adenosine derivatives that compete with NAD⁺ binding while also occupying the selectivity cleft.

On the basis of adenosine as a lead with an IC₅₀ of about 50 nM careful, structure-based design studies^{77–80} have resulted in adenosine derivatives that exhibit a 250 000 gain in affinity for parasite glyceraldehyde-3-phosphate dehydrogenase and do not inhibit the human enzyme.³⁷ These selective inhibitors project a benzamido group into the selectivity cleft through C2' substitution of the ribose. Simultaneously they fill up a hydrophobic groove near the purine N6 with a 1-methylnaphthyl group or variant thereof (Fig. 3). The designed compounds stunt the growth of cultured *T. brucei* and *T. cruzi* parasites at low micromolar concentration.³⁷

In another approach, conformationally restricted analogues of glyceraldehyde 3-phosphate were designed so that they would present a reactive electrophilic center towards the essential cysteine residue at the enzyme's active site. Such compounds indeed bound irreversibly to this cysteine as

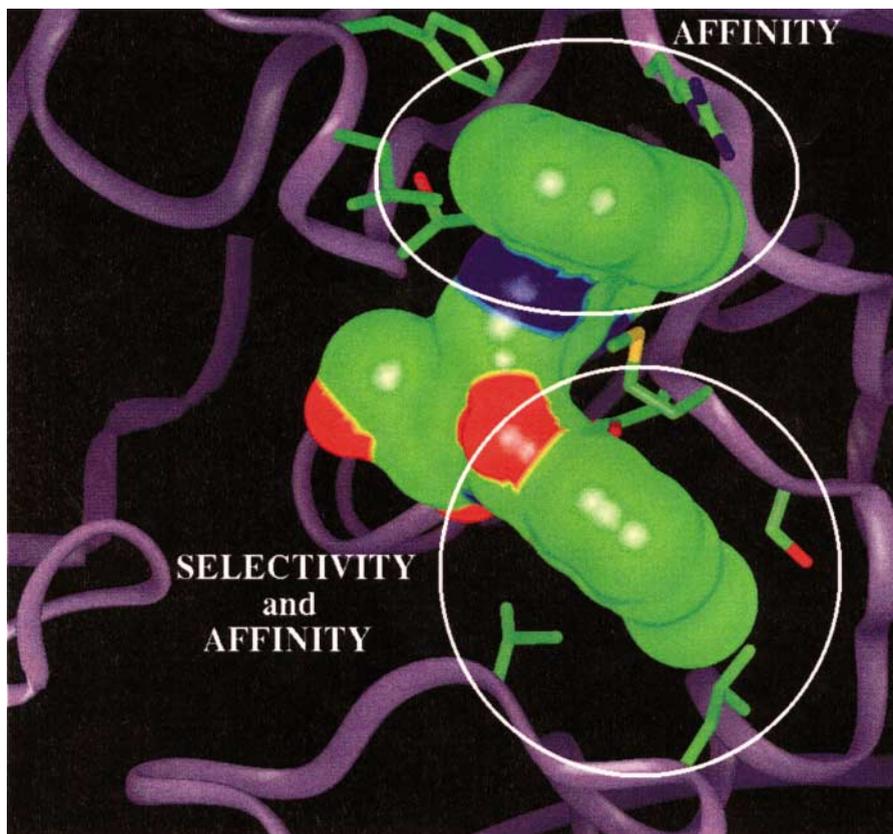


Fig. 3 Model of *L. mexicana* glyceraldehyde-3-phosphate dehydrogenase complexed with N6-(1-naphthylmethyl)-2'-deoxy-2'-(3-methoxybenzamido)-adenosine.³⁷ The 3-methoxybenzamido provides selectivity because the equivalent region in the human enzyme is sterically occluded.

evidenced by kinetic assays involving protection with the substrate, NMR experiments and titration of sulfhydryl groups.⁸¹ The best inhibitor of the *T. brucei* enzyme is 3-(diethylphosphono)propenal, with a K_i of 66 μM , almost 600 times lower than the K_i for the rabbit-muscle enzyme, and a dynamic inactivation constant of 54 min^{-1} . This high selectivity factor is striking because of the considerable conservation of the active site. However, it can be accounted for by the higher hydrophobic character of the active site of the parasite enzyme. The compound kills cultured trypanosomes with a LD_{100} 0.3 μM . This compound was synthesized with a protected phosphate group to improve the ability to cross the membrane. Inside the trypanosome, cytosolic esterases should deprotect the phosphorus headgroup. A similar strategy aimed at analogues of the product of the enzyme reaction, 1,3-bisphosphoglycerate, also led to compounds that exhibit a high selectivity towards the *T. brucei* enzyme.⁸² For instance, the 1,1-bisfluoro analogue of 1,3-bisphospho-D-glyceric acid has an affinity constant of 0.06 mM whereas the same compound has no effect on the rabbit-muscle enzyme at a concentration of 5 mM. Co-crystals recently obtained between this analogue and the *T. cruzi* enzyme, should lead to a better understanding of the residues involved in this selectivity.

Phosphoglycerate kinase

Phosphoglycerate kinase, a monomeric enzyme, catalyses the transfer of a phospho group from 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. During catalysis Mg^{2+} is used as a cofactor. Amino-acid sequences of the enzymes from *T. brucei*, *L. mexicana* and *L. major* have been determined. They are 75–76% identical to each other and 45–47% to the human phosphoglycerate kinase. Of the trypanosomatids only the crystal structure of the *T. brucei*

enzyme has been solved.⁴⁷ The enzyme possesses two domains. The substrate 1,3-bisphosphoglycerate binds to the N-terminal domain, the nucleotide to the C-terminal domain. During catalysis a hinge-bending motion occurs between the domains, bringing the substrates together.

The structure of human phosphoglycerate kinase has not been determined, but the porcine structure⁸³ is probably a valid substitute as the two enzymes share 97% sequence identity. Again most aspects of the active sites of the mammalian and parasite enzymes are conserved. However, the adenosine binding pockets for ADP/ATP exhibit significant differences. Thus far, adenosine derivatives with C2 and/or N6 substitution have been obtained that show up to 100-fold higher affinity than adenosine itself; the IC_{50} of the best compound was 30 μM .⁸⁴ For one of these inhibitors 2-amino-N6-(p-hydroxyphenethyl)adenosine, molecular modelling gave clues that this inhibitor could not fit sterically in the enzyme if the canonical ADP/ATP binding mode was maintained. The X-ray crystallographic structure with bound inhibitor was therefore solved,²⁷ and showed that the adenosine portion of the inhibitor binds in a flipped way, with the N2-amino group located where the N6-amino group normally sits. In addition, the ribose ring is in a syn conformation with respect to the purine ring instead of trans (Fig. 4).⁸⁴ At present, none of the inhibitors shows significant selectivity versus the trypanosomatid enzymes over the mammalian enzyme. However, some of them stunt the growth of *T. brucei* and *T. cruzi* parasites in culture.

Phosphoglycerate mutase

Two classes of phosphoglycerate mutases can be distinguished, both catalysing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. One type requires 2,

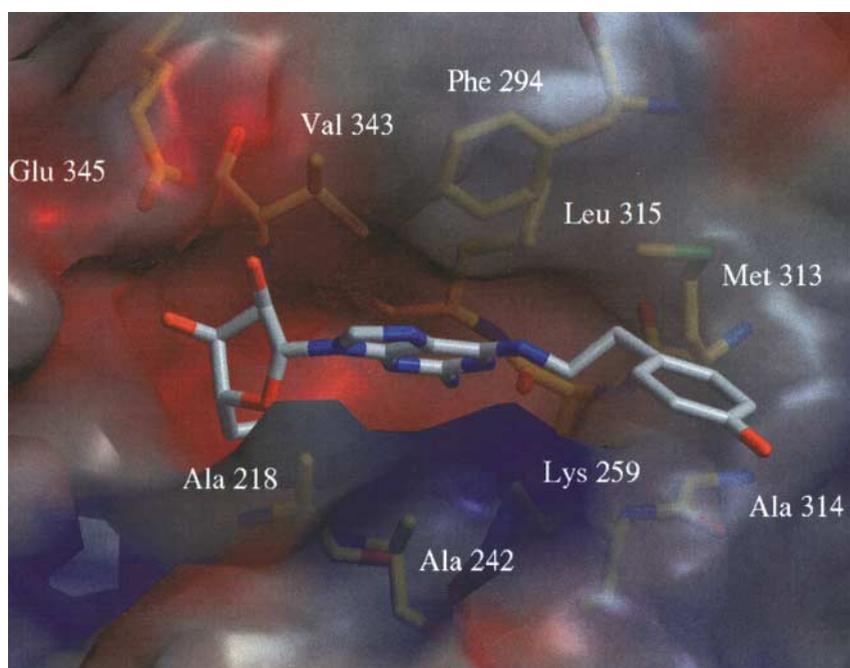


Fig. 4 Crystallographic binding mode of 2-amino-N6-(p-hydroxyphenethyl)adenosine to *T. brucei* phosphoglycerate kinase.⁸⁴

3-bisphosphoglycerate as cofactor, whereas the other does not.³⁹ The two types are not homologous; their structural folds and their reaction mechanisms are entirely different. Humans, like all vertebrates possess a cofactor-dependent mutase, whereas it was recently shown that the phosphoglycerate mutase of trypanosomes belongs to the cofactor-independent class.⁸⁵ This presence in the parasite of an essential enzyme that is not homologous to the corresponding enzyme of its human host offers an excellent possibility for developing selective inhibitors as lead drugs. Recently, the first report of a high-resolution crystal structure of a cofactor-independent enzyme, from *B. stearothermophilus*, was made.⁸⁶ The availability of this structure will aid in the design of such inhibitors. Meantime, work is in progress to over-express trypanosomatid phosphoglycerate mutases for crystallographic studies.

Enolase

Enolase catalyses the Mg²⁺-dependent dehydration of 2-phosphoglycerate to phosphoenolpyruvate. In all eukaryotes, enolase is a homodimeric enzyme with subunits of 45 kDa. In vertebrates, three isoenzymes (α , β and γ) occur of which the expression is regulated developmentally and tissue-specifically.³⁹ The *T. brucei* enolase gene was recently cloned.⁸⁷ The encoded polypeptide has 59–62% identity with the different human enolases. The kinetic properties of the enzyme from the parasite and mammals (rabbit muscle) are very similar; the only striking difference is the activation of the mammalian enzyme by K⁺, whereas this ion has no effect on the trypanosomal enzyme. No human and trypanosomatid crystal enolase structures are available as yet. Structure modelling based on the available structures of yeast⁸⁸ and lobster⁸⁹ enolases indicated that the overall conformation of the active site of the trypanosomal enzyme is very similar to those of the enzymes of other organisms. However, there are some atypical residues close to the *T. brucei* active site which could possibly be exploited for the irreversible binding of selective inhibitors: one Lys and two Cys residues. Current research is devoted to exploring this possibility.

Pyruvate kinase

Pyruvate kinase catalyses a phospho transfer from phosphoenolpyruvate to ADP, forming ATP and pyruvate. In trypanosomatids pyruvate kinase is allosterically regulated by fructose 2,6-bisphosphate, an effector that promotes the active R-state of the enzyme.⁹⁰ Recently, the crystal structure of the *L. mexicana* enzyme was determined.⁴⁸ The enzyme is a homotetramer with each monomer possessing four domains. The active site is located between a central (β/α)₈ barrel and a small β barrel domain, with a binding site for the allosteric effector located within a third domain with α/β topology. An N-terminal helix occupies a pivotal position in the quaternary structure. The determined crystal structure is of the effector-free inactive T- state.

Mammals possess four isoenzymes of pyruvate kinase: M1 mostly in skeletal muscle, M2 in many tissues, L in the liver, and R in red blood cells.³⁹ The sequences of *T. brucei*⁹¹ and *L. mexicana*⁹² pyruvate kinase, which are 74% identical to each other, display 47–51% identity to the various human isoenzymes. Mammalian pyruvate kinases differ in their activity

regulation from the trypanosomatid enzyme. The M1 isoenzyme does not exhibit allosteric properties, while a different effector, namely fructose 1,6-bisphosphate, regulates the three other isoenzymes. No structure of a human pyruvate kinase has been determined, but the structures of the cat⁹³ and the rabbit M1 enzyme,⁹⁴ which exhibit 93% sequence identity with the human M1 and M2 pyruvate kinases and approximately 70% with the human R and L isoenzymes, are valid substitutes for drug design. In addition, structural studies on the yeast and *Escherichia coli* enzymes have provided insight into the allosteric regulation.⁹⁵ From this information the unique features of the trypanosomatid effector site become apparent. The conformations of loops comprising residues 443–453 and 480–489 are strikingly different. Each of these loops provides a putative ligand for the 2-phospho group of fructose 2,6-bisphosphate, namely Lys453 and His480.⁴⁸ Clearly, pyruvate kinase of trypanosomatids has unambiguous selectivity features that make it a target for drug design.

Support for the notion that the effector-binding site is a good target comes from the observation that fructose 6-phosphate analogues such as 2,5-anhydro-D-mannitols (earlier developed as inhibitors of phosphofructokinase,⁶⁵ see above) inhibit the trypanosomatid enzyme by binding at this regulatory site as evidenced by kinetic studies. The formation of a quaternary complex involving the two substrates, the inhibitor and the enzyme has been shown kinetically; the inhibition constant of the best inhibitor presently available is approximately 75 μ M. Modelling of such fructose 6-phosphate analogues in the crystal structure of the *L. mexicana* enzyme (Fig. 5) suggested that reactive residues in the effector-binding pocket, such as Lys453, specific for trypanosomatid pyruvate kinase and Arg456, are properly positioned to be exploited for the development of irreversible, selective inhibitors.

Glycerol-3-phosphate dehydrogenase

Glycerol-3-phosphate dehydrogenase catalyses the interconversion of dihydroxyacetone phosphate and L-glycerol 3-phosphate. A crystal structure has been determined, but only for the *L. mexicana* enzyme in the absence and in the presence of the cofactor NAD⁺.⁴⁹ The enzyme is a homodimer, and each monomer is composed of two domains: an N-terminal NAD⁺-binding domain with the classical Rossmann nucleotide-binding fold, and a C-terminal substrate-binding domain. Remarkably, the substrate-binding domain shares structural similarity with plant acetoxyacid isomeroreductase. The crystal structure also revealed the presence of unexpected long and slender electron density buried in the C-terminal domain. The shape of this density together with its position adjacent to a Cys residue might be indicative of a post-translational modification, possibly with a fatty acid. This intriguing feature that might also be associated with biological function, perhaps membrane association, is under further investigation.

The human enzyme shares only 30% sequence identity with the *L. mexicana* enzyme. For the *T. brucei* enzyme, which has 63% identity with the *Leishmania* enzyme, the number is even lower, about 28%.⁹⁶ It bodes well for selective inhibitor design that the adenosine portion of the NAD⁺-

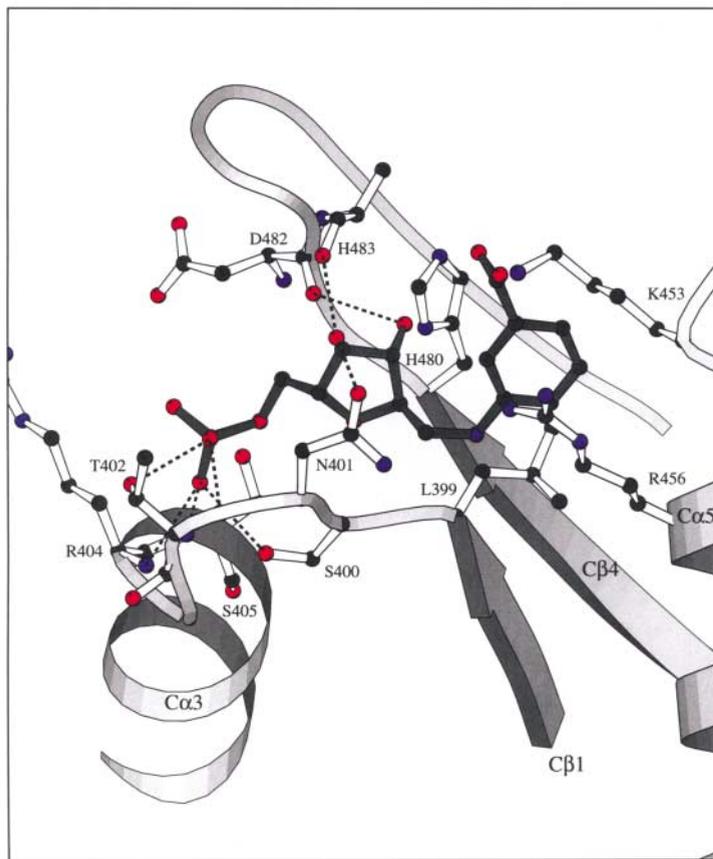


Fig. 5 Fructose 6-phosphate analogue modelled in the effector-binding site of *L. mexicana* pyruvate kinase. Specificity for the binding of the ligand to the parasite enzyme is provided by the carboxylic group borne by the aromatic ring, which is in close contact with the parasite-specific residues Lys453 and His480. The modelling and figure courtesy of Dr D. J. Rigden.

binding site exhibits a 12-residue insertion in the human enzyme, which probably means that the parasite enzyme can accommodate larger ligands in that area than the human one.

Glycerol kinase

Glycerol kinase usually catalyses the ATP-dependent phosphorylation of glycerol. The reverse reaction would only be thermodynamically feasible at high ADP/ATP and glycerol 3-phosphate/glycerol ratios. Such conditions would be difficult to attain in the entire cell, but can occur in a separate small compartment such as the glycosome.^{4,32} It is assumed that in the trypanosome glycerol kinase fulfils both roles: phosphorylation of glycerol as an alternative carbon source when sugars are limited, and production of ATP and glycerol under anaerobic conditions for the reasons explained above. In the human host, the enzyme is only involved in glycerol phosphorylation.

The amino-acid sequences of human and *T. brucei* glycerol kinase are 50% identical.⁹⁷ Inspection of these sequences and the available 3-D structure of *E. coli* glycerol kinase⁹⁸ showed that the structure is well conserved. This analysis, and site-directed mutagenesis studies, suggest that a specific Ser-Ala substitution close to the active site, and specific for all trypanosomatids which can produce glycerol,

may be responsible for the 10-fold reduced affinity of the parasite enzyme for glycerol and glycerol 3-phosphate compared to the enzyme from other sources. Whether these or other specific features are amenable for the development of selective inhibitors remain to be determined. It is unlikely that this enzyme, under most physiological conditions, plays an essential role in the trypanosome's metabolism, and is sub optimal as a drug target. But it may be an important target when inhibited in conjunction with other enzymes, most notably glycerol-3-phosphate oxidase.

Glycerol-3-phosphate oxidase

The *T. brucei* glycerol-3-phosphate oxidase in the inner mitochondrial membrane is a complex comprising a FAD-linked glycerol-3-phosphate dehydrogenase, a ubiquinone pool and a SHAM-sensitive ubiquinol: oxygen oxidoreductase, also known as the trypanosome alternative oxidase (TAO).⁴ No sequence or structural information is as yet available for the mitochondrial glycerol-3-phosphate dehydrogenase of the trypanosome. The TAO activity resides in a single protein of 39 kDa, of which the gene has been characterised.⁹⁹ It is phylogenetically related to the alternative oxidase of plants, fungi and yeasts,²⁴ but no homologues exist in animals. The absence of a related protein in humans, and the recent

finding that the TAO is essential for the survival of cultured bloodstream-form trypanosomes should stimulate efforts to develop specific inhibitors with higher affinity than the currently available SHAM. The recently described TAO inhibitor ascofuranone may be a promising candidate.²¹

Transporters

Trypanosomatid glycolysis might also be blocked by inhibition any of the membrane transport steps involved: the transporter mediated uptake of glucose or efflux of pyruvate through the plasma membrane, or any of the metabolite fluxes through the glycosomal membrane. However to date, no glycosomal membrane transporters for glycolytic intermediates have been identified; their existence has been inferred from the various data suggesting that the glycosomal membrane is a permeability barrier.^{13,14,53,100} Targeting of the transporters present in the plasma membrane may have the advantage that they are readily accessible for externally added substances.

The glucose transporters of trypanosomatids have been well-characterized.¹⁰¹ *T. brucei* contains a multigenic family of glucose transporters, consisting of two isoforms: THT1 and THT2 (for Trypanosome Hexose Transporter). The transporters all belong to the glucose transporter superfamily exemplified by the mammalian erythrocyte transporter GLUT1. THT1 is a facilitated diffusion transporter with a relatively low affinity ($K_m \approx 1$ mM) for glucose. It is only expressed in bloodstream-form *T. brucei*. THT2 is a relatively high-affinity ($K_m \approx 0.05$ mM) transporter specific for the insect-stage parasites. Similar transporters have been found in other trypanosomatids. The members of the *T. brucei* THT1 multigene family are 99% identical, and display 30–85% identity with the various THT isoforms of other trypanosomatids. The identity with the human GLUT1 transporter is 19%. No crystal structure information is available for any of these transporters, but the secondary structure prediction indicates a conserved topology with 12 transmembrane domains. With regard to substrate specificity, THT1 of *T. brucei* and other trypanosomatids shows some important differences when compared with human GLUT1. The parasite transporter is 1000-fold less sensitive to cytochalasin B, and, unlike GLUT1 can transport D-fructose and D-galactose with a relative low affinity. In contrast to the GLUT1, *T. brucei* THT1 does not recognize analogues of glucose with substitutions at its C4 position. These differences are being exploited for the design of selective inhibitors, but so far no such inhibitors with high affinity for the trypanosome transporter have been obtained.

The pyruvate produced by the bloodstream-form of *T. brucei* is excreted into host bloodstream by a facilitated diffusion carrier in symport with a proton.^{18,102} This pyruvate-proton symport is necessary for the regulation of the intracellular pH. Inhibition of the transporter resulted in accumulation of pyruvate within the trypanosomes and acidification of the cytosol, and death by osmotic destabilization of the cells.¹⁸ Based on the sensitivity for inhibitors, it was concluded that the trypanosome transporter may be related to the host monocarboxylate carriers. However, some quantitative differences exist in sensitivity towards inhibitors and specificity for pyruvate analogues and metabolically related

substances.^{18,102} Importantly, the *T. brucei* transporter does not transport L-lactate. The gene of the transporter remains to be identified, and no information whatsoever is available about the structure of the protein. Corresponding proteins are not considered as drug targets for *T. cruzi* and *Leishmania*, because no significant quantities of pyruvate are produced as metabolic end-product in these latter parasites.⁷

Glycosomes

Glycosomes are peroxisome-like organelles into which the matrix proteins are imported by the activity of a variety of proteins called peroxins.¹⁰³ The translocation of the matrix proteins across the peroxisomal membrane is a complicated cascade process in which different peroxins undergo reversible interactions with each other. Inhibitors interfering with these interactions are expected to prevent the synthesis of functional glycosomes and thus kill the parasites. The design of selective inhibitors seems feasible because of the very low level of conservation of peroxins. For the five *T. brucei* peroxins characterised to date identities with human counterparts of only maximally 32% have been found.^{104,105} Two cytosolic peroxins, Pex5 and Pex7 recognize a specific sequence in the matrix protein to be imported into the organelle. This sequence is called PTS (Peroxisome-Targeting Signal). There are two well-defined signals: PTS1, the C-terminal tripeptide, and PTS2, a nonapeptide close to the N-terminus, recognized by Pex5 and Pex7, respectively.¹⁰³ Although the amino-acid motifs of the targeting signals are rather well conserved, the PTSs may offer great opportunities for the design of trypanocidal compounds when they are considered in the structural context. For example, in the tetrameric *T. brucei* and *L. mexicana* aldolases, the N-termini, each with a PTS2 sequence, from two different subunits form two closely intertwined structures.⁴⁰ Any inhibitor binding to this specific structure would interfere with the PTS2-Pex7 interaction and thus prevent aldolase from entering glycosomes. Human aldolase is a cytosolic protein and does not contain a PTS2 sequence. Selectivity, to prevent inhibition of the import into peroxisomes of any host PTS2-bearing proteins could be introduced by the design of compounds that also recognise trypanosomatid-specific aldolase features on the surface neighbouring the PTS2 dimer (Fig. 6). Indeed, the groove flanking the PTS dimer contains a number of unique residues that could be exploited. A similar approach may be considered for each of the other glycolytic enzymes present in the glycosome.

CONCLUSIONS AND PERSPECTIVES

Identification of selective inhibitors of enzymes involved in trypanosomatid glycolysis which could be used as leads for new drugs is a promising approach. Despite the fact that glycolysis is a relatively well conserved pathway, important structural and/or mechanistic differences have been identified in most trypanosomatid enzymes compared to their human equivalents. In a variety of cases these differences have already allowed the design and synthesis of selective inhibitors. Some of these inhibitors have been shown to block glycolysis in the parasites, and to retard their growth in

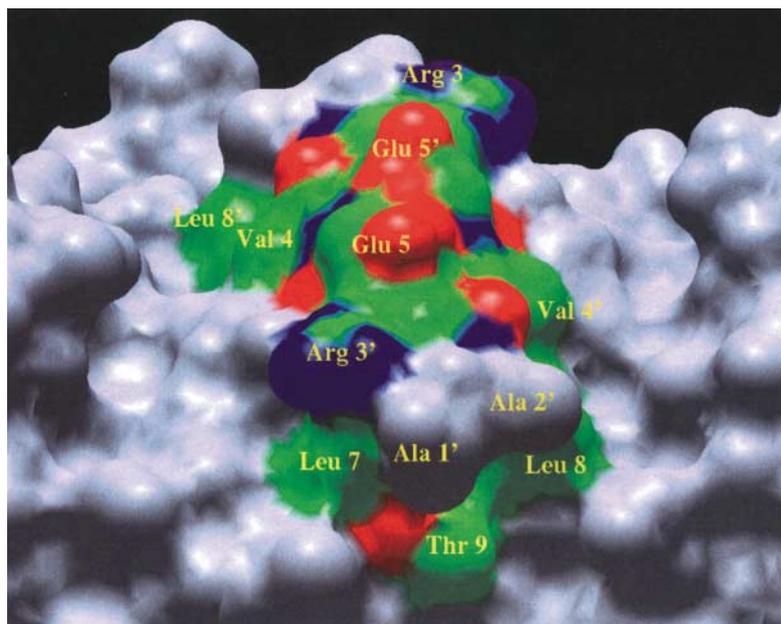


Fig. 6 Molecular surface of the *T. brucei* aldolase PTS2 dimer.⁴⁰ PTS2 residues have been coloured by element type (carbon = green, nitrogen = blue, oxygen = red) while the neighbouring residues are in grey. Residues originating from the second PTS2 are labelled prime. Notice the deep grooves on both sides of the dimer that might be targets for drug design. Such drugs would prevent interaction with PEX7, either directly by steric occlusion, or by allosteric inhibition in case a PTS2 conformation different from the dimer needs to be recognised.

culture without any effect on the growth of cultured mammalian cells. Future research will further optimize the affinity and selectivity of these inhibitors, to make them suitable for assays of *in vivo* activity, toxicity and pharmacokinetics. In addition, prodrugs of such inhibitors are being developed in which charged groups are masked to facilitate crossing of the plasma membrane. Deprotection of these prodrugs should occur by esterases abundantly present in the parasite's cytosol. For other enzymes, of which the analysis started more recently, lead inhibitors have still to be designed or to be selected from combinatorial libraries. Some very promising targets fall in this latter category: phosphofructokinase and particularly phosphoglycerate mutase.

For those cases analysed, the conservation of enzymes among the Trypanosomatidae seems sufficiently high to expect that selective inhibitors developed for one parasitic species will also often be active on the corresponding enzyme of a different species. Indeed, for the group of best inhibitors developed against glyceraldehyde-3-phosphate dehydrogenase and aldolase cross reactivity occurs.

The inhibitors discussed in this paper were all designed as compounds binding at, or near the active site of an enzyme, thus interfering with substrate binding or catalytic mechanism. Other strategies to interfere with trypanosomatid glycolysis seem feasible as well, and are also pursued. Most enzymes of the glycolytic pathway of the parasites are sequestered inside glycosomes. Both experimental data and computer simulation of trypanosomatid metabolism have provided strong support for the notion that the presence of intact glycosomes and proper compartmentation of the

enzymes within these organelles is essential for glycolysis in the parasites and their survival.^{12-14,100} Therefore, any inhibitors that interfere indirectly with glycolysis by preventing the import of the enzymes into the organelle may also be suitable as leads for anti-trypanosome drugs.

In summary, the importance of glycolysis for Trypanosomatidae, the peculiar organization of the pathway in these parasites, and the occurrence of unique features in the structure and/or mechanism of many of the enzymes involved in this metabolic process offer plenty of opportunities for the design of new anti-trypanosome drugs. Considerable progress has already been made in the long route toward the development of such drugs.

Acknowledgements

The research on anti-trypanosomatid drug design in the laboratories of the various authors is financially supported through grants from the European Commission (STD and INCO-DC programmes) and the National Institutes of Health, USA (grant A144199). We are grateful to Dr D. J. Rigden, Bioinformatics Laboratory, Universidade Catolica de Brasilia, Brazil for preparing Figure 5.

Correspondence to: Paul Michels, ICP-TROP 74.39, Avenue Hippocrate 74, B-1200 Brussels, Belgium. Tel: +32 2 7647473; Fax: +32 2 7626853; E-mail: michels@trop.ucl.ac.be

References

1. Atouguia J, Costa J. *Mem Inst Oswaldo Cruz* 1999; 94: 221–224.
2. UNDP/World Bank/WHO. In: Fourteenth Programme Report of the UNDP/World Bank/WHO. Special Programme for Research and Training in Tropical Diseases. Geneva:World Health Organisation, 1999.
3. Barrett MP, Mottram JC, Coombs GH. *Trends Microbiol* 1999; 7: 82–88.
4. Opperdoes FR. *Annu Rev Microbiol* 1987; 41: 127–151.
5. Clayton CE, Michels P. *Parasitol Today* 1996; 12: 465–471.
6. Tielens AGM, Van Hellemond JJ. *Parasitol Today* 1998; 14: 265–271.
7. Cazzulo JJ. *FASEB J* 1992; 6: 3153–3161.
8. Fernandes AP, Nelson K, Beverley SM. *Proc Natl Acad Sci USA* 1993; 90: 11608–11612.
9. Opperdoes FR, Borst P. *FEBS Lett* 1977; 80: 360–364.
10. Nwagwu M, Opperdoes FR. *Acta Tropica* 1982; 39: 61–72.
11. Cronin CN, Tipton KF. *Biochem J* 1985; 227: 113–124.
12. Bakker BM, Mensonides FIC, Teusink B, Van Hoek P, Michels PAM, Westerhoff HV. *Proc Natl Acad Sci USA* 2000; 97: 2087–2092.
13. Michels PAM, Hannaert V, Bringaud F. *Parasitol Today* 2000; 16: 482–489.
14. Helfert S, Bakker BM, Michels PAM, Clayton CE. 2000, submitted.
15. Seyfang A, Duszenko M. *Eur J Biochem* 1991; 202: 191–196.
16. Duszenko, M, Mecke D. *Mol Biochem Parasitol* 1986; 19: 223–229.
17. Barnard JP, Reynafarje B, Pedersen PL. *J Biol Chem* 1993; 268: 3654–3661.
18. Wiemer EAC, Michels PAM, Opperdoes FR. *Biochem J* 1995; 312: 479–484.
19. Fairlamb AH, Opperdoes FR, Borst P. *Nature* 1977; 265: 270–271.
20. Van der Meer C, Versluis-Broers JAM, Opperdoes FR. *Exp Parasitol* 1979; 48: 126–134.
21. Minagawa N, Yabu Y, Kita K, Nagai K, Ohta N, Meguro K, Sakajo S, Yoshimoto A. *Mol Biochem Parasitol* 1997; 84: 271–280.
22. Engel JC, Franke de Cazzulo BM, Stoppani AOM, Cannata JJB, Cazzulo JJ. *Mol Biochem Parasitol* 1987; 26: 1–10.
23. Hart DT, Coombs GH. *Exp Parasitol* 1982; 54: 397–409.
24. Van Hellemond JJ, Simons B, Millenaar FF, Tielens AGM. *J Eukaryot Microbiol* 1998; 45: 426–430.
25. Bakker BM, Michels PAM, Opperdoes FR, Westerhoff HV. *J Biol Chem* 1999; 274: 14551–14559.
26. Bakker BM, Westerhoff, HV, Opperdoes FR, Michels PAM. *Mol Biochem Parasitol* 2000; 106: 1–10.
27. Sjoerdsma A. *Clin Pharmacol Ther* 1980; 30: 3–22.
28. Morrison JF, Walsh CT. In: Meister A (Ed). *Advances in Enzymology*, John Wiley and Sons, Toronto, 1988; 61: 201–299.
29. Schloss JV. *Acc Chem Res*. 1988; 21: 348–353.
30. Varghese JN, Smith PV, Sollis SL, Blick TJ, Sahasrabudhe A, McKimm-Breschkin JL, Colman PM. *Structure* 1998; 6: 735–746.
31. Bakker BM, Westerhoff HV, Michels PAM. *J Bioenerg Biomembr* 1995; 27: 513–525.
32. Bakker BM, Michels PAM, Opperdoes FR, Westerhoff HV. *J Biol Chem* 1997; 272: 3207–3215.
33. Visser N, Opperdoes FR. *Eur J Biochem* 1980; 103: 623–632.
34. Misset O, Bos OJM, Opperdoes FR. *Eur J Biochem* 1986; 157: 441–453.
35. Eisenthal R, Cornish-Bowden A. *J Biol Chem* 1998; 273: 5500–5505.
36. Bakker BM, Michels PAM, Walsh MC, Opperdoes FR, Westerhoff HV. In: Cornish-Bowden A, Cardenas ML (Eds). *Technological and Medical Implications of Metabolic Control Analysis*, Kluwer Academic Publishers, Dordrecht, 2000, pp. 157–164.
37. Aronov AM, Suresh S, Buckner FS, Van Voorhis WC, Verlinde CLMJ, Opperdoes FR, Hol WGJ, Gelb MH. *Proc Natl Acad Sci USA* 1999; 96: 4273–4278.
38. Verlinde CLMJ, Hol WGJ. *Structure* 1994; 7: 577–587.
39. Fothergill-Gilmore LA, Michels PAM. *Prog Biophys Mol Biol* 1992; 59: 105–235.
40. Chudzik DM, Michels PAM, de Walque S, Hol WGJ. *J Mol Biol* 2000; 300: 697–707.
41. Wierenga RK, Noble ME, Vriend G, Nauche S, Hol WGJ. *J Mol Biol* 1991; 220: 995–1015.
42. Maldonado E, Soriano-Garcia M, Moreno A et al. *J Mol Biol* 1998; 283: 193–203.
43. Williams JC, Zeelen JP, Neubauer G et al. *Protein Eng* 1999; 12: 243–250.
44. Vellieux FM, Hajdu J, Verlinde CLMJ et al. *Proc Natl Acad Sci USA* 1993; 90: 2355–2359.
45. Souza DH, Garratt RC, Araujo AP et al. *FEBS Lett* 1998; 424: 131–135.
46. Kim H, Feil IK, Verlinde CLMJ, Petra PH, Hol WGJ. *Biochemistry* 1995; 34: 14975–14986.
47. Bernstein BE, Michels PAM, Hol WGJ. *Nature* 1997; 385: 275–278.
48. Rigden DJ, Phillips SE, Michels PAM, Fothergill-Gilmore LA. *J Mol Biol* 1999; 291: 615–635.
49. Suresh S, Turley S, Opperdoes FR, Michels PAM, Hol WGJ. *Struct Fold Des* 2000; 8: 541–552.
50. Cardenas ML, Cornish-Bowden A, Ureta T. *Biochim Biophys Acta* 1998; 1401: 242–264.
51. Aleshin AE, Zeng C, Bourenkov GP, Bartunik HD, Fromm HJ, Honzatko RB. *Structure* 1997; 6: 39–50.
52. Rosano C, Sabini E, Rizzi M et al. *Structure* 1999; 7: 1427–1437.
53. Willson M, Alric I, Périé J, Sanejouand YH. *J Enzym Inhib* 1997; 12: 101–121.
54. Trinquier M, Boisdon MJ, Périé J, Willson M. *Spectrochim Acta A* 1998; 54: 367–373.
55. Willson M, Périé J. *Spectrochim Acta A* 1999; 55: 911–917.
56. Marchand M, Kooystra U, Wierenga RK et al. *Eur J Biochem* 1989; 184: 455–464.
57. Nyame K, Do-Thi CD, Opperdoes FR, Michels PAM. *Mol Biochem Parasitol* 1994; 67: 269–279.
58. Sun YJ, Chou CC, Chen WS, Wu RT, Meng M, Hsiao CD. *Proc Natl Acad Sci USA* 1999; 96: 5412–5417.
59. Jeffery CJ, Bahnson BJ, Chien W, Ringe D, Petsko A. *Biochemistry* 2000; 39: 955–964.
60. Michels PAM, Chevalier N, Opperdoes FR, Rider MH, Rigden DJ. *Eur J Biochem* 1997; 250: 698–704.
61. Evans PR, Hudson PJ. *Nature* 1979; 279: 500–504.
62. Shirakihara Y, Evans PR. *J Mol Biol* 1988; 204: 973–994.
63. Claustre S, Bringaud F, Azema L, Périé J, Willson M. *Carbohydr Res* 1999; 315: 339–344.
64. Gamblin SJ, Davies GJ, Grimes JM, Jackson RM, Littlechild JA, Watson, HC. *J Mol Biol* 1991; 219: 573–576.
65. Dalby A, Dauter Z, Littlechild JA. *Protein Sci* 1999; 8: 291–297.
66. Périé J, Rivière-Aldric I, Blonski C, Willson M, Opperdoes FR. *Pharmac Ther* 1994; 60: 347–365.
67. Gefflaut T, Blonski C, Périé J. *Bioorg Med Chem* 1996; 4: 2043–2054.
68. Gefflaut T, Blonski C, Périé J, Willson M. *Progr Biophys Mol Biol* 1995; 63: 301–340.
69. Blonski C, De Moissac D, Périé J, Sygusch J. *Biochem J* 1997; 323: 71–77.

70. Wierenga RK, Noble ME, Postma JP, Groendijk H, Kalk KH, Hol WGJ, Opperdoes FR. *Proteins* 1991; 10: 33–49.
71. Mande SC, Mainfroid V, Kalk KH, Goraj K, Martial JA, Hol WGJ. *Protein Sci* 1994; 3: 810–821.
72. Verlinde CLMJ, Rudenko G, Hol WGJ. *J Comput-Aided Mol Design* 1991; 6: 131–147.
73. Verlinde CLMJ, Witmans CJ, Pijning T, Kalk KH, Hol WGJ, Opperdoes FR. *Protein Sci* 1992; 1: 1578–1584.
74. Gomez-Puyou A, Saavedra-Lira E, Becker I, Zubillaga RA, Rojo-Dominguez A, Perez-Montfort R. *Chem Biol* 1995; 2: 847–855.
75. Perez-Montfort R, Garza-Ramos G, Alcantara GH et al. *Biochemistry* 1999; 38: 4114–4120.
76. Mercer WD, Winn SI, Watson HC. *J Mol Biol* 1976; 104: 277–283.
77. Verlinde CLMJ, Callens M, Van Calenbergh S et al. *J Med Chem* 1994; 37: 3605–3613.
78. Van Calenbergh S, Van Den Eeckhout E, Herdewijn P et al. *Helv Chim Acta* 1994; 77: 631–644.
79. Van Calenbergh S, Verlinde CLMJ, Soenens J et al. *J Med Chem* 1995; 38: 3838–3849.
80. Aronov AM, Verlinde CLMJ, Hol WGJ, Gelb MH. *J Med Chem* 1998; 41: 4790–4799.
81. Willson M, Lauth N, Périé J, Callens M, Opperdoes FR. *Biochemistry* 1994; 33: 214–222.
82. Ladame S, Bardet M, Périé J, Willson M. *Bioorg Med Chem* 2000; in press.
83. Harlos K, Vas M, Blake CF. *Proteins* 1992; 12: 133–144.
84. Bressi JC, Choe J, Hough MT et al. *J Med Chem* 2000; in press.
85. Chevalier N, Rigden DJ, Van Roy J, Opperdoes FR, Michels PAM. *Eur J Biochem* 2000; 267: 1464–1472.
86. Jedrzejewski MJ, Chander M, Setlow P, Krishnasamy G. *EMBO J* 2000; 19: 1419–1431.
87. Hannaert V, Brinkmann H, Nowitzki U et al. *Mol Biol Evol* 2000; 17: 989–1000.
88. Lebioda L, Stec B, Brewer JM. *J Biol Chem* 1989; 264: 3685–3693.
89. Duquerroy S, Camus C, Janin J. *Biochemistry* 1995; 34: 12513–12523.
90. Van Schaftingen E, Opperdoes FR, Hers HG. *Eur J Biochem* 1985; 53: 403–406.
91. Allert S, Ernest I, Poliszczak A, Opperdoes FR, Michels PAM. *Eur J Biochem* 1991; 200: 19–27.
92. Ernest I, Callens M, Opperdoes FR, Michels PAM. *Mol Biochem Parasitol* 1994; 64: 43–54.
93. Muirhead H, Clayden DA, Barford D, Lorimer CG, Fothergill-Gilmore LA, Schiltz E, Schmitt W. *EMBO J* 1986; 5: 475–481.
94. Larsen TM, Laughlin LT, Holden HM, Rayment I, Reed GH. *Biochemistry* 1994; 33: 6301–6309.
95. Mattevi A, Bolognesi M, Valentini G. *FEBS Lett* 1996; 389: 15–19.
96. Kohl L, Drmota T, Do-Thi CD, Callens M, Van Beeumen J, Opperdoes FR, Michels PAM. *Mol Biochem Parasitol* 1996; 76: 159–173.
97. Kralova I, Rigden DJ, Opperdoes FR, Michels PAM. *Eur J Biochem* 2000; 267: 2323–2333.
98. Hurley JH, Faber HR, Worthylake D, Meadow ND, Roseman S, Pettigrew DW, Remington SJ. *Science* 1993; 259: 673–677.
99. Chaudhuri M, Hill GC. *Mol Biochem Parasitol* 1996; 83: 125–129.
100. Blattner J, Helfert S, Michels P, Clayton C. *Proc Natl Acad Sci USA* 1998; 95: 11596–11600.
101. Tetaud E, Barrett M, Bringaud F, Baltz T. *Biochem J* 1997; 325: 569–580.
102. Vanderheyden N, Wong J, Docampo R. *Biochem J* 2000; 346: 53–62.
103. Elgersma Y, Tabak HF. *Biochim Biophys Acta* 1999; 1286: 269–283.
104. Lorenz P, Maier AG, Baumgart E, Erdmann R, Clayton C. *EMBO J* 1998; 17: 3542–3555.
105. De Walque S, Kiel JAKW, Veenhuis M, Opperdoes FR, Michels PAM. *Mol Biochem Parasitol* 1999; 104: 107–119.

Author Query

Please provide the article titles for all journal references and chapter titles for all book references.
Please check the insertion of text A in page no. 11