Metabolic enzymes of helminth parasites: potential as drug targets

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Abstract

Metabolic pathways which extract energy from carbon compounds are essential for an organism's survival. Therefore, inhibition of enzymes in these pathways represents a potential therapeutic strategy to combat parasitic infections. However, the high degree of similarity between host and parasite enzymes makes this strategy potentially difficult. Nevertheless, several existing drugs to treat infections by parasitic helminths (worms) target metabolic enzymes. These include the trivalent antimonials which target phosphofructokinase and Clorsulon which targets phosphoglycerate mutase and phosphoglycerate kinase. Glycolytic enzymes from a variety of helminths have been characterised biochemically, and some inhibitors identified. To date none of these inhibitors have been developed into therapies. Many of these enzymes are externalised from the parasite and so are also of interest in the development of potential vaccines. Less work has been done on tricarboxylic acid cycle enzymes and oxidative phosphorylation complexes. Again, while some inhibitors have been identified none have been developed into drug-like molecules. Barriers to the development of novel drugs targeting metabolic enzymes include the lack of experimentally determined structures of helminth enzymes, lack of direct proof that the enzymes are vital in the parasites and lack of cell culture systems for many helminth species. Nevertheless, the success of Clorsulon (which discriminates between highly similar host and parasite enzymes) should inspire us to consider making serious efforts to discover novel anthelminthics which target metabolic enzymes.

Keywords: Glycolysis, neglected tropical disease, Clorsulon, trematode, nematode, cestode

Introduction: the need for new drugs to combat helminth infections

Infections with helminth (worm) parasites are a major burden on humanity. It is estimated that approximately one third of the human population are currently infected with parasitic helminths [1]. The majority of these individuals live in developing countries and the infections are classed as neglected tropical diseases. Many of these individuals are infected with several helminths or with other infectious agents such as HIV, the malarial parasite or bacteria [2]. In many cases, the helminth infections are not immediately life-threatening. However, in general, they result in significant ill health and may have longer term consequences even if the actual infection is cleared. The consequences are not just felt by the infected individuals: reduced productivity of those infected can have drastic consequences for families and communities. This is, perhaps, especially severe in largely agricultural communities which rely heavily on human labour. There is also a world-wide burden on farm animals. Again, this reduces productivity and, consequently, income. Almost twenty years ago, the costs of helminth infections to agriculture in Australia was estimated at one billion US dollars [3]. The current, global cost is likely to be many times that amount.

The main groups of helminth parasites of importance to mammals are from the phyla Platyhelminthes (flat worms) and Nematoda (round worms). Within the Platyhelminthes the trematoda (flukes) and cestoda (tapeworms) are the most important classes and within Nematoda soil transmitted and filarial nematodes are the most important groups. By numbers of humans infected, the roundworm *Ascaris lumbricoides* is the most significant with an estimated 807 million people infected [1]. Whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* and *Ancylostoma duodenale*) each infect in excess of 500 million people [1]. Among the trematodes, the blood flukes (*Schistosoma spp.*) infect over 200 million people and are second only to the malarial parasites in terms of number of humans killed globally each year [1, 4, 5]. Many of these parasitic infections are zoonotic, being passed to humans from domestic animals [6].

To date, there are no effective vaccines for helminth infections and treatment relies entirely on chemotherapy. While many anthelminthic drugs (e.g. triclabendazole, praziquantel) are cheap, effective and generally considered safe, not all diseases can be treated effectively. Resistance to these drugs is also emerging. Resistance to the triclabendazole (the treatment of choice for *Fasciola spp* infections) has been documented in many countries in farm animals [7-14]. Recently the first confirmed case of a human infected with triclabendazole resistant *F. hepatica* has been reported [15]. Although resistance to praziquantel (used to treat *Schistosoma spp* infections) has not been conclusively demonstrated in the field, it has been shown to occur under laboratory conditions [16, 17]. Ivermectin (a broad spectrum anthelminthic) resistance has also been documented [18-22]. There is even resistance to drugs which have been relatively recently introduced. Monepantel is a broad spectrum drug for the treatment of nematode infections in livestock. It was introduced in 2009 and resistant isolates have already been reported [23]. The emergence of resistance to these drugs is not surprising and its further spread should be expected. Although it may be slowed by improved dosing and prescribing policies, it will not be stopped. Resistance is an almost inevitable evolutionary response by the organism once it is placed under severe selective pressure by the widespread use of anthelminthic drugs. While longer term solutions may come from the use of vaccines or improved living conditions in the developing world, the immediate need is for new drugs [24].

The potentials and pitfalls of targeting metabolic enzymes

Central carbon metabolism (glycolysis, Krebs' tricarboxylic acid cycle, the electron transport chain and associated pathways) is necessary for energy production in the majority of eukaryotes. Inhibition of these processes generally results in reduced ATP production and can lead to cell death. A number of common poisons target elements of carbon metabolism. For example, cyanide ions inhibit cytochrome coxidase and arsenate ions can substitute for phosphate ions reducing the ATP

yield from glycolysis and the tricarboxylic (citric) acid cycle (TCA) [25, 26]. These toxins have broad species specificity and affect the majority of organisms tested. Consequently, they have no potential in the treatment of pathogens such as helminth parasites. Nevertheless, the effectiveness of these toxins suggests that inhibition of enzymes in these metabolic pathways is likely to result in death of the parasite.

Further evidence for the critical importance of carbon metabolism comes from inherited metabolic diseases resulting from mutations in the genes encoding enzymes from these pathways. These diseases tend to be rare, but the symptoms are often severe. Examples include triose phosphate isomerase deficiency (OMIM #615512), phosphoglycerate kinase 1 deficiency (OMIM # 300653), hereditary fructose intolerance (aldolase B deficiency; OMIM #22960) and classic galactosemia (galactose 1-phosphate uridylyltransferase deficiency; OMIM #230400) [27-31]. In addition to reduced energy production, these diseases often have additional phenotypes. For example, triosephosphate isomerase deficiency results in a build-up of dihydroxyacetone phosphate which is believed to be toxic, a result which is recapitulated by inhibitors of the enzyme [27, 32]. Therefore, it is expected that inhibition of key metabolic enzymes in helminth pathogens would lead to numerous deleterious pharmacological effects which would kill (or severely harm) the organism.

The pathways of carbon metabolism are highly conserved through evolution, as are the enzymes which catalyse the individual steps. Thus, there are few structural differences between the host and parasite enzymes which can be exploited for the design of species-specific inhibitors. For example, the crystal structures of human and *S. mansoni* hexokinase I have a root mean squared deviation of approximately 1 Å [33]. Nevertheless, there was considerable interest in the 1950s, 1960s and 1970s in studying the metabolic pathways and enzymes of parasites. This interest was, in part, motivated by a desire to discover biochemical or structural differences between host and parasite enzymes which could be exploited in drug discovery. Over the subsequent two to three decades interests waned and only in the last 10 or so years has this interest in helminth metabolic enzymes

recovered. The loss of interest coincided with the widespread use of highly effective anthelminthics such as praziquantel and triclabendazole. The re-emergence of interest coincided with the realisation that resistance to these compounds is a growing threat and to increased interest in targeting metabolism in other pathogens and in cancer [34-39].

Prior to the discovery of praziquantel, the main treatment for schistosome infections was antimony-based compounds. These treatments were somewhat unsatisfactory since they cause considerable side-effects in the patient [40]. These trivalent organic antimonials (e.g. Stibophen, Scheme 1) target the glycolytic enzyme phosphofructokinase (PFK, EC 2.7.1.11) [41, 42]. A similar mechanism, combined with inhibition of a second enzyme, aldolase occurs in filarial worms [43]. The use of these compounds in schistosomiasis has been largely superseded by praziquantel. They are still used to treat leishmaniasis. However, the mode of action in *Leishmania spp* is believed to involved disruption of nucleotide metabolism rather than effects on glycolysis [44, 45].

Clorsulon (MK-401, 4-amino-6-trichloroethenyl 1,3-benzenedisulfonamide; Scheme 1) is used to treat farm animals infected with *Fasciola spp* [46, 47]. Its mode of action is the selective, competitive inhibition of the glycolytic enzymes phosphoglycerate kinase (EC 2.7.2.3) and phosphoglyceromutase (EC 5.4.2.11) [48-51]. It also has weak activity against carbonic anhydrase (EC 2.4.1.1) in the host and, most likely, the parasite [52, 53]. The drug causes widespread damage to the ultrastructure of *F. hepatica* [54-57].

The broad spectrum benzimidazole anthelminthic mebendazole (Scheme 1) has been shown to inhibit the TCA enzyme malate dehydrogenase (EC 1.1.1.37) from *Ascaris suum*, *Fasciola hepatica* and *Moniezia expansa* [58]. Two related compounds, fenbendazole and thiabendazole (Scheme 1) inhibit the malate dehydrogenase of *Trichuris globulosa*. In addition, these compounds also inhibit glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and triacylglycerol lipase (EC 3.1.1.3) from this species [59]. The main target of benzimidazoles is generally considered to be the cytoskeletal protein β-tubulin [60]. It remains to be discovered how significant the inhibition of metabolic

enzymes is in their overall mode of action. Nevertheless, the existence of these examples of proven anthelminthics which target metabolic enzymes suggests that there is considerable scope for further advances in this area.

Although the main pathways of carbon metabolism, and the enzymes which catalyse the individual steps, show high similarity between the parasites and hosts, there are some metabolic adaptations which are unique to the parasites. Most likely, these reflect the life cycles of the worms. In general, these life cycles involve an intermediate, invertebrate host (often a snail or other mollusc) as well as the definitive, mammalian host. In addition, there may be free-living stages. This means that the parasite must adjust to a range of temperatures and oxygen levels [61]. Thus, we would expect that helminth parasite enzymes should be able to be active over wider temperature ranges than the host's (or that helminths would be able to express different isoenzymes which are adapted to particular temperature conditions). Unlike the hosts, the parasite must be able to survive for extended periods under anaerobic conditions. Most eukaryotic species rely largely on glycolytic ATP production under low oxygen conditions. In mammals, this can only be sustained for short periods of time since the energy yield is too low and lactate levels increase, altering the pH of the blood [62].

Many helminth parasites have adaptations to their metabolic pathways which permit increased ATP production under anaerobic conditions. The TCA is modified so that part of it can run partly "in reverse" (Figure 1) [63]. In *F. hepatica*, phospho*enol*pyruvate is carboxylated and reduced, converting it to malate. This conversion occurs in the cytoplasm and the malate is transported to the mitochondrial matrix. Similar reactions occur in most aerobic organisms to replenish TCA intermediates when the cycle is used for biosynthesis rather than energy production. In the fluke, there are two fates of the mitochondrial malate [64]. Some molecules are oxidatively decarboxylated to pyruvate, which is subsequently converted to acetyl coenzyme A [65, 66]. This process produces two molecules of NADH. The acetyl coenzyme A then reacts with succinate releasing acetate and generating succinyl coenzyme A. Conversion of this succinyl coenzyme A to

succinate is coupled to the direct production of ATP (Figure 1) [67-69]. The remaining malate molecules are reduced to succinate (via fumarate) in a reversal of the TCA reactions. The production of succinate is catalysed by fumarate reductase (EC 1.3.5.4), rather than succinate dehydrogenase (EC 1.3.5.1), the enzyme which catalyses the "forward" reaction under aerobic conditions. The succinate reacts with propionyl coenzyme A to generate methylmalonyl coenzyme A (via succinyl coenzyme A). The regeneration of propionyl coenzyme A from this compound is coupled to the production of one molecule of ATP [70, 71]. Thus, under anaerobic conditions the fluke is able to dismutate malate, producing ATP without net oxidation or reduction (Figure 1). Further ATP is produced from coupling the reduction catalysed by fumarate reductase to an electron transport chain [61, 63, 72]. The electrons required for this reaction are provided by reduced rhodoquinone, generated by a modified form of mitochondrial complex I. In the fluke and under anaerobic conditions, complex I transfers electrons from NADH to rhodoquinone and couples this reaction to the transport of protons across the inner mitochondrial membrane [73]. This generates a proton motive force and enables the synthesis of a limited amount of ATP, even in the absence of oxygen (Figure 1). Rhodoquinone (Scheme 2) is a mobile, low redox potential electron carrier found in helminths (and some bacteria) which are able to generate proton motive forces in the absence of a terminal electron acceptor such as oxygen [73-77]. The synthetic pathway for rhodoquinone appears to be very similar to that of ubiquonone; indeed, it has been speculated that only the final reaction differs [78].

Although *F. hepatica* remains one of the best characterised helminth parasites in terms of its anaerobic metabolic adaptations, similar pathways have been demonstrated or inferred in a variety of other species. These include the trematodes *Schistosoma mansoni* [79], *Calicophoron ijimai* [80] and *Paragonimus westermani* [81], the nematodes *Ascaris suum* [82-85], *Setaria digitata* [86] and *Haemonchus contortus* [87] and the cestodes *Spirometra mansonoides* (*Diphyllobothrium mansonoides*) [88], *Hymenolepis diminuta* [89], *Hymenolepis microstoma* [90] and *Taenia crassiceps* [91].

Helminth glycolytic enzymes: current state of knowledge

Although genome sequences are available for some helminths, our level of knowledge about the structures and enzymology of helminth enzymes lags substantially behind that for humans and other well-studied species. In particular, detailed comparative studies between parasite and host enzymes are relatively rare. However, the identification of subtle differences in structure, mechanism or other biochemical properties may reveal aspects which could be targeted in the design of inhibitors specific to parasite proteins.

The first committed step in glycolysis is catalysed by hexokinase (EC 2.7.1.1). RNAi, overexpression and enzymological studies have validated this enzyme as a target in *Trypanosoma brucei*. In this organism, a 60% reduction in hexokinase activity resulting from silencing the corresponding gene by RNAi was sufficient to kill the parasite [92]. Sequencing of hexokinase from *Haemonchus contortus* revealed two insertions (compared to the human sequence) located on the surface of the enzyme which were suggested as possible sites that could be targeted by inhibitors [93]. However, no biochemical work has been done to follow up this proposal. *Brugia malayi* hexokinase is tetrameric (compared to the monomeric and dimeric forms more commonly seen in mammals) and has different kinetic parameters to the human enzyme [94, 95]. *S. mansoni* hexokinase is inhibited by 5-thio-D-glucose (Scheme 3) [96]. However, this compound also inhibits mammalian hexokinase [82]. Modified monosaccharides have been suggested as general inhibitors or antimetabolites targeting hexokinase [97]; however, little work has been done to exploit this suggestion with hexokinases from helminth parasites.

Glucose 6-phosphate isomerase (phosphoglucose isomerase; EC 5.3.1.9) catalyses the conversion of glucose 6-phosphate to fructose 6-phosphate. In the cestode *Echinococcus multilocularis*, the enzyme is involved in pathogen-host interactions as well as glycolysis. In order to interact with the

host, the enzyme must be released from the cells and, as a consequence, host animals develop an immune response towards the enzyme [98]. This dual role suggests that antagonism of the enzyme's actions may disrupt both the energy metabolism of the organism and the interaction with the host. Sugar phosphates (and related compounds) inhibit *S. mansoni* glucose 6-phosphate isomerase; 5-phospho-D-arabinonate (Scheme 3) was the most potent inhibitor tested [99]. However, this compound also inhibits the human enzyme to a similar extent [100].

Phosphofructokinase is a proven target of trivalent antimonials (see above). In the filarial parasite *Setaria cervi*, the enzyme may act as a secondary target for the anthelminthic suramin (Scheme 3) [101]. The enzyme has been characterised from a number of other species, including the nematodes *Teladorsagia circumcincta* [102] and *A. suum* [103, 104], the trematode *F. hepatica* [105-107] and the cestode *Hymenolepis diminuta* [108]. To date, no compounds have been identified which mimic the worm clearing effectiveness of the trivalent antimonials but lack the side-effects. A similar picture emerges for aldolase (EC 4.1.2.13), the enzyme which catalyses the splitting of fructose 1,6-*bis*phosphate. Aldolase from the filarial nematode *Onchocerca volvulus* is secreted from the organism and induces an immune response in the host [109]. The enzyme from a number of helminths including *S. mansoni, A. suum*, *Heterodera glycines* and *Globodera rostochiensis* has also been characterised [110-112]. The viability of the plant parasitic nematode *Heterodera glycines* is reduced when the organism infects genetically modified plants which express RNAi targeted against its aldolase gene [113], thus demonstrating that this enzyme has potential as a drug target.

Triose phosphate isomerase (TPI; EC 5.3.1.1) catalyses the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, thus ensuring that all six carbon atoms from glucose are ultimately converted to pyruvate by glycolysis. TPI from the cestode *Taenia solium* shows few biochemical differences from the enzyme from one of the parasite's main hosts, the pig [114, 115]. This enzyme can be noncompetitively inhibited by antibodies (and Fab fragments derived therefrom) raised against it [116]. TPI from the trematodes *F. hepatica* and *S. mansoni* have both been

biochemically characterised [117, 118]. Phospho*enol*pyruvate (Scheme 3) is an inhibitor of *F. hepatica* TPI; this inhibition is approximately 10-fold weaker than observed with the human enzyme [117, 119]. The structures of the enzyme from both species have been modelled and the *S. mansoni* enzyme contains a small loop on the surface (Ser-157 to Asp-159) which is not present in the human enzyme; this may be a site for immune recognition of the parasite enzyme [118, 120]. Otherwise, the fluke enzymes show high predicted structural similarity to the human ones [117, 118]. In a number of unicellular parasites, covalent modification of TPI at surface-exposed cysteine residues has proved to be a successful strategy for the identification of species-specific TPI inhibitors [121-128]. One of these residues is conserved in *S. mansoni* TPI (as Cys-221), suggesting that there is potential for a similar approach. An alternative strategy, which has also been employed in unicellular parasites, has been to discover reagents which disrupt the dimer interface [129-131]. This may also have potential in parasitic helminths. TPI is externalised in a number of species and may play a role in host-parasite interactions. As such it has attracted attention as a vaccine candidate; for examples, see [132-135].

Glyceraldehyde 3-phosphate is oxidised and phosphorylated in a reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12). Interestingly, RNAi knockdown of the *GAPDH* gene in *S. mansoni* resulted in no detectable phenotypic changes. This was ascribed to metabolic plasticity of the organism [136]. Alternatively, it is possible that the protein product has a relatively long half-life in the organism and so GAPDH activity was not greatly affected by reduction in mRNA levels. The GAPDH from *H. contortus* is highly stable, both to thermal denaturation and pH [137]. The enzyme from *F. hepatica* behaves differently. In the absence of bound ligands, the enzyme is relatively unstable towards thermal denaturation but addition of glyceraldehyde 3-phospate substantially increases the protein's stability [138]. GAPDH from *F. hepatica* also has interesting oligomeric properties. Addition of either glyceraldehyde 3-phosphate or NAD^+ shifts the tetramer-dimer equilibrium towards the dimer. The mammalian enzyme undergoes a similar transition, but both substrates are required [138, 139]. The dimeric form of the

rabbit enzyme is inactive as a dehydrogenase [139]. Further investigations are required to determine if the dimeric form of the *F. hepatica* enzyme is active. If, like the rabbit enzyme, it proves not to be, reagents which promote or stabilise the dimeric form over the tetramer may represent a potential strategy for antagonism of GAPDH activity in the parasite. GAPDH from *S. mansoni* is recognised and inhibited by antisera from infected animals [140]. It is one of the antigens recognised by humans who are more resistant to infection by the parasite [141]. Thus, GAPDH is also externalised by the parasite and may have some potential as a vaccine candidate [142-146]. In *H. contortus*, GAPDH interacts with the C3 complement subunit, inhibiting its function [147]. Antagonism of this interaction may also be a fruitful strategy for chemotherapy.

The first production of ATP in glycolysis is catalysed by the enzyme phosphoglycerate kinase. Vaccination with *F. hepatica* phosphoglycerate kinase gave some protection against infection which was ascribed to blockage of the fluke's energy metabolism [148]. However, no direct evidence of enzyme inhibition has been obtained to date. It is equally probably that the protection following vaccination results from the disruption of some interaction between surface-expressed enzyme and the host. A tegumental location for the enzyme in *Clonorchis sinensis* supports the latter hypothesis [149]. In most eukaryotes, phosphoglyceromutase requires a tightly bound 2,3-*bis*phospho-D-glycerate cofactor. However, nematodes have a cofactor-independent phosphoglyceromutase (EC 5.4.2.12) [150]. In addition to the cofactor requirement the two types of phosphoglyeromutase differ in sequence, structure and catalytic mechanism. The nematode enzyme has attracted considerable attention as a potential drug target due to these differences from the host enzyme [151-154]. A number of potential inhibitors have been identified by computational chemistry methods (Scheme 4); however these have yet to be tested *in vitro* or *in vivo* [151]. In the non-parasitic nematode, *Caenorhabditis elegans*, RNAi studies have demonstrated that the enzyme is essential for viability [150]. However, recent high throughput screening has thrown doubt onto the "druggability" of this enzyme. Although some inhibitors were discovered from the screen (Scheme 4), there were generally of relatively low affinity (IC_{50}>10 μM) [155]. Trematodes and cestodes have

cofactor-dependent (i.e. host-like) phosphoglyceromutases and relatively few of these enzymes have been characterised; one exception is the enzyme from *C. sinensis* [156]. It is an interesting paradox that the host-like enzymes in *F. hepatica* can be successfully and selectively inhibited by Clorsulon (see above), yet there appears to be issues with the druggability of the unusual nematode enzymes. This suggests that even subtle differences between host and parasite enzymes can be exploited and that, perhaps, the search for novel molecules which inhibit trematode and cestode phosphoglycerate kinase and/or phosphoglycerate mutase should be intensified.

Like the majority of glycolytic enzymes, enolase (EC 4.2.1.11), is externalised in many parasitic helminths and may play a role in host-pathogen interactions, particularly those involving the blood clotting system. Consequently the majority of efforts have focussed on testing the enzyme's potential as a vaccine candidate [157-166]. Silencing of the enolase gene in *C. sinensis* by RNAi resulted in significantly decreased survival of the worms, suggesting that the protein has potential as a drug target [167]. A modest reduction in survival (approximately 20%) was seen in similar experiments in *A. suum* [168]. In *S. mansoni*, enolase is a binding partner of the antimalarial drug mefloquine (Lariam; Scheme 3) [169]. This drug has some potential for the treatment of schistosomiasis and it is possible that enolase is a pharmacologically important target [169-171]. Interestingly, the mode of action in the malarial parasite is not well defined. The drug is believed to interact with haem (or its precursors) or to inhibit one of the enzymes involved in the synthesis if haem, possibly haem polymerase [172-175].

The final reaction of glycolysis results in the second generation of ATP and is catalysed by pyruvate kinase (EC 2.7.1.40). Where phospho*enol*pyruvate is used to generate oxaloacetate (see above) this reaction is omitted and the helminth pyruvate kinases are relatively understudied. The *F. hepatica* *Moniezia expansa* and *Dicrocoelium dendriticum* enzymes display positive cooperativity towards phospho*enol*pyruvate and are inhibited by malate (Scheme 3) [176-178]. Malate inhibition does not

appear to have been detected in the mammalian enzyme. Since pyruvate kinase is unlikely to be active for the anaerobic parts of the parasite's life cycle its potential as a drug target may be limited.

Tricarboxylic acid cycle and oxidative phosphorylation: current state of knowledge

Glycolysis and the TCA are linked by a reaction catalysed by pyruvate dehydrogenase enzyme complex (EC 1.2.4.1, EC 2.3.1.12 and EC 1.8.1.4). Pyruvate is oxidatively decarboxylated generating carbon dioxide, acetyl coenzyme A and NADH. The complex from *A. suum* has been extensively characterised [179-185]. The pyruvate dehydrogenase complex (PDC) is inactivated by phosphorylation (catalysed by pyruvate dehydrogenase kinase, EC 2.7.11.2) in higher eukaryotes [186]. However, it appears that this inactivation is more subtle and graded in *A. suum*. Whereas a single phosphorylation can result in complete inactivation in the human PDC, a greater degree of phosphorylation is required for inactivation in *A. suum* [187, 188]. This enables the complex to retain some activity even under relatively anaerobic conditions. In general, cestode PDCs are less sensitive to inhibition by NADH than those from aerobic organisms, including their hosts [179, 189]. Similarly the activation of pyruvate dehydrogenase kinase by increased NADH levels is less in cestodes compared to mammals [190, 191].

Compared to the glycolytic enzymes, the TCA enzymes from helminth parasites have, in general, received less attention. A recent gene knockdown study has shown that down-regulation of the genes encoding citrate synthase (EC 2.3.3.1), aconitase (EC 4.2.1.3) and malate dehydrogenase block mitosis in *C. elegans* suggesting that selective inhibition of this pathway may be viable in the treatment of nematode infections [192]. An initial characterisation of citrate synthase from *F. hepatica* has been carried out, but this was hampered by difficulties in purifying the recombinant protein [193]. Mammalian α-ketoglutarate dehydrogenase (EC 1.2.4.2) is up-regulated by calcium ions, but the enzyme from *F. hepatica* is not [194, 195]. The precise molecular nature and

mechanisms of this difference has not been elucidated, nor has it been exploited in the discovery of selective inhibitors. Fumarase (EC 4.2.1.2) from *A. suum* has been recombinantly expressed and purified in good yield, but no inhibitors of this enzyme have been reported [196]. Similarly, recombinant mitochondrial malate dehydrogenase from *C. sinensis* has been produced and partially characterised[197, 198]. 4,4'-*Bis*dimethylaminodiphenylcarbinol (Michler's hydrol; Scheme 5) inhibits the mitochondrial enzyme from this species but not the cytoplasmic isoform [198]. The mechanism of this inhibition has not been determined. Filarin (vinorelbine; Scheme 5), a cancer chemotherapeutic synthesised from precursors derived from the rosy periwinkle *Catharanthus roseus,* inhibits malate dehydrogenase from the filarial worm, *Setaria digitata* [199].

Other metabolic pathways

Some other metabolic pathways have received attention over the years. Of particular interest is the "shunt" which links cytoplasmic phospho*enol*pyruvate to mitochondrial oxaloacetate. Phospho*enol*pyruvate-carboxykinase (PEPCK; EC 4.1.1.32) catalyses the first step of this shunt. The enzyme from the cestode *Raillietina echinobothrida* is activated by a variety of plant extracts [200]. PEPCK from another cestode, *Hymenolepis diminuta*, is inhibited by α-ketoglutarate (Scheme 6) [201]. The mammalian enzyme is also inhibited by this TCA intermediate [202].

Nafuredin (Scheme 6) inhibits mitochondrial complexes I and II in helminths. When assayed against complex I it competed with rhodoquinone. It demonstrated anthelminthic activity against *H. contortus* in trials with experimentally infected sheep and against mice infected with the cestode *Hymenolepis nana* [203]. A related γ-lactone compound nafuredin-γ (Scheme 6) also has activity against complex I [204]. *E. multilocularis* complex I is inhibited by quinazoline (Scheme 6) and this compound kills the parasite [205]. This compound, and its derivatives, also inhibit mammalian

mitochondrial complex I [206]. So, the challenge would be to identify derivatives which selectively inhibit the complex from helminths.

The Leloir pathway converts galactose into the glycolytic intermediate glucose 6-phosphate. This pathway is essential in unicellular parasites from the genus *Trypanosoma* since they are unable to take up galactose from the environment and rely on "reversing" some of the Leloir pathway reactions in order to generate galactose for glycoprotein synthesis [207-211]. Thus, inhibition of GALE in these species has potential for the development of novel therapeutics [212-215]. Six inhibitors of *F. hepatica* UDP-galactose 4-epimerase (EC 5.1.3.2) have been identified; two showed good selectivity over the human enzyme [216]. These compounds were 5-fluoroorotate (5-FOA) and a peptide derivative, N-[(benzyloxy)carbonyl]leucyltryptophan (Scheme 6). Interestingly, orotate (which differs from 5-FOA only by the absence of the fluorine atom) demonstrated essentially no selectivity between the human and parasite enzymes. This loss of selectivity arises from two factors: a 20-fold drop in the affinity for the fluke enzyme and 50-fold increase in the affinity for the human one [216]. The rationale for targeting GALE was based on the devastating symptoms of severe forms of GALE deficiency (type III galactosemia) in humans [217]. However, it remains to be formally demonstrated that targeting the enzyme in helminth parasites will result in sufficient levels of impairment. Unlike in trypanosomes, there is no evidence that the enzyme is essential for the production of galactose. However, the tegument of many helminths contains complex glycoproteins and it is likely that the disruption of the Leloir pathway would lead to alterations in this structure potentially altering parasite-host interactions [218-226]. Like many of the glycolytic enzymes, GALE may also have potential as a vaccine against helminth infections [227].

Carnitine palmitoyl transferase 1 (CPT1; EC 2.3.1.21) is a key enzyme in the transfer of fatty acids into the mitochondria prior to their β-oxidation. Inhibition of CPT1 in *S. mansoni* by etomoxir (Scheme 6) resulted in complete inhibition of egg production [228]. Etomoxir inhibits CPT1 in a variety of species, including humans [229]. The etomoxir treated worms themselves remained

viable, although with decreased oxygen consumption resulting from lower rates of oxidative phosphorylation [228]. Thus, it can be concluded that oxidative phosphorylation of NADH resulting from β-oxidation of fatty acids is essential for egg production in this species. Blocking this process may have value in breaking infection cycles and reducing the pathological effects associated with egg production. However, it seems unlikely that it would kill adult worms.

Arginase (EC 3.5.3.1) from *S. mansoni* is a rare example of a helminth metabolic enzyme for which the crystal structure in the presence of an inhibitor is known [230]. The enzyme catalyses the hydrolysis of arginine to produce urea and ornithine. In helminths, this reaction is thought to be important in proline biosynthesis [231]. It has also been hypothesised that it reduces the amount of arginine in the parasite. Doing so may assist in immune evasion since arginine is a key precursor of nitric oxide (NO) which is produced in macrophages and stimulates the cytotoxic activities of these cells [232, 233]. A druggable region of the protein has been identified and this site can accommodate a range of amino acid-like molecules [230]. Of particular interest is a cleft in this region which can accommodate substituents on the α-carbon of amino acids. This enables the protein to bind to α,α-disubstituted amino acids, such as (R)-2-amino-6-borono-2-[2-(piperidin-1-yl)ethyl]hexanoic acid (Scheme 6). The cleft in the *S. mansoni* enzyme is more non-polar than the equivalent cleft in the human enzyme and it has been suggested that species specific reagents could be designed by exploiting this fact [230].

Unmet research needs[U1]

Fundamental research on parasitic helminths is limited in compared to fields such as cancer, heart disease or HIV research. This results in a relatively small knowledge base to exploit in the discovery of novel therapeutics. Despite differences in metabolic pathways being elucidated in the 1970s and 1980s, few attempts have been made to exploit these in drug discovery. The number of crystal

structures of helminth proteins is small. Although there has been some successful inhibitor discovery based on molecular models (e.g. [216]), experimental structures are generally considered superior to models. Homology models, by their nature, are based on known structures. Thus, while they predict the overall fold very well, they are poor at estimating the structures of regions of the protein which differ markedly from the templates. Yet it is these very differences that may provide the key to the design of reagents which specifically inhibit helminth enzymes. Therefore, a[U2] critical research need is a larger number of experimental structures of helminth metabolic enzymes.

While it may be self-evident to biochemists that disrupting central carbon metabolism pathways (especially, perhaps, glycolysis) is highly likely to result in death of the organism, drug discovery scientists may require more convincing. Therefore, experiments demonstrating the vital nature of key metabolic enzymes are required. Although RNAi is often considered to be the "gold standard" in such experiments, a note of caution should be sounded. RNAi reduces the amount of mRNA transcript for a specific gene: it has not direct effect on the levels of protein. Protein concentrations will only decrease as the molecules are degraded and not replaced (since there is no mRNA). Metabolic enzymes are typically long-lived and may, therefore, persist for days (or longer) in the cell [234]. Therefore, lack of a phenotype after only a few days' exposure to RNAi is not conclusive evidence that the protein is not vital. In such experiments it will be necessary to verify that protein levels are depleted (through activity assays or western blotting). Long exposures to the RNAi may also be necessary and, in some cases, this may not be practical. If so, alternative approaches may be necessary such as enzyme inhibition *in vivo*. This may require us to rediscover the "old" literature: the discovery of many metabolic pathways relied, in part, on the use of specific enzyme inhibitors (for a famous example, see [235]).

Since single point inhibition of pathways can result in compensatory mechanisms within the cell, it may be better to consider pathways as a whole. Thus the development of systems biology approaches to helminth metabolism is desirable. Much of the data to build the models already

exists. Many helminth metabolic enzymes were purified from native sources in the 1970s and 1980s and their kinetic parameters determined. This is complemented by more recent data derived from studies on recombinant proteins.

The anaerobic metabolism of many helminths relies on the partial reversal of the TCA and the use of rhodoquinone in a modified electron transport pathway (Figure 1; Scheme 2). A greater understanding of this process, in particular the synthesis of rhodoquinone would be useful. Targeting the synthesis of rhodoquinone (which does not occur in the host) would, in theory, prevent this anaerobic electron transport without damaging the host.

Ultimately, if existing knowledge and new work is to lead towards new drugs to treat helminth infections, selective inhibitors need to be discovered. This is likely to come from a combination of high throughput screening and computational chemistry approaches. However, as in other therapeutic areas, it is likely that the majority of inhibitors discovered will be useless as drugs. Either the compounds will fail to penetrate cells (and thus never have the opportunity to bind to the target) or they will prove equally toxic to the host and parasite. In the majority of helminths, the only way to test the efficacy of compounds is on live worms in culture or on artificially infected animals. Ideally, cell culture systems would be available to enable intermediate stage testing and a relatively low cost method to eliminate some compounds before proceeding to more complex test systems. Thus, the development of parasitic helminth cell culture systems is another significant, unmet research need.

Conclusions

Inhibition of metabolic pathways in parasitic helminths would, almost certainly, result in reduced growth or death of the pathogen. The major challenges are a relative lack of knowledge about the biochemistry of the enzymes involved and the (perceived) difficulty of discovering reagents which

are selective for parasite enzymes over the host ones. In this respect, the case of phosphoglyceromutase (described above) represents a cautionary tale. The nematode enzyme has many features of an "ideal" drug target. It is likely to be essential to the organism, it is readily assayable *in vitro* and, critically, it differs in structure, sequence and mechanism from the host enzyme. In theory, compounds which block this enzyme's activity should kill the helminth without affecting the host[U3]. Yet, a recent study considered the enzyme to be "undruggable" [155]. In contrast, the trematode phosphoglyceromutase is similar in sequence (and, therefore, presumably structure and mechanism) to the mammalian enzyme. This enzyme is a target of the anthelminthic drug Clorsulon[U4] [51]. If this tale teaches us anything, it is that sometimes dogma should be avoided and that there may be merit in attempting to find the next Clorsulon, i.e. a selective inhibitor of a parasitic helminth metabolic enzyme.

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Figure and scheme legends

Figure 1: **Schematic diagram of the metabolism of helminths under anaerobic conditions.** CoA, coenzyme A; AcCoA, acetyl coenzyme A; MeMalCoA, methylmalonyl coenzyme A; PropCoA, propionyl coenzyme A; RQ, rhodoquinone; RQH, reduced rhodoquinone. The grey shaded box represents mitochondrial membrane complex I, the dashed line transport of malate across the mitochondrial membranes and the large, open arrow the tranlocation of protons between the matrix and the intermembrane space of the mitochondria. The outward translocation by complex I generates a proton motive force which can be utilised by ATP synthase (grey circle; EC 3.6.3.14) to generate ATP.

Scheme 1: Structures of drugs known to target helminth metabolic enzymes

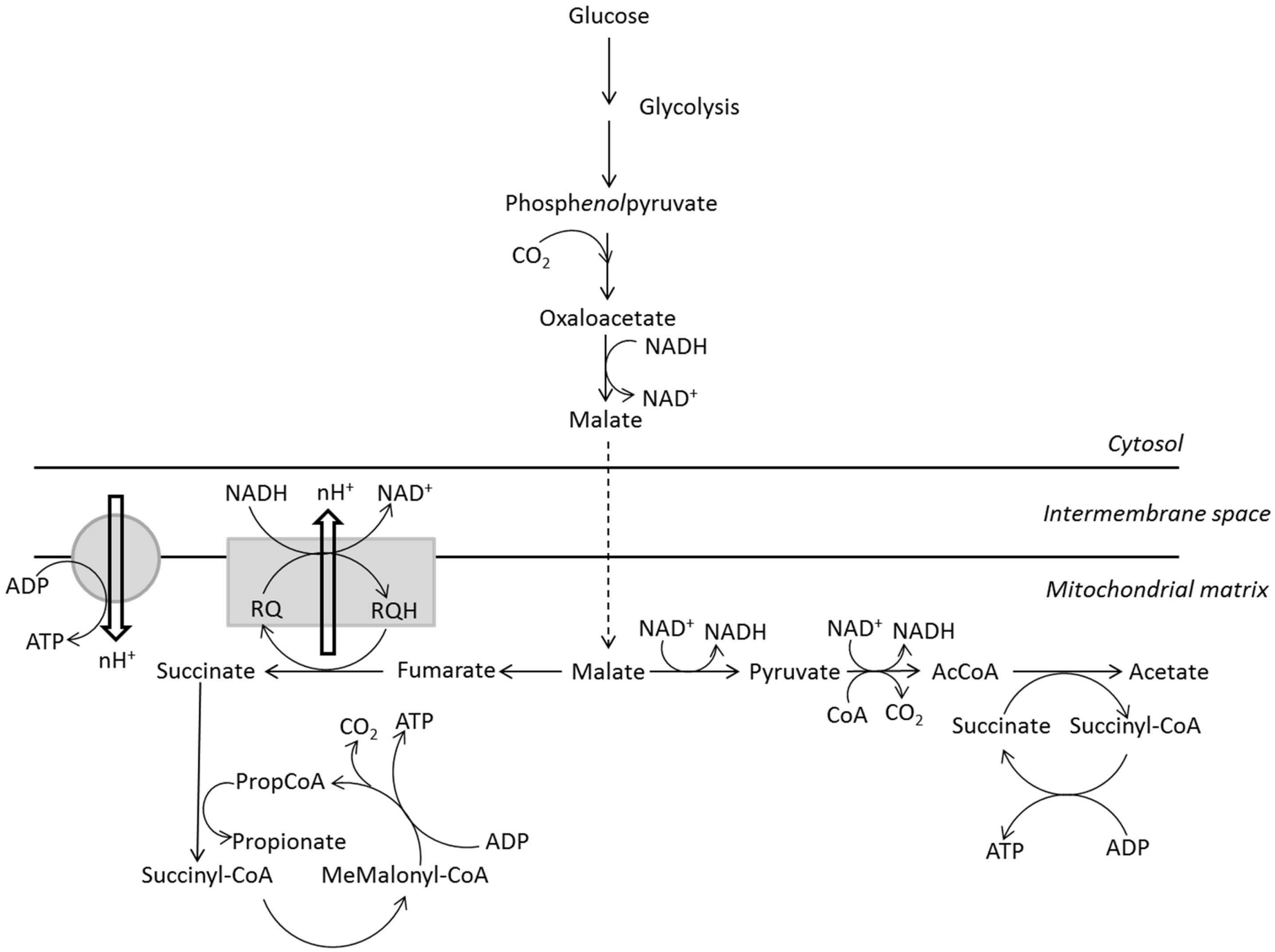
Scheme 2: Structure of rhodoquinone

Scheme 3: Structures of inhibitors of helminth glycolytic enzymes

Scheme 4: Structures of experimentally and computationally identified inhibitors of nematode phosphoglycerate mutase

Scheme 5: Structures of inhibitors of helminth TCA and oxidative phosphorylation enzymes

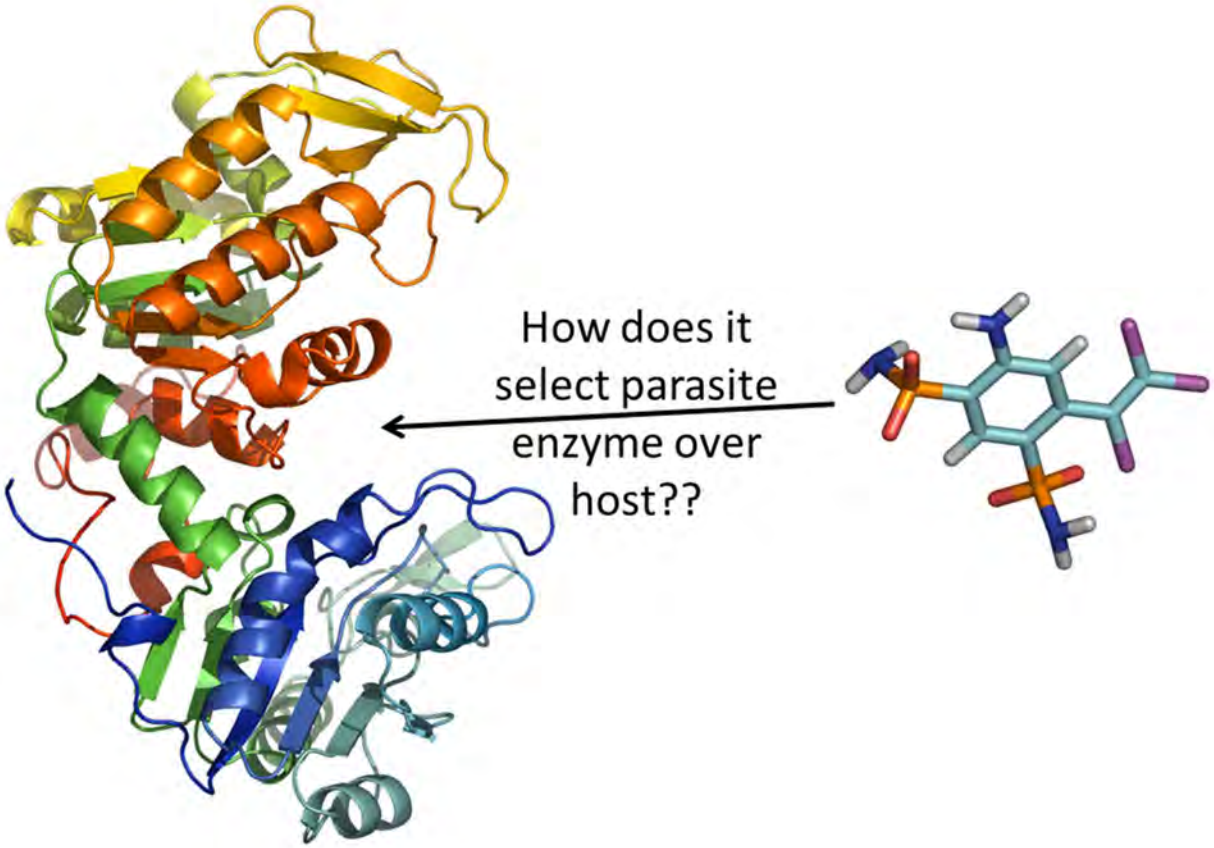
Scheme 6: Structures of inhibitors of helminth β-oxidation, Leloir pathway and amino acid metabolism pathways

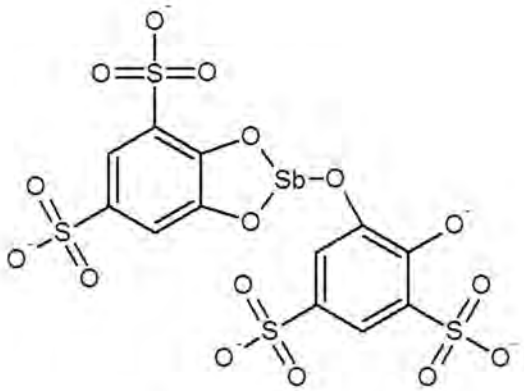

Glucose
Glycolysis
Phosphenolpyruvate
CO2
Oxaloacetate
NADH
NAD+
Malate
Cytosol
Intermembrane space
Mitochondrial matrix
NADH
nH+
NAD+
ADP
ATP
nH+
RQ
RQH
Succinate
Fumarate
Malate
NAD+
NADH
Pyruvate
NAD+
NADH
AcCoA
Acetate
CoA
CO2
Succinate
Succinyl-CoA
ATP
ADP
CO2
ATP
PropCoA
Propionate
ADP
Succinyl-CoA
MeMalonyl-CoA

Metabolic enzymes of helminth parasites: potential as drug targets

David J. Timson

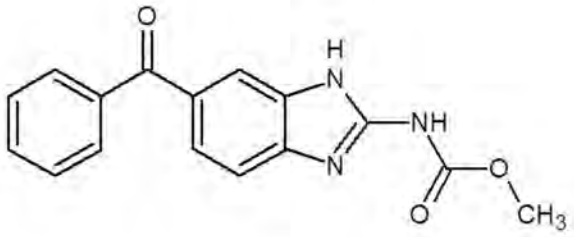
ABSTRACT: Clorsulon is a widely used anthelminthic which targets two glycolytic enzymes. The challenge is to find more molecules which target metabolism.



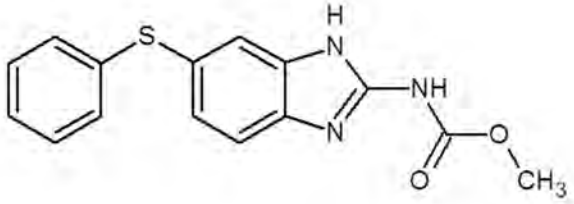


Stibophen
(A trivalent antimonial)

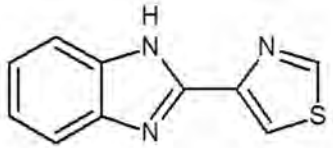
Clorsulon



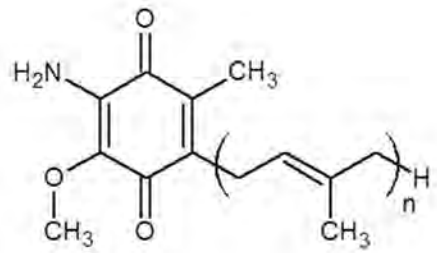
Mebendazole



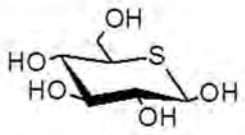
Fenbendazole



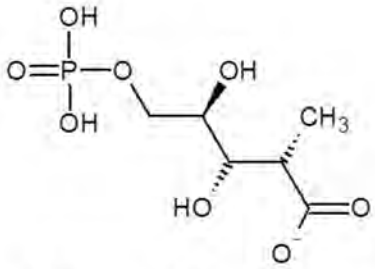
Thiabendazole



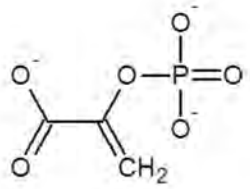
Rhodoquinone
E°'=67 mV



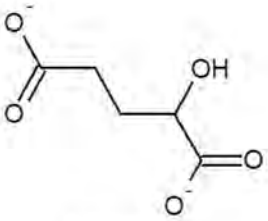
5-thioglucose



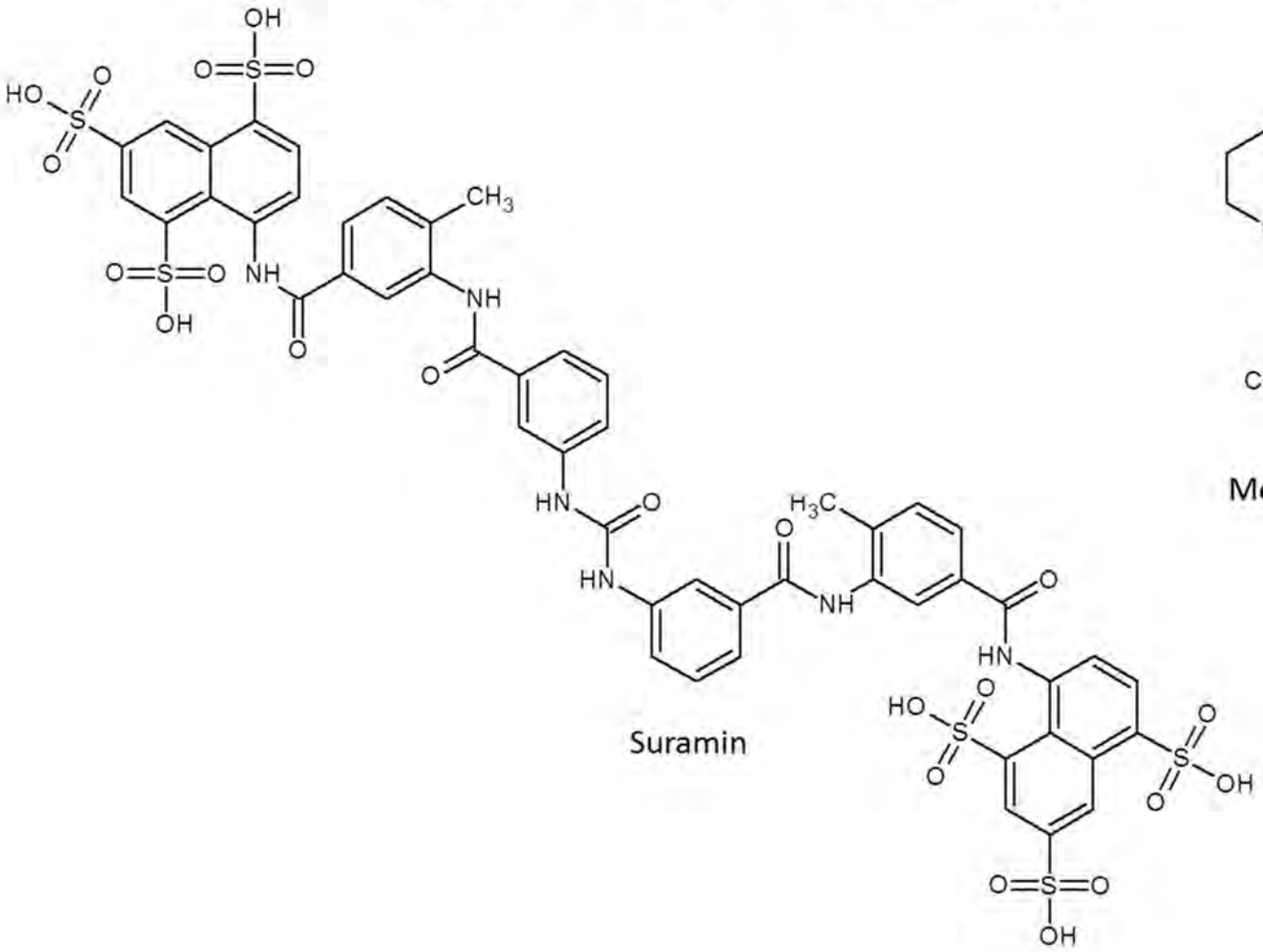
5-phospho-D-arabinoate



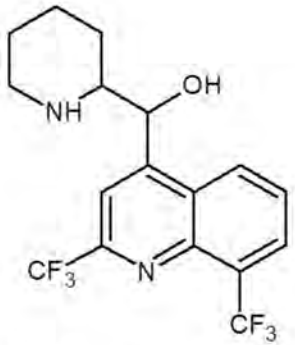
Phospho*enol*pyruvate



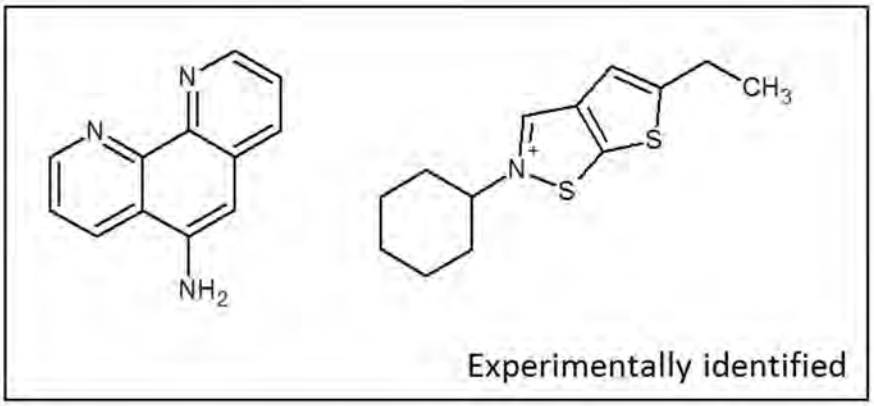
Malate

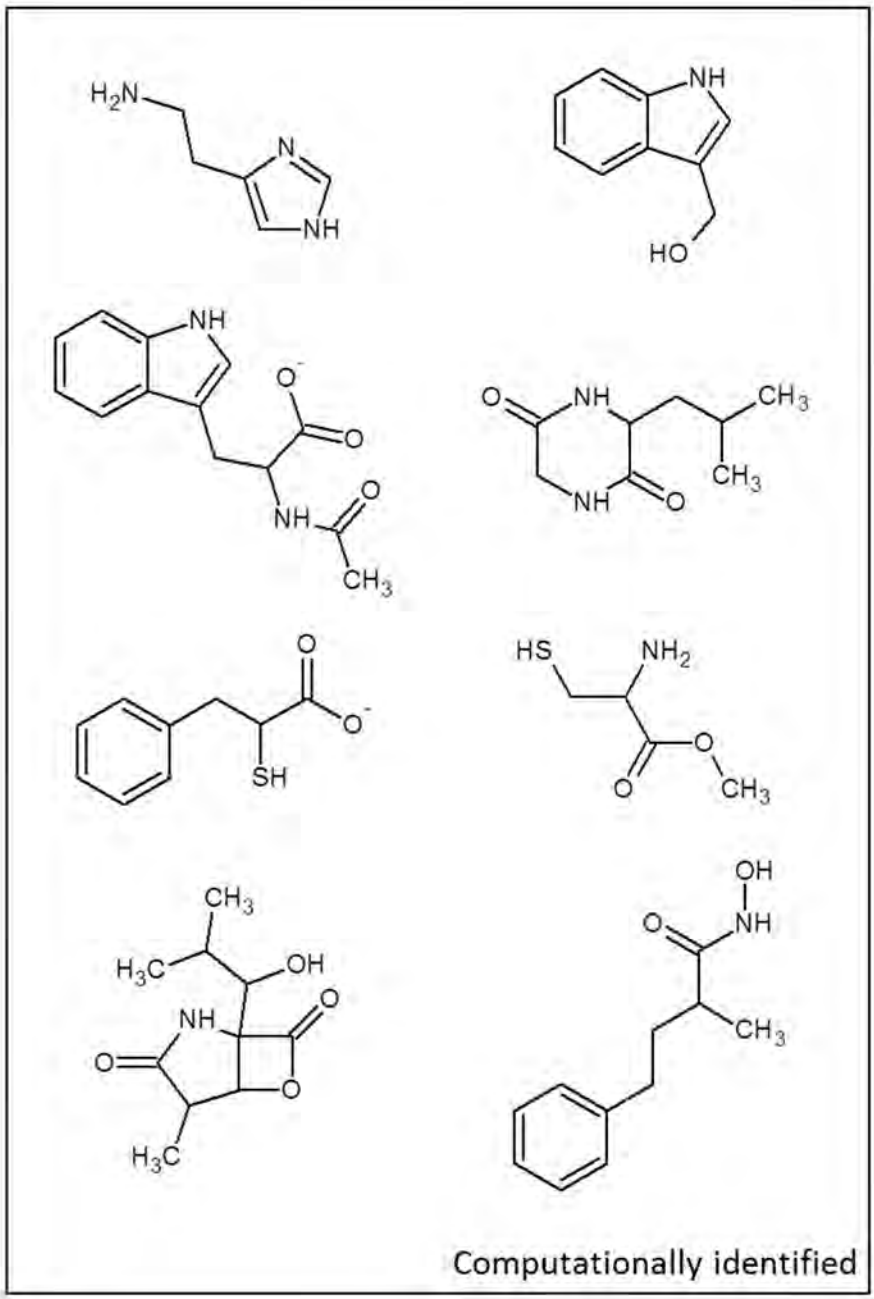


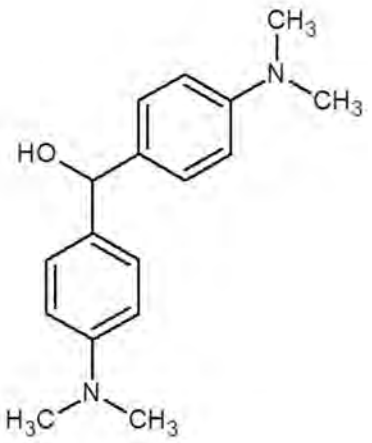
Suramin



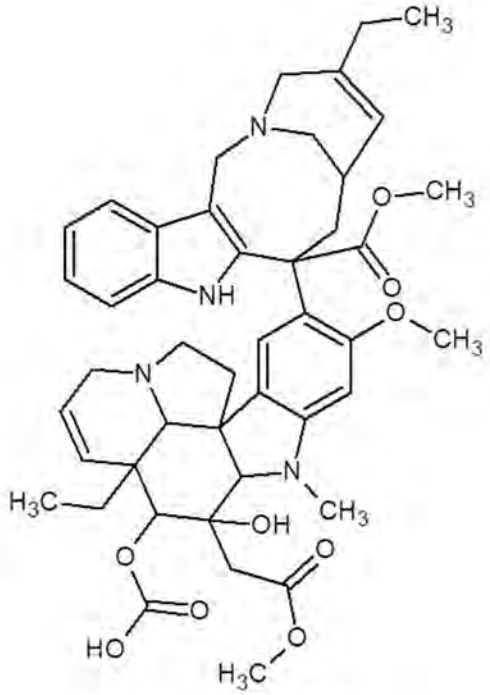
Mefloquine (Larium)


N
N
NH2
CH3
S
N+
S
Experimentally identified

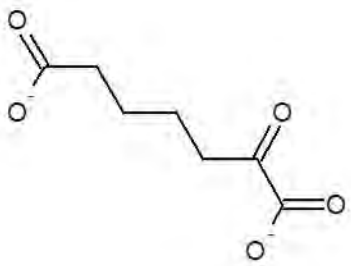

H2N
N
NH
NH
HO
NH
O-
O
NH
O
CH3
O
NH
CH3
NH
O
CH3
O
O-
SH
HS
NH2
O
O
CH3
CH3
H3C
OH
NH
O
O
O
H3C
OH
NH
O
CH3
Computationally identified



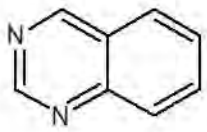
4,4'-*bis*dimethylaminodiphenylcarbinol
(Michler's hydrol)



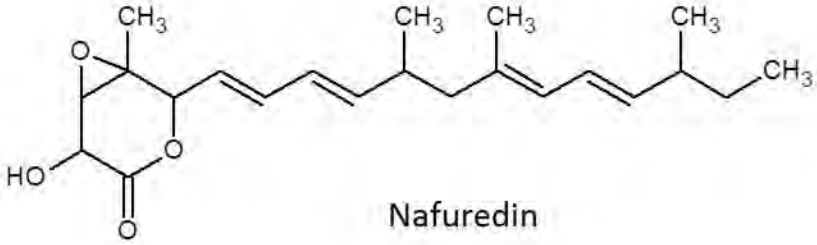
Filarin (vinorelbine)



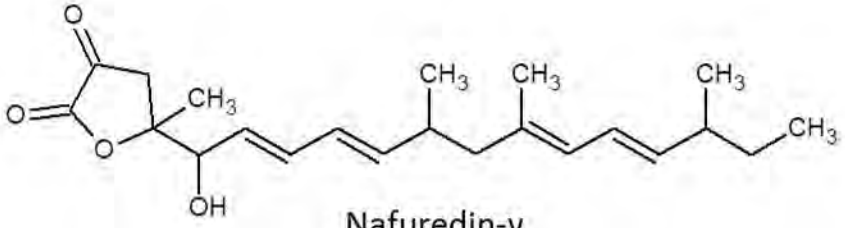
α-Ketoglutarate



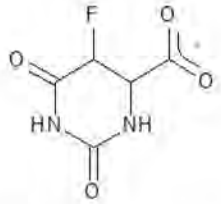
Quinazoline



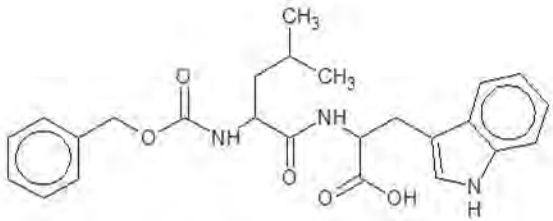
Nafuredin



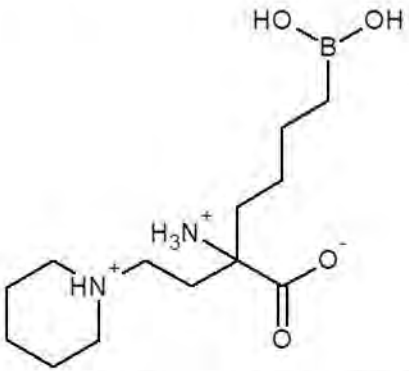
Nafuredin-γ



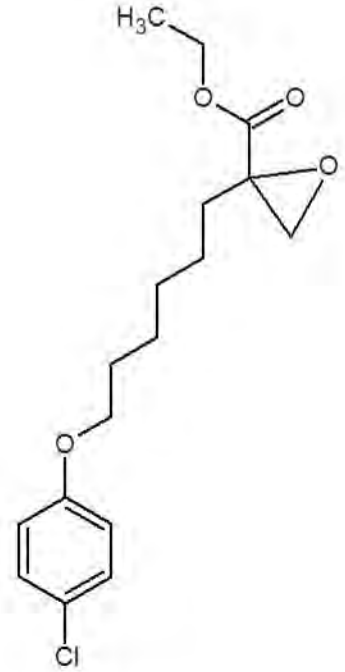
5-Fluoroorotate



N-[(benzyloxy)carbonyl]leucyltryptophan



(R)-2-amino-6-borono-2-[2-(piperidin-1-yl)ethyl]hexanoic acid



Etomoxir