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Perspective

Parasite Enzymes as Potential Targets for Antiparasitic Chemotherapy

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Introduction

The word "parasite", derived from ancient Greek, means one frequenting the tables of the rich and earning welcome by flattery. It should include all the known infectious agents, such as viruses, bacteria, fungi, protozoa, and helminths, even though the infected host may not be flattered and the infectious agents may not be exactly welcome. During the early 20th century, however, the study of protozoa and helminths emerged as a new discipline termed "parasitology" based on some obscure scientific arguments. This was quite unfortunate, since infectious diseases caused by these newly defined parasites in recent decades have been largely a problem in the Third World. The parasites are sitting at the tables of the poor and causing 3 billion people malnutrition, blindness, debility, disfiguration, and death. Although it is the foremost human health problem in the world today, it has also been the most neglected human health problem for various economical and political reasons. There has not been enough emphasis on the research efforts and, consequently, not enough new agents discovered in the last 30 years to cope with the spreading of parasitic infections.

Ironically, the parasitic infections should be, in theory, relatively easy to deal with because the cause of the disease is already well identified in every case. The advancement in cell culturing techniques has enabled us to witness the successful in vitro cultivation of essentially all the important parasites in recent time.

Among the helminths, *Schistosoma mansoni*[1] and the filaria *Dipetalonema viteae*[2] can be developed to the adult stages in vitro. *Trypanosoma brucei*, the African trypanosome,[3] *Trypanosoma cruzi*, the American trypanosome,[4] *Leishmania donovani*,[5] *Trichomonas vaginalis*,[6] *Giardia lamblia*,[7] and *Entamoeba histolytica*,[8] to name but a few protozoan parasites, can all grow in vitro in axenic media without the presence of any host tissues or cells. Even the intracellular parasites, such as the human malarial parasite *Plasmodium falciparum* in its erythrocytic phase,[9] *Toxoplasma gondii*,[10] *T. cruzi* amastigotes,[11] and *Leishmania mexicana* amastigotes,[12] are all routinely cultivated in vitro in cell cultures and can be harvested in pure and viable forms free of contaminating host cellular materials. The capabilities have not only ended the traditional belief in a certain mysterious dependence of parasite on host but also enabled us to study the parasites in the same ways as we used to investigate the bacteria. This technical breakthrough has been singularly responsible in bringing modern biochemistry, molecular biology, and immunology into research on parasites in recent years.

For the interest in a rational approach to antiparasitic chemotherapy, comparative biochemical investigations on the host and parasite are important in pointing out potentially different metabolic activities for selective inhibition. One would ordinarily expect that the parasite would have many deficiencies in its metabolism that underline its parasitic nature. This is certainly true in many instances; the oversimplified metabolic pathways are usually indispensable for survival of the parasite and, thus, represent points of its vulnerability. However, this aspect is not the only opportunity for attack. The parasite may be living in a luxurious environment and becoming lazy, but the environment is not exactly friendly. To defend itself against immunological attacks, proteolytical digestion, etc. from the host, the parasite has to have its defense mechanism. These extra capabilities are obviously essential for survival of the parasite. In some instances, necessary nutrients are not supplied to the parasite from the host, although the latter can obtain the same nutrients from the diet. We are thus occasionally facing the peculiar situation where the parasite has acquired the extra synthetic activity needed for its survival. Finally, the mere evolutionary distance between the host and parasite provides ample differences among individual enzymes or functional pathways to allow specific inhibition of the parasite. I shall try to cite a few specific examples in each of the three categories of antiparasitic opportunites and try to have an overall assessment on the probable future fruitful ap-

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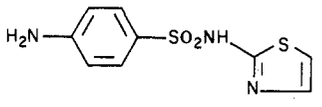
proaches to antiparasitic chemotherapy.

Unique Enzymes Found Only in the Parasites

These enzymes are, for the researchers, the cleanest targets for chemotherapy. Like those in the synthesis of the bacterial cell wall, inhibition of these enzymes should have no side effect on the host. In reality, however, these types of enzymes are rare among the parasitic protozoa and helminths. Among the few discovered, their usefulness as chemotherapeutic targets is sometimes limited because of the development of drug-resistance problems.

Enzymes for Dihydropteroate Synthesis. The intracellular sporozoan parasites, such as the *Plasmodia*,[13] *Toxoplasma*,[14] and *Eimeria*[15] species, have long been known to respond to sulfonamides and sulfones. This drug sensitivity has led to the assumption that the sporozoa species has to synthesize its own folate to survive. But it was not until 1971 that Walter and Königk[16] demonstrated the reaction of 2-amino-4-hydroxy-6-(hydroxymethyl)dihydropteridine diphosphate with *p*-aminobenzoate to form 7,8-dihydropteroate in cell-free extracts of the rodent malarial parasite *Plasmodium chabaudi*. 2-Amino-4-hydroxy-6-(hydroxymethyl)dihydropteridine pyrophosphokinase and 7,8-dihydropteroate synthase have since been identified, isolated, and purified some 1000-fold from *P. chabaudi*[17] and another rodent parasite, *Plasmodium berghei*.[18] The two enzymic activities appear to be associated with the same enzyme complex with a molecular weight of about 200 000. *p*-Aminobenzoate had an estimated K_m value of 1.5×10^{-6} M, whereas sulfathiazole,



sulfathiazole

sulfaguanidine, and sulfanilamide acted as competitive inhibitors with estimated K_i values of 1.4×10^{-5}, 6.9×10^{-5}, and 13.0×10^{-5} M, respectively.[17]

Similar 7,8-dihydropteroate synthase activities have since been demonstrated in other malarial parasites, e.g., *Plasmodium knowlesi*, *Plasmodium gallinaceum*, and *Plasmodium lophurae*.[19] There is no knowledge yet, however, on the origin of 2-amino-4-hydroxy-6-(hydroxymethyl)dihydropteridine in plasmodia. There has been also no success in demonstrating dihydrofolate synthetase activity in the parasites, which raises the possibility that 7,8-dihydropteroate may have substituted for dihydrofolate in malarial parasites. This possibility is interesting, since the "dihydrofolate reductase" of *P. berghei* is known to differ from the mammalian dihydrofolate reductase in many aspects; e.g., the parasite enzyme is in a bifunctional protein containing also the thymidylate synthetase activity.[20] The parasite reductase does not recognize folate as substrate,[21] which agrees with a previous observation[22] that crude extract of *P. berghei* was incapable of converting folate to folinate but could do so with dihydrofolate. The latter observation may explain why the parasite needs to synthesize its own 7,8-dihydropteroate, because it cannot utilize the exgonenous folate from the host.[22] Similar lack of recognition of folate as a substrate was also observed in the dihydrofolate reductase of *Eimeria tenella*,[23] which, together with thymidylate synthetase, also turned out to be in a bifunctional protein.[24]

The lack of utilization of exogenous folate may not, however, fully explain the indispensable nature of the apparent synthesis of 7,8-dihydropteroate in *Plasmodia*, *Toxoplasma*, and *Eimeria*, all of whom reside inside host cells. It is known that most of the folate molecules are linked with polyglutamates in the cytoplasms of mammalian cells and are very difficult to transport across cell membranes.[25] This difficulty may have compounded the problem of supplying 7,8-dihydropteroate or dihydrofolate to the parasites and made all the enzymes involved in their synthesis very attractive targets for antisporozoan parasite chemotherapy.

The sulfones and sulfonamides synergize with the inhibitors of dihydrofolate reductase, and the combinations have been very effective in controlling malaria, toxoplasmosis, and coccidiosis.[13-15] Although some incidents of drug resistance, especially among coccidia,[26] have been reported, the combinations remain largely effective against malaria and toxoplasmosis. Fansidar, a combination of sulfadixine and pyrimethamine, has been successful in controlling chloroquine-resistant *Plasmodium falciparum* malaria.[27]

Pharmacological properties of parasite 7,8-dihydropteroate synthetases may differ from those of the bacterial enzymes. For instance, metachloridine[28] and 2-ethoxy-*p*-aminobenzoate[29] are both ineffective against sulfa-sensitive bacteria. But the former has antimalarial activity, and the latter is effective against *Eimeria acervulina* infection; both activities can be reversed by *p*-aminobenzoate. The enzymes involved in the synthesis of dihydropteroyl derivatives in the sporozoan parasites thus remain much to be explored for possible new approaches of antiparasitic chemotherapy.

Pyruvate:Ferredoxin Oxidoreductase. Certain anaerobic protozoan parasites lack mitochondria and mitochondrial activities. They possess, instead, ferredoxin-like or flavodoxin-like low redox potential electron-transport proteins to convert pyruvate to acetyl-CoA.[30] In trichomonad flagellates[31] and rumen ciliates,[32]

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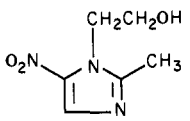
the process takes place in a membrane-bounded organelle, the hydrogenosome.[33] By the actions of pyruvate:ferredoxin oxidoreductase and hydrogenase in the hydrogenosome, H_2 is produced by these organisms under anaerobic conditions as the major means of electron disposition. *Entamoeba* species and *Giardia lamblia* have no hydrogenosome, but a ferredoxin was isolated from *E. histolytica*,[34] and the presence of iron–sulfur and flavin centers was indicated by electron paramagnetic resonance studies on *G. lamblia*.[35] These proteins and centers can be specifically reduced by pyruvate, suggesting their physiological function as pyruvate:ferredoxin oxidoreductase.

This enzyme has no counterpart in the mammalian system. In contrast to the mammalian pyruvate dehydrogenase complex, the pyruvate:ferredoxin oxidoreductase is incapable of reducing pyridine nucleotides because of its low redox potential (ca. −400 mV). On the other hand, however, this low potential can transfer electrons from pyruvate to the nitro groups of metronidazole

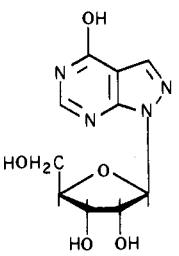


metronidazole

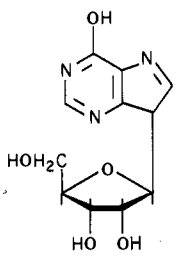
and other 5-nitroimidazole derivatives to form cytotoxic reduced products, which bind to DNA and proteins.[36] This is apparently why these anaerobic protozoan species are highly susceptible to the compounds. Despite the recent development of drug-resistance problems in *Trichomonas vaginalis*[37] and the suspected carcinogenic property,[38] metronidazole still remains the drug of choice for treating anaerobic protozoan parasite infections today.

The hydrogenosomal ferredoxin of *Tritrichomonas foetus* has been purified and characterized recently.[39] It is an iron–two sulfur protein with an estimated molecular weight of 12 000. It functions as an electron-transport component in the reduction of metronidazole by pyruvate:ferredoxin oxidoreductase. Further understanding of this electron-transport system may lead to future design of specific inhibitors as another possible way of controlling the parasites, or an electron-trapping agent may be developed, which in the reduced form could be cytotoxic without binding nonspecifically to DNA.

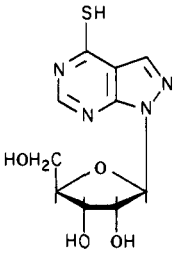
Nucleoside Phosphotransferases. All the protozoan parasites studied thus far have turned out to be deficient in de novo synthesis of purine nucleotides.[40] The functioning of various purine salvage pathways in the parasites has thus become essential for their survival and growth. Among the *Leishmania* spp., a unique salvage enzyme, purine nucleoside phosphotransferase, was identified[41] that could transfer the phosphate group from a variety of monphosphate esters, including *p*-nitrophenyl phosphate, to the 5′-position of purine nucleosides. This particular enzyme also phosphorylates purine nucleoside analogues, such as allopurinol riboside,[41] formycin B,[42] thiopurinol



allopurinol riboside



formycin B



thiopurinol riboside

riboside,[43] etc., to convert them to the corresponding nucleotides. These nucleotides are either further converted to triphosphates and eventually incorporated into nucleic acid of *Leishmania*[41,44] or become inhibitors of other essential enzymes in purine metabolism.[43] Consequently, allopurinol riboside, formycin B, and thiopurinol riboside all turned into potent antileishmanial agents in both in vitro and in vivo tests.[42–45] These compounds are particularly interesting, since they are remarkably nontoxic to the mammalian host due to its lack of the same enzyme. Further investigation of the purine nucleoside phosphotransferase in *Leishmania* is apparently an effective way of finding new chemotherapeutic treatments of leishmaniasis.

Among the trichomonad flagellates, there has been ample evidence suggesting deficiency in de novo synthesis of both purines and pyrimidines.[46,47] Pyrimidine salvage thus becomes indispensable for these parasites. Among them, *Tritrichomonas foetus*, *Trichomonas vaginalis*, and *Giardia lamblia* were found to be also lacking dihydrofolate reductase and thymidylate synthetase, which enables them to grow normally in the presence of 0.5 mM methotrexate, the most potent antifolate.[46,47] This metabolic deficiency leads to the apparent isolation of the supplying route of TMP from the rest of the pyrimidine ribonucleotides; TMP is provided by a single salvage pathway of converting exogenous thymidine to TMP via the action of a phosphotransferase.[46,47] This enzyme activity has been isolated from *T. foetus* and *T. vaginalis*. It resides in the particulate fraction pelletable by 10^5g centrifugations and is soluble in 1% Triton X-100. It has demonstrated high substrate specificity but low stringency on the phosphate donors; *p*-nitrophenyl phosphate, as well as many nucleoside monophosphates, act equally well. The enzyme activity is not affected by thymidine kinase inhibitors, such as acyclovir, but is inhibitable by guanosine or 5-fluorodeoxyuridine; both compounds also inhibit the in vitro growth of the parasites. This enzyme is a very attractive target for chemotherapeutic treatment of the anaerobic protozoan parasites. One could design either a false substrate or an inhibitor of the phosphotransferase; DNA synthesis in the parasites may be arrested in either case.

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Indispensable Enzymes Found in the Parasites

Due to the many metabolic deficiencies among the parasites, there are at times parasite enzymes whose functions may be essential for the survival of parasites, but the same enzymes in the host are not necessarily indispensable. This discrepancy opens up opportunities for antiparasitic chemotherapy, albeit it does not always represent a significant difference of properties between the parasite and the host enzymes.

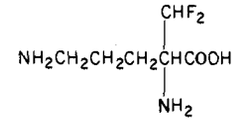
Purine Phosphoribosyltransferases. The absence of de novo purine nucleotide synthesis in protozoan parasites, as well as in *Schistosoma mansoni*,[48] has been reflected by the relative importance of hypoxanthine–guanine phosphoribosyltransferase in many of them. That hypoxanthine is the obligatory base for purine nucleotide synthesis has been demonstrated in *Crithidia fasciculata*,[49] four species of *Leishmania*,[49] *Plasmodium berghei*,[50] *Eimeria tenella*,[51] *Schistosoma mansoni*,[52] and *Tritrichomonas foetus*.[53] Although specific design of potent inhibitors of hypoxanthine–guanine phosphoribosyltransferase for antiparasitic actions has not been met with great success in the past,[54] some unusual substrate specificity of the parasite hypoxanthine–guanine phosphoribosyltransferase has been noted recently. The enzymes in *Leishmania donovani*[55] and *Trypanosoma cruzi*[56] can recognize allopurinol as the substrate and convert it to allopurinol ribotide, which then accumulates in the parasite and becomes aminated, turned into triphosphate, and finally incorporated into RNA as has been described previously for the incorporation of allopurinol riboside into *Leishmania* nucleic acids. Since allopurinol is an extremely poor substrate for the mammalian hypoxanthine–guanine phosphoribosyltransferase, it has demonstrated not only significant antileishmanial[55] and antitrypanosomal[56] activities in vitro but also good antiparasitic therapeutic effects in vivo.[57] It has no apparent toxicity in man; it does not inhibit human hypoxanthine–guanine phosphoribosyltransferase and, thus, does not cause Lesch–Nyhan type syndrome known to be associated with the enzyme deficiency. It will, however, almost certainly suppress the gout syndrome in the treated patient because it is known to inhibit xanthine oxidase.

The parasite hypoxanthine–guanine phosphoribosyltransferase has thus satisfied two criteria to be qualified as a good target in the parasites for chemotherapy: (1) its function is essential for the survival of the parasite, and (2) its substrate specificity differs from that of the host. Recent studies have mounted more convincing indications that for this particular enzyme isolated from various parasites, each one has its own substrate specificity. *Eimeria tenella* consists of an enzyme using hypoxanthine and guanine, as well as xanthine, as substrates.[51] It has been purified to homogeneity from the unsporulated oocysts of the parasite by GMP–agarose affinity column chromatography and found to have a relatively low subunit molecular weight of 23 000.[51] Allopurinol is also a competitive inhibitor of this enzyme with an estimated K_i/K_m ratio of 100 vs. hypoxanthine. This modest enzyme inhibition was reflected in the allopurinol inhibition of *E. tenella* development in cell cultures, an effect reversible by adding more hypoxanthine to the growth medium.[51] In *Leishmania donovani* promastigote, three separate enzymes, hypoxanthine–guanine, xanthine, and adenine phosphoribosyltransferase, have been identified and partially purified.[55] In addition to the finding that allopurinol was a more efficient substrate for the *Leishmania* hypoxanthine–guanine phosphoribosyltransferase, the xanthine phosphoribosyltransferase in *L. donovani* has found no counterpart in mammalian systems, and, thus, could be another interesting target for antileishmanial chemotherapy.

Recent studies have also indicated that *Giardia lamblia* has an exceedingly simple scheme of purine salvage. It possesses only two pivotal enzymes, the adenine and guanine phosphoribosyltransferases, which convert exogenous adenine and guanine to the corresponding nucleotides.[58] There is no salvage of hypoxanthine, xanthine, or any purine nucleosides, and there is no interconversion between adenine and guanine nucleotides in the parasite. Thus, the functions of the two phosphoribosyltransferases are both essential for the survival and development of *G. lamblia*. The guanine phosphoribosyltransferase is an interesting enzyme because it does not recognize hypoxanthine, xanthine, or adenine as substrate.[58] This substrate specificity distinguishes the *Giardia* enzyme from the mammalian enzyme, which uses hypoxanthine, and the bacterial one, which has xanthine as substrate. Design of a highly specific inhibitor of this enzyme is thus possible.

Ornithine Decarboxylase. Polyamines, found among essentially all living organisms, are required for cellular proliferation and differentiation.[59] Ornithine decarboxylase, the enzyme that controls the formation of putrescine in numerous eukaryotic cells, is a unique enzyme because of its striking inducibility and very short half-life.[60] Among the many species of trypanosomatids, putrescine and spermidine form the major pool of polyamines,[61] which are synthesized rapidly from ornithine but are taken up much more slowly from extracellular sources.[62] Intracellular levels of the polyamines fluctuate during the rapid growing cycle of the protozoa.

α-(Difluoromethyl)ornithine, a suicidal inhibitor of ornithine decarboxylase with known antitumor activities,[63] was recently found to possess good activities against Af-



α-(difluoromethyl)ornithine

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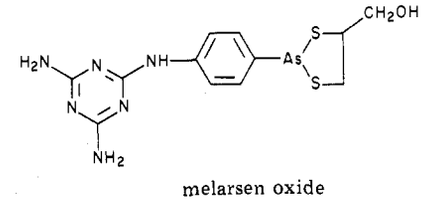
rican trypanosomes in infected animal models.[64-66] This in vivo antitrypanosomal activity can be specifically reversed by putrescine, spermidine, or spermine.[67] *Trypanosoma brucei* bloodstream form, recovered from infected rats following a 12-h drug treatment, indicated total absence of ornithine decarboxylase activity and more than 90% depletion of putrescine in the parasite.[68] In *T. brucei* cells treated with the drug for 36 h, DNA synthesis was totally stopped, while RNA synthesis decreased more than 50%.[68] It is thus most likely that the antitrypanosomal activity of α-(difluoromethyl)ornithine is attributed to the inhibition of ornithine decarboxylase. This same compound has been also found effective in controlling *Eimeria tenella* infection in chickens,[69] the in vitro schizogony of *Plasmodium falciparum*,[65] and the in vitro growth of *Giardia lamblia*.[70] All these activities have been demonstrated reversible by the polyamines.[65,69,70]

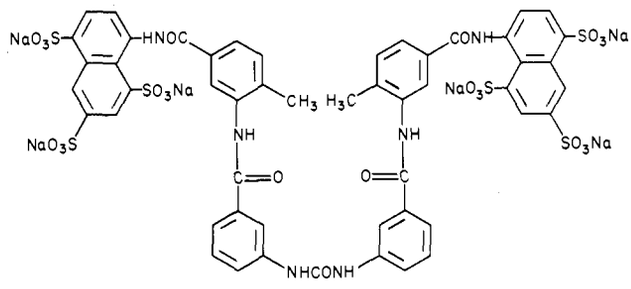
It is thus apparent that because of the fast rates of proliferation among the protozoan parasites, an effective inhibition of the parasite ornithine decarboxylase may control their growth without damaging the host metabolism. Further studies may uncover more enzymes involved in the polyamine metabolism of protozoan parasites as suitable targets of chemotherapy.

Glycolytic Enzymes. In the bloodstream form of African trypanosomes, such as *Trypanosoma brucei*, the single mitochondrion has been reduced to a peripheral canal containing no cytochrome and no functioning Kreb's cycle. It is entirely dependent on glycolysis for its production of energy. Under aerobiosis, one glucose molecule can generate only two ATP molecules, with two pyruvate molecules excreted into the host bloodstream as the only end product.[71] This low energy yield makes it necessary to have the glycolysis in *T. brucei* proceeding at an extremely high rate of 85 nmol of glucose min^{-1} (mg of protein)$^{-1}$ in order to enable the trypanosome to divide once every 7 h.[72] The high glycolytic rate, 50 times of that in the mammalian host, is made possible not only by the abundant glucose supply in the host blood but also by the clustering of most of the glycolytic enzymes in the parasite in membrane-bound microbody-like organelles with a diameter of 0.3 μm. The organelle, termed the glycosome,[73] has been identified among all the *Trypanosomatids* examined thus far.[74] Since lactate dehydrogenase is absent from the parasite, the regeneration of NAD from NADH in the glycosome during glycolysis depends on a dihydroxyacetone phosphate:glycerol 3-phosphate shuttle plus a glycerol-3-phosphate oxidase.[71] Thus, under anaerobic conditions glycerol 3-phosphate cannot be oxidized back to dihydroxyacetone phosphate and becomes accumulated inside the glycosome.[75] This accumulation, together with the accumulation of ADP due to depletion of NAD, eventually drives the reversed glycerol kinase catalyzed reaction in the glycosome to generate glycerol and ATP.[75] Thus, anaerobiosis of *Trypanosoma brucei* generates only one ATP from each glucose molecule and excretes equimolar pyruvate and glycerol as end products.

It is obvious that this delicate and indispensable glycolytic system ought to be an attractive target for antitrypanosomal chemotherapy. Recent studies have demonstrated very convincingly that one can inhibit the glycerol-3-phosphate oxidase with salicylhydroxamic acid (SHAM) to bring *T. brucei* under anaerobic condition and then stop the glycolysis by inhibiting the reversed glycerol kinase catalyzed reaction with added glycerol[76-78] This SHAM plus glycerol combination can lyse the African trypanosome bloodstream forms in vitro within minutes and is very effective in suppressing parasitemia in vivo.[76,77] Some of the well-known antitrypanosomal agents, discovered by random screens in the past, have been also found recently to act by inhibiting glycolysis in the parasite. For instance, melarsen oxide has been found highly



melarsen oxide



suramin

inhibitory of *T. brucei* pyruvate kinase,[79] whereas the mechanism of action of suramin is most likely by inhibition of glycerol-3-phosphate dehydrogenase.[80]

Another interesting aspect of the glycolysis, which may be included in future chemotherapeutic consideration, is the aggregation of hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glycerol kinase, glycerol-3-phosphate dehydrogenase, glyceraldehyde phosphate dehydrogenase, and phosphoglycerate kinase in the glycosome.[73] This close physical arrangement of the glycolytic enzymes may enable sub-

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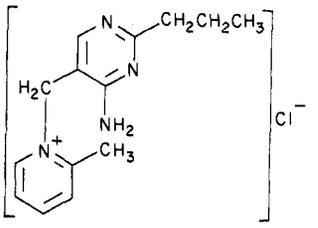
strate channeling through the chain of reactions that makes the high glycolytic rate possible. The channeling effect may turn a weak enzyme inhibitor into a very potent one if the inhibitor happens to be the product of the preceding enzyme in the reaction chain.

Enzymes with Different Pharmacological Properties Found in the Parasites

These parasite enzymes have been identified as probable targets for antiparasitic chemotherapy not because of the parasite nature of the organism but, more likely, because of the long evolutionary distance separating the parasites and the hosts. It is thus difficult to discover these targets by studying the metabolic deficiency or the special nutrient requirement of the parasite. They have been mostly found by investigating the modes of action of some well-established antiparasitic agents discovered by try-and-err means in the past. Since the research efforts usually start from a single drug, the eventually identified target of the drug action may not be always a single, well-defined enzyme but rather sometimes a transporter, receptor, cellular structural component, or any other specific function essential for the survival of the parasite.

Thiamine Transporter. Carbohydrate metabolism provides the main energy source in coccidia.[81] When chickens were put on a diet deficient in thiamine, riboflavin, or nicotinic acid, all cofactors in carbohydrate metabolism, suppresion of *Eimeria tenella* and *Eimeria acervulina* infection resulted.[82] A thiamine analogue, amprolium (1-[(4-amino-2-propyl-5-pyrimidinyl)-

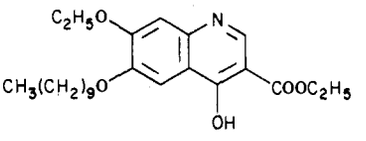


amprolium

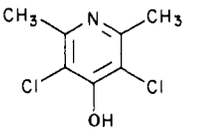
methyl]-2-picolinium chloride), has long been used as an effective anticoccidial agent[83] with relatively low toxicity to the host.[84] The antiparasitic activity of amprolium is reversible by thiamine.[84] However, it was only until recently that the mechanism of amprolium action was proven to be by inhibition of thiamine transport in the parasite.[85] *Eimeria tenella* has only a slightly higher substrate affinity (K_m = 0.07 μM) than the host cell (K_m = 0.36 μM) in thiamine transport. Amprolium, however, has a K_i value of 7.6 μM for *E. tenella* thiamine transport and a K_i of 362.5 μM for that of chicken intestine, a difference of 50-fold. This finding has clearly qualified the thiamine transporter in *Eimeria* spp. as the target for chemotherapy. The only puzzling aspect is the rather limited spectrum of antiparasitic activities found in amprolium; other than the anticoccidial activities in chickens and cattle, amprolium has no activity against toxoplasmosis, a closely related parasitic infection. It is an interesting assumption that only the thiamine transporter in *Eimeria* spp. possesses some particular pharmacological properties.

Mitochondrial Electron Transporter. Mitochondria of *Eimeria tenella* have the unusual difference spectra of cytochromes, which suggest the absence of cytochrome *c* and the presence of cytochrome o.[86] Pharmacologically, a family of 4-hydroxyquinoline derivatives, which have long been known as the relatively nontoxic, effective anticoccidial agents, have been found to act on the parasites by inhibiting their mitochondrial respiration.[87] Direct investigation on the isolated, intact *Eimeria tenella* mitochondria indicated that the 4-hydroxyquinolines had no effect on NADH oxidase or succinoxidase activity but had extremely potent inhibition on the NADH- or succinate-induced mitochondrial respiration.[86] On the other hand, however, the ascorbate-induced *E. tenalla* mitochondrial respiration was totally unsusceptible to these 4-hydroxyquinolines.[86] Thus, the block by the anticoccidial agents may be located between the oxidases and cytochrome *b* in the electron-transport chain. A certain component(s) at the location must be essential for mediating the electron transport, as well as be highly sensitive to the 4-hydroxyquinolines. This component(s) must also be a very specific chemotherapeutic target in *Eimeria* spp., since the 4-hydroxyquinolines are totally without effect on chicken liver and mammalian mitochondrial respiration and completely without activity against any parasites other than the *Eimeria* spp.[86]

Another anticoccidial compound, meticlorpindol, a py-



decoquinate



metichlorpindol

ridone derivative, has been also found to inhibit *Eimeria tenella* mitochondrial respiration, although the potency is much lower.[81] Synergism between meticlorpindol and the 4-hydroxyquinolines in anticoccidial activities has been observed often,[88] and collateral senstivities to the 4-hydroxyquinolines have been also found among coccidia resistant to meticlorpindol.[89] It is thus likely that coccidial mitochondria may have a branched electron-transport chain, one susceptible to 4-hydroxyquinolines and the other inhibitable by meticlorpindol.

Microtubules. Microtubules, the important part of the cytoskeleton and mitotic spindle, constituted by the α- and β-tubulin subunit proteins, have been regarded as evolutionarily conserved. However, recent comparisons of α- and β-tubulins from several species of wide taxonomic separation, bovine kidney, sea urchin eggs, squid brain, *Pteridium* sperm flagella, *Chlamydomonas* flagella,[90] and the parasite nematode in chickens, *Ascaridia galli*,[91] indicated that while the β-tubulins of these organisms appeared to have the same electrophoretic migration, the α-tubulins varied widely. This variation is not only interesting in pointing out the evolutionary relations among eukaryotic cells but also useful in classifying the microtubules in parasites as potential targets for antiparasitic chemotherapy. There is a large group of benzimidazole derivatives that has long been established as highly ef-

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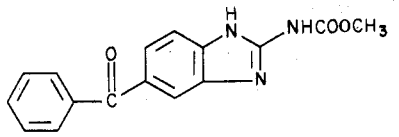
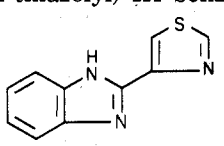
fective anthelmintics.[92] Mebendazole (methyl 5-



mebendazole

benzoylbenzimidazole-2-carbamate) was among the first of such anthelmintics found to act primarily by blocking transport of secretory granules and movement of other subcellular organelles which coincided clearly with the disappearance of cytoplasmic microtubules from the intestinal cells of the worm.[93] On the other hand, the microtubular system of the host cells was unaffected by the treatment. Mebendazole and another anthelmintic benzimidazole, fenbendazole, were later shown to compete with colchicine binding to *Ascaris lumbricoides* embryonic tubulins with 250–400 times higher potencies than the binding competition to bovine brain tubulins.[94] The differential binding affinities may thus explain the selective toxicity of the benzimidazoles toward the parasitic nematodes. Another anthelminitic benzimidazole derivative, thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole], which



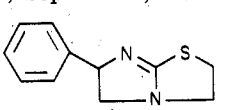
thiabendazole

also posseses antifungal activities,[95] is found most likely acting by binding to the fungal tubulins.[96] Mutants of *Aspergillus nidulans* resistant to thiabendazole were isolated and studied genetically.[97] The mutations conferring drug resistance were usually acompanied by mitotic temperature sensitivity and were found mostly in one genetic locus *benA*, which was identified as the gene coding for β-tubulin.[97] The revertants could be obtained either by a back-mutation in *benA* or by a mutation in the α-tubulin gene *tubA* to suppress the drug resistance caused by the *benA* mutation.[98] Apparently both α- and β-tubulin are involved in binding with thiabendazole.

Nerves. Invertebrates, such as the helminths and the arthropods, differ from vertebrates mainly by their characteristic nervous systems. The nerve axons in invertebrates, for instance, are unmyelinated and, thus, are more susceptible to disturbances of nerve membranes. The individual muscle fiber in an arthropod is innervated with an excitatory synapse using L-glutamic acid as the neurotransmitter and an inhibitory nerve with γ-aminobutyric acid (GABA) as the transmitter, whereas cholinergic nerves are concentrated in the central system.[99] For the nematodes, whether it is a free-living soil species, *Caenorhabditis elegans*,[100] or a gastrointestinal parasitic kind, *Ascaris lumbricoides*,[101] their entire neuronal systems appear to be identical. There are cholinergic and GABAergic synapses at the neuromuscular junctions, as well as in the central ventral cords,[102] of the worms. The mammalian hosts, on the other hand, have mainly nicotinic cholinergic receptors at the neuromuscular junctions, and the GABA nerves are all confined within the central nervous system protected by the blood–brain barrier.[103]

We know that all the major classes of insecticides today are neurotoxicants. The chlorinated hydrocarbons, such as DDT, act on nerve membranes. The organophosphates and carbamates are inhibitors of acetylcholine esterase. The pyrethroids, reputed to be the least toxic insecticides,[104] are known to have two types of activities.[105] The type I action, caused by pyrethroids without an α-cyano group, induces prolonged inward sodium current in nerve axons.[106] The type II action of cyanopyrethroids is primarily on the GABA receptor–ionophore complex functioning as an antagonist.[107] The onset of type II symptom, occuring among mammals in their central nervous system, can be delayed by pretreatment with the GABA agonist diazepam.[108]

Since the anthelmintics, if they are to be neurotoxicants to the parasites, have to be first administered to the mammalian hosts before reaching the parasitic nematodes, they have to be nontoxic to the host. Furthermore, they have to be able to penetrate the thick cuticles of the nematodes in order to be effective. It is thus much harder to find a useful anthelmintic acting on the nervous system of the parasite. However, the records indicate that, other than the tubulin-binding benzimidazoles mentioned previously, the majority of commercially available anthelmintics act on the nerves of the nematodes. They can be classified into two groups: (1) those acting as ganglionic nicotinic acetylcholine agonists and (2) those acting directly or indirectly as GABA. The first category includes levamisole, pyrantel, bephenum, etc.[109] The cholinergic



levamisole

receptors at the nematode neuromuscular junctions are of the ganglionic nicotinic type; these agonists are extremely effective in causing muscular contraction of the worms. The ganglionic nicotinic antagonist mecamylamine can revert the action of these anthelmintics. However, since mecamylamine cannot penetrate the cuticles, it has to be injected into the lumen of the nematode in order to show its effect.

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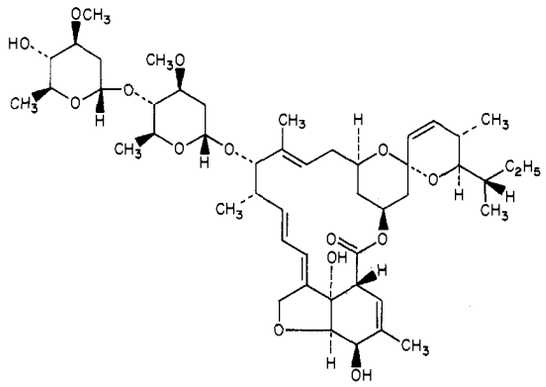
The second category includes piperazine, which apparently acts as a GABA agonist at the neuromuscular junction and causes flaccid paralysis of the nematode.[102] A much more exciting family of natural compounds, the milbemycins and the avermectins,[110] however, have been



avermectin B_{1a}

recently discovered. They act indirectly to intensify the GABA action on the ventral cord interneuron and motoneuron junction to immobilize the nematodes[111] and at the neuromuscular junction of arthropods to cause paralysis.[112] As it could be anticipated, both milbemycins and avermectins are potent anthelmintics, insecticdes, and antiectoparasite agents[110] with no cross-resistance problems with agents acting on the cholinergic systems.[110] Picrotoxin, a specific blocker of the chloride ion channel controlled by postsynaptic GABA binding, can reverse all the physiological activities of the drugs.[111,112]

Avermectins have little effect on the mammalian central nervous system because they cannot pass through the blood–brain barrier readily.[110] However, when the mammalian brain synaptosomes and synaptic membranes were used as models for investigation, specific high-affinity binding sites for the avermectins were identified with the GABA nerves.[113] The drug binding stimulated, specifically, GABA release from the presynaptic end of the GABA nerve[114] and enhanced the postsynaptic GABA binding.[115] It also has a stimulatory effect on benzodiazepine binding[116] and an in vivo enhancement of diazepam muscle relaxant activity.[117] There is little doubt that milbemycins and avermectins act on the GABA nervous system and, contrary to the type II action of cyanopyrethroids, amplify the GABA function. The GABA nerves among the invertebrates are thus very attractive targets for chemotherapy.

Little is known about the nervous systems in cestodes and trematodes except that they may be quite different from that of the nematodes, because milbemycins and avermectins have no effect on them.[110] On the other hand, a newly discovered, highly effective antischistosomal and anti-tape-worm agent, praziquantel,[118,119] known to enhance

Table I. Identified Targets in Different Parasites for Chemotherapy

targets	parasites
enzymes for dihydropteroate synthesis	sporozoa
pyruvate:ferredoxin oxidoreductase	anaerobic protozoa
nucleoside phosphotransferase	flagellated protozoa
purine phosphoribosyltransferase	protozoa and trematode
ornithine decarboxylase	protozoa
glycolytic enzymes	kinetoplastida
thiamine transporter	coccidia
mitochondrial electron transporter	coccidia
microtubules	helminth
nerves	helminth and ectoparasite

Ca^{2+} influx and induce muscular contraction in the parasites,[120] exerts no action on the nematodes or insects. Some benzodiazepine derivatives also have activities similar to praziquantel, but the activities are unrelated to the anxiolytic activities in the mammalian central nervous system.[121] Thus the nerves and muscles in schistosomes and tape worms are interesting for future chemotherapeutic studies.

Summary

I have thus far listed a total of 10 potential targets for antiparasitic chemotherapeutic consideration. This is by no means a completed list. Many more will be added to it with time and with more future findings. Among these 10 targets (summarized in Table I), however, one may gain some insight and see a few interesting general trends: (1) Nucleic acid metabolism and carbohydrate-energy metabolism in protozoan parasites appear to be targets for fruitful chemotherapeutic attacks. Their being useful targets results generally from the deficient metabolism in the protozoan parasites. Thus, the main vulnerability among the protozoan parasites is closely associated with their parasitic nature. (2) Microtubules and nervous systems appear to be the main chemotherapeutic targets in helminths. They differ from those in the host not because of their parasitic nature but, more likely, because of the evolutionary distance separating the mammalian hosts and the primitive metazoa. Thus, free-living nematodes, such as *Caenorhabditis elegans*, have their microtubules just as suceptible to the benzimidazole anthelmintics as those from the parasitic worms. The motoneuronal map of *C. elegans* is identical with that of *Ascaris lumbricoides*. Both worms are similarly immobilized by levamisole, piperazine, avermectins, etc. The dual insecticidal and antiexoparasite activities found in the avermectins and milbemycins may also suggest that the free-living insects and the ticks and lice may have the same GABA nervous system.

This main discrepancy between protozoan parasites and metazoan parasites may be partly attributable to the higher mutation rates and higher frequencies of genetic recombination among the protozoa, evidenced by the higher rates of development of drug resistance among them. The fast adaptation to a new environment may be essential for survival, but it would also lead to metabolic

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deficiencies after the protozoa lived in a luxurious environment for a while. This revelation may suggest that future chemotherapeutic studies on parasitic helminths can utilize free-living helminths as models to eliminate many unnecessary technical difficulties. Also, there perhaps could be a further classification among the parasites to term the protozoa "true parasites" and the helminth "pseudo-parasites" from the viewpoint of chemotherapy.

Communications to the Editor

(*E*)-2-(3,4-Dimethoxyphenyl)-3-fluoroallylamine: A Selective, Enzyme-Activated Inhibitor of Type B Monoamine Oxidase

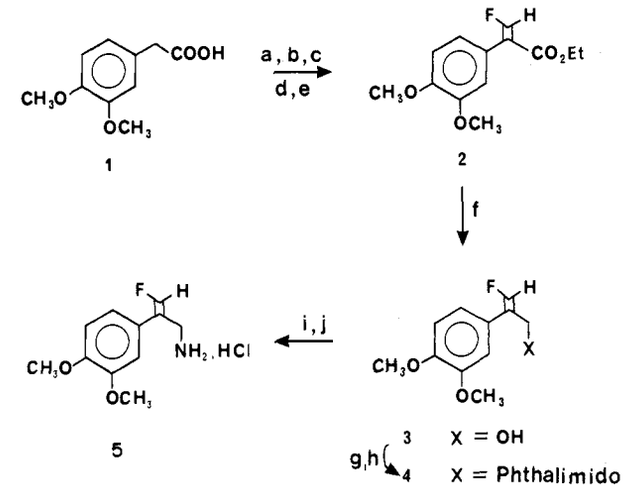
Sir:

The search for clinically effective inhibitors of monoamine oxidase [amine:oxygen oxidoreductase (deaminating, flavin-containing); EC 1.4.3.4; MAO] has been one of the continuing themes of drug research for the past 3 decades.[1] While the therapeutic advantages of MAO inhibitors are generally well accepted,[2] one problem that has not yet been satisfactorily resolved is the so-called "cheese effect".[3]

An attractive approach to the design of clinically safe MAO inhibitors is to take advantage of the occurrence of two forms of MAO: type A and type B.[4] On the basis of the substrate specificity of MAO A and B and the relative distribution of the two enzyme forms in different body organs, it has been reasonably postulated that a selective inhibitor of the B form would be largely free of the cheese effect.[5] This concept has played a role in the development of some selective inhibitors[6] of MAO.

We have prepared a series of substituted allylamines[7] as enzyme-activated inhibitors of MAO. Of most interest is (*E*)-2-(3,4-dimethoxyphenyl)-3-fluoroallylamine (**5**),[8] synthesized according to the route in the Scheme I. The preparation of **2**[9] from commercially available 1 followed essentially known chemistry developed in our laboratory[10] and elsewhere.[11] Selective reduction of **2** was achieved with diisobutylaluminium hydride in hexane, followed by acidic workup. The resulting alcohol **3**[9] was most conveniently converted to the phthalimide **4**[9] via the bromide. Deprotection afforded **5**, which was purified as its hydrochloride salt[9] (mp 216–217 °C). The *E* configuration of the double bond was established on the basis of NMR data and confirmed by X-ray structural analysis.[12]

Scheme I[a]



[a] a = *tert*-butyl acetate, $HClO_4$; b = LDA, $ClCO_2Et$; c = sodium *tert*-butoxide, $ClCHF_2$; d = CF_3CO_2H; e = NaOH; f = DIBAL; g = PBr_3; h = potassium phthalimide; i = NH_2NH_2; j = HCl.

Incubation of a preparation of rat brain mitochondrial MAO[13] with varying concentrations of **5** resulted in a time-dependent loss of enzyme activity, which followed pseudo-first order kinetics for more than 2 half-lives (Figure 1). The minimum half-life (τ_{50}) at saturating concentration and the apparent dissociation constant (K_I) of **5**, determined at 10 °C according to the method of Kitz and Wilson,[14] are 14.5 min and 130 μM and 1.7 min and 40 μM for the A and B forms of MAO, respectively. Protection against inactivation of either the A or the B form of the enzyme can be demonstrated by preincubation with the corresponding substrate 5-HT (type A) or benzylamine (type B). Enzyme activity is not recovered after extensive dialysis or after treatment with benzylamine,[7b] indicating that the inhibition is irreversible. These results strongly suggest that **5** is an enzyme-activated irreversible

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(12) An X-ray structural analysis of **5** was kindly undertaken by Professor R. Weiss at the Laboratoire de Cristallochimie, Institut Le Bel, Université Louis Pasteur, Strasbourg.
(13) Rat brain mitochondria were prepared[16] in 0.1 M phosphate buffer (pH 7.2). Aliquots of mitochondrial suspension were preincubated at 37 or at 10 °C for different times with a range of concentrations of **5**. After extensive dilution (100- to 250-fold), the remaining MAO activity of the type A and type B forms of the enzyme were determined with 5-hydroxy[^{14}C]-tryptamine (10 μM) and [^{14}C]phenethylamine (5 μM) as selective substrates for type A and type B, respectively.
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