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Pyrazolopyrimidine Metabolism in Parasitic Protozoa

Buddy Ullman[1]

Abstract: The pyrazolopyrimidines are purine analogs that are cytotoxic toward and metabolized by several genera of parasitic protozoa, including the *Leishmania* and the *Trypanosoma*. Examples of pyrazolopyrimidines that are selectively metabolized by these parasites include allopurinol, allopurinol riboside, 4-thiopurinol, 4-thiopurinol riboside, and formycin B. These pathogenic protozoa are capable of efficient conversion of the pyrazolopyrimidines to the nucleotide level. The pyrazolopyrimidine metabolites which are isomers of inosine monophosphate are subsequently aminated and incorporated as the adenylate analog into RNA. Mammalian cells are incapable of these metabolic transformations. The sulfur containing pyrazolopyrimidines, however, are neither aminated nor incorporated into nucleic acid. The selective metabolism of the pyrazolopyrimidines by the intracellular metabolic machinery of the parasites of the Trypanosomatidae family offers a rational approach to the chemotherapy of the diseases caused by these pathogenic hemoflagellates.

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Table I *The Trypanosomatidae:* The pathogenic hemoflagellates, their morphological forms, and the diseases they cause.

Genus	Species	Stages	Disease
Leishmania	*donovani*	promastigote	visceral leishmaniasis
	braziliensis	(insect form)	mucocutaneous leishmaniasis
	mexicana	amastigote	cutaneous leishmaniasis
	tropica	(intracellular)	cutaneous leishmaniasis
Trypanosoma	*cruzi*	epimastigote (insect form)	Chagas disease
	brucei gambiense	trypomastigote (blood stream)	African sleeping sickness
	brucei rhodesiense	amastigote (intracellular)	African sleeping sickness
	brucei brucei		nagana (not infective to humans)

[a]The amastigote form of the Afrcan trypanosomes, although generally not recognized, has been reported in experimentally infected rodents and in monkey heart (81, 82).

Twelve genera of parasitic protozoa are known to cause infection in people or their domestic animals. Parasitic infections stand as a major obstacle to world health and economic progress in developing countries and are becoming a prevalent health problem in many western countries, as well. While there have been major advances in combating infectious disease of bacterial origin, until recently there has been a relative lack of interest by western scientists and public health officials in parasitic diseases. The pathogenic hemoflagellates of the family Trypanosomatidae consist of the heteroxenous *Leishmania* and *Trypanosoma* and are the causative agents of leishmaniasis and trypanosomiasis, respectively. Depending on which subspecies or strain, *Leishmania* can cause cutaneous, mucocutaneous, or visceral leishmaniasis. The trypanosomes fall into two major categories: the South American *Trypanosoma cruzi,* the causative agent of Chagas disease, and the African trypanosomes, such as *Trypanosoma brucei brucei, Trypanosoma brucei rhodeniense* and *Trypanosoma brucei gambiense*, which cause African sleeping sickness. The organisms, their morphological forms, and the diseases they cause are listed in Table I.

The establishment of an effective parasite-specific chemotherapeutic regimen for the treatment of parasite-mediated diseases depends on a fundamental understanding of the various metabolic processes of the cell. Although all the biochemistry has not been elucidated, most of the major metabolic pathways in the parasitic protozoa, including those pathways for carbohydrate, protein and lipid metabolism, are similar to those of the mammalian host (1, 2). However, there is one exception. Whereas mammalian cells can synthesize purine nucleotides both *de novo* and by salvage from preformed purines, all the parasitic protozoa examined to date are incapable of *de novo* synthesis of the purine ring (3–9). This evidence is based on the failure of these organisms to incorporate radiolabelled precursors such as glycine or formate into nucleotides and on the requirement for exogenous purines for growth in completely defined minimal media. In order to scavenge purines from the host, the Trypanosomatidae have evolved a unique series of purine salvage enzymes for which no identical mammalian counterpart exists (10–17). The unique purine salvage enzymes suggest that the purine pathway in the pathogenic hemoflagellates might be amenable to pharmacologic manipulation by chemotherapeutic agents which require these purine salvage enzymes for metabolic activation. The pyrazolopyrmidines offer such a rational approach.

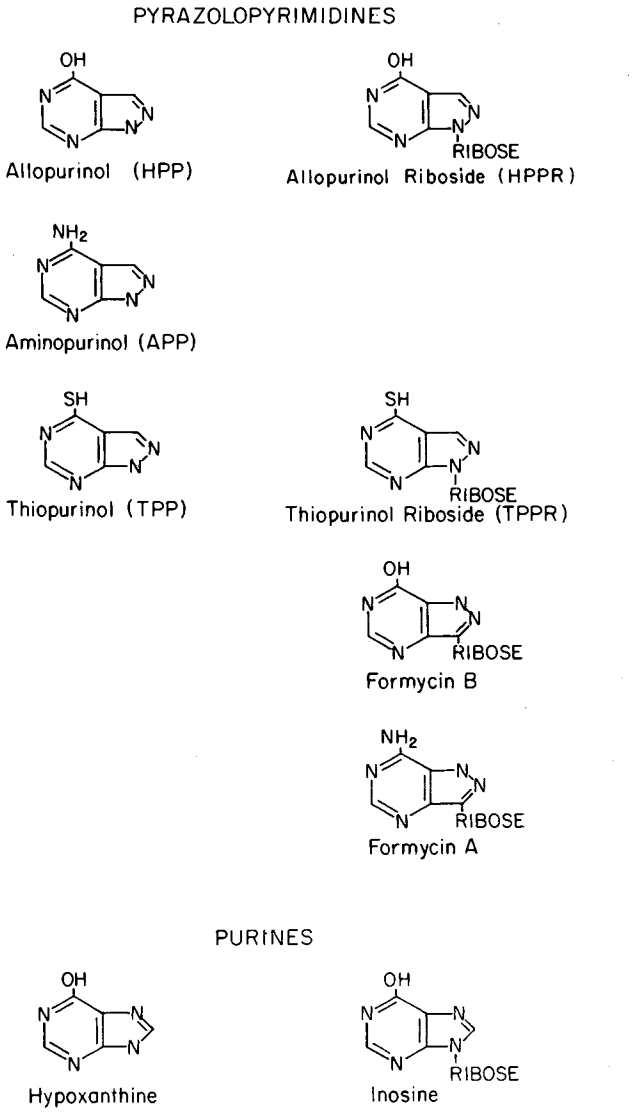

Fig. 1 Chemical structures of the pyrazolopyrimidines and their naturally occurring purine analogs.

Chemical Strucutres of the Pyrazolopyrimidines

Pyrazolopyrimidines are structural analogs of purines in which there is an inversion of the nitrogen from either the 7 or 9 position of the purine ring to what corresponds to the 8 position. The pyrazolopyrimidines discussed in this review which are effective against the pathogenic hemoflagellates include allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), allopurinol riboside (4-hydroxypyrazolo[3,4-*d*]pyrimidine riboside), 4-thiopurinol (4-thiopyrazolo[3,4-*d*]pyrimidine), 4-thiopurinol riboside (4-thiopyrazolo[3,4-*d*]pyrimidine riboside), and formycin B (7-hydroxy-3-ß-D-ribofuranosyl-pyrazolo[4,3-*d*]pyrimidine), Figure 1.

Allopurinol

Allopurinol metabolism in humans and mammals

Allopurinol (HPP) is a relatively non-toxic hypoxanthine isomer that is used extensively in humans for the treatment of hyperuricemia and gout (18–21). Originally designed for its potentiation of the antitumor effects of 6-substituted purines (22, 23), HPP was shown to be both a substrate and an inhibitor of the enzyme xanthine oxidase (23–26). Much of the HPP in humans is converted to oxypurinol (4,6-dihydroxypyrazolo-[3,4-*d*]pyrimidine) by xanthine oxidase, the latter being a far more effective inhibitor of the xanthine oxidase (23–28). The remainder is converted to allopurinol-1-ribonucleoside (HPPR) or excreted in the urine (24). Nanomolar to micromolar levels of allopurinol riboside monophosphate (HPPR-MP) have been detected in tissues of rats fed HPP (29, 30). Although HPPR-MP inhibits both PRPP-amidotransferase (31) and orotidylate decarboxylase (32, 33) activities, the amounts of HPPR-MP accumulated in mammalian tissues do not induce a purine or pyrimidine nucleotide starvation (29, 30). HPP does not get incorporated into nucleic acids in mammalian cells (29).

Effects of Allopurinol on Leishmania Promastigotes

In 1970, Frank et al. reported that HPP can inhibit the growth of the insect parasite, *Crithidia fasciculata* (34). Subsequently, Marr and coworkers demonstrated that HPP also inhibits the growth of 3 *Leishmania* species, *L. braziliensis, L. donovani,* and *L. mexicana* (35, 36). These workers initially hypothesized that the antiparasitic activity of HPP was mediated either through the inhibition of pteridine (34) or pyrimidine nucleotide (35) synthesis. However, neither *C. fasciculata* (35, 37) nor *L. braziliensis* (35) possess xanthine oxidase activity, implying that HPP does not exert its cytotoxic effect by interfering with the interconversion of pteridines, and pyrimidines do not restore normal growth to all three leishmanial species exposed to HPP (36). In all three *Leishmania* species the growth inhibitory effects of HPP can be reversed by adenine, suggesting that HPP might induce an adenylate nucleotide starvation, perhaps mediated by inhibition of the protozoan adenylosuccinate synthetase (36). However, HPP does not specifically or rapidly deplete intracellular adenylate nucleotide pools (38). The growth inhibitory and cytotoxic effects of HPP on all four leishmanial species in completely defined growth medium (39) are shown in Figure 2.

Nelson et al. investigating the metabolism of HPP in promastigotes of *L. braziliensis* and *L. donovani* found that these organisms are capable of concentrating unmetabolized HPP against a gradient and can accumulate millimolar concentrations of HPPR-MP from exogenous HPP (38). HPPR-MP levels were several fold greater than the cellular ATP pools. Moreover, HPPR-MP was subsequently aminated to 4-amino-pyrazolopyrimidine riboside 5'-monophosphate (APPR-MP), phosphorylated to the di- and triphosphate levels, and finally incorporated in small amounts into RNA, but not into DNA, Figure 3 (38). Mammalian cells on the other hand can only accumulate HPPR-MP in minute amounts and are incapable of aminating HPPR-MP or incorporating radiolabelled HPP into RNA (24, 29, 40, 41).

The reversibility of HPP cytotoxicity by adenine or hypoxanthine is probably due to the ability of the nucleobases to interfere with the transformation of HPP to the nucleotide level (38). Neither purine base interferes with the transport of HPP across the pellicular membrane, since intracellular HPP levels are even augmented in the presence of adenine (38).

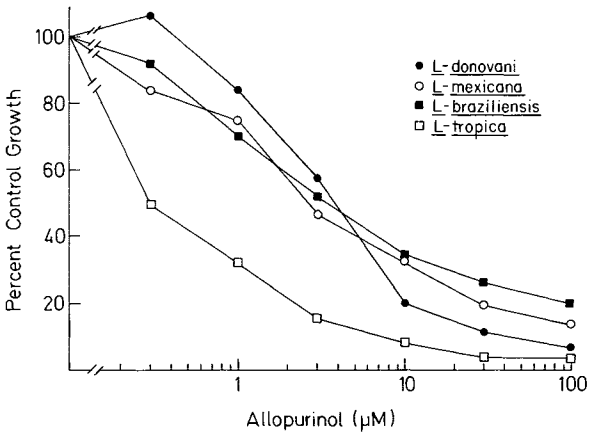

Fig. 2 The growth inhibitory and cytotoxic effects of allopurinol on all four species of *Leishmania*. The growth inhibitory effects of HPP were determined on all four leishmanial species in Costar multiwell plates (24 wells) as follows: 1.00 ml aliquots of completely defined growth medium (39) containing 10^6 cells were placed in separate wells with varying HPP concentrations. Two control wells lacking the drug were maintained in parallel. After approximately 5–7 days, the cell density was determined in a Coulter counter Model ZB1. During this incubation period, the control wells normally reached a density of 2–4 x 10^7 cells/ml. The initial cell density was subtracted from the final cell density, and the resulting difference expressed as a percentage of the control growth and plotted as a function of the concentration of HPP.

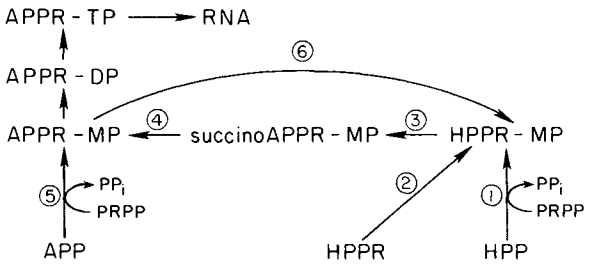

Fig. 3 Metabolic transformations of HPP and HPPR in the pathogenic hemoflagellates. The metabolism of HPP and HPPR in *Leishmania* and *Trypanosoma* is depicted. The relevant enzymes are numbered as follows: 1) hypoxanthine-guanine phosphoribosyltransferase, 2) nucleoside phosphotransferase, 3) adenylosuccinate synthetase, 4) adenylosuccinate lyase, 5) adenine phosphoribosyltransferase, and 6) adenylate deaminase.

Hypoxanthine, a more effective substrate than HPP for the leishmanial hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) activity (10), inhibits HPP phosphoribosylation, while adenine reverses the cytotoxic effects of HPP subsequent to its conversion to hypoxanthine, a reaction catalyzed by the leishmanial enzyme, adenine deaminase (13). Furthermore, addition of adenine 12 hours after exposure to HPP does not reverse the cytotoxic effects of the drug (36, 38), suggesting that adenine and hypoxanthine prevent the metabolic transformation of HPP to HPPR-MP rather than circumventing a block in adenylate nucleotide synthesis.

This selective accumulation of HPPR-MP by protozoa is mostly due to the unique substrate specificity of the HGPRTase activity in these organisms (10). The calculated apparent K_m value for HPP of the partially purified HGPRTase activity from *Leishmania donovani* is approximately 230 μM, 30- and 60-fold higher than the apparent K_m values for hypoxanthine and guanine, respectively, Table II. HPP is a much better substrate for the protozoan HGPRTase than for the human erythrocytic HGPRTase (10, 42). Moreover, the specific activity of the HGPRTase activity in *L. donovani* promastigotes is much higher than that in mammalian cells (42–47). Other factors that may contribute to the selective ability of *Leishmania* to accumulate large quantities of HPPR-MP are the absence of xanthine oxidase (35) and the ability of the promastigotes to concentrate unmetabolized HPP from the culture medium (38).

Approximately 10% of the accumulated HPPR-MP in *L. donovani* promastigotes is subsequently converted to APPR-MP (38), Figure 3. Thus, the adenylosuccinate synthetase and adenylosuccinate lyase activities must be capable of aminating HPPR-MP. The selective ability of *Leishmania* to convert HPPR-MP to APPR-MP lies in the substrate specificity of its adenylosuccinate synthetase (48). Spector and coworkers have shown that the partially purified adenylosuccinate synthetase from *L. donovani* is capable of forming succino-APPR-MP from HPPR-MP (48). Conversely, purified adenylosuccinate synthetase from rabbit muscle does not accept HPPR-MP as an alternative substrate for IMP (49). The apparent K_m values of the leishmanial adenylosuccinate synthetase are 12 μM for IMP and 340 μM for HPPR-MP, Table II (48). Despite the unfavorable kinetic parameters, the millimolar concentrations of HPPR-MP accumulated by *Leishmania* promastigotes in the presence of low endogenous cellular IMP pools ensure a favorable conversion of HPPR-MP to APPR-MP. The adenylosuccinate lyase activity from *Leishmania* is capable of cleaving both adenylosuccinate and succino-APPR-MP with apparent K_m values of 3.3 μM and 11 μM, respectively (48). The specific ability of *Leishmania* to convert HPPR-MP to APPR-MP lies however in the broad specificity of the adenylosuccinate synthetase reaction, since the adenylosuccinate lyases from mammalian and protozoan sources have similar substrate specificities.

The mechanisms of HPP toxicity are still not proven. Once formed, APPR-MP is further phosphorylated by *Leishmania* to APPR-DP and APPR-TP and eventually incorporated into RNA, Figure 3. Because of the relatively low concentrations of the APPR-XP's compared to that of HPPR-MP (38), it is not clear what role amination of HPPR-MP plays in the antileishmanial activity of HPP. HPPR-MP itself is also a potent inhibitor of the leishmanial GMP reductase activity, as are the naturally occurring nucleotides IMP, XMP and GMP (50, 51). The apparent K_i value of the leishmanial GMP reductase for HPPR-MP is 37 μM (50, 51), whereas the K_i value for the same enzyme obtained from human erythrocytes is 3.6 mM (51, 52). Thus, GMP reductase activity should be completely inhibited by the millimolar concentrations of HPPR-MP that accumulate intracellularly (38). These observations suggest that leishmanial GMP reductase could be a potential target site for chemotherapy. Nevertheless complete inhibition of GMP reductase by HPP might not be expected to play an important role in HPP cytotoxicity, since it is unlikely that GMP reductase is essential for purine salvage by the parasite. The predominant purine supply in mammalian cells is the adenylate

Table II Kinetic parameters of purine enzymes relevant to pyrazolopyrimidine metabolism in *Leishmania donovani*.
The apparent Km values are given for the ribose-phosphate acceptor of HGPRTase, for the nucleotide substrate of adenylosuccinate synthetase, and for the phosphate acceptor of the nucleoside phosphotransferase. The apparent Ki values for nucleotide or nucleotide analog are given for GMP reductase and also for TPPR-MP with adenylosuccinate synthetase. Ki values *in italics*. All kinetic parameters were obtained on enzymes from *L. donovani*. All values are micromolar, and the appropriate references have been placed in parentheses.

Substrate	HGPRTase	Adenylosuccinate Synthetase	GMP Reductase	Nucleoside Phosphotransferase
Hypoxanthine	7.6 (10)			
Guanine	3.8 (10)			
HPP	230 (10)			
TPP	440 (10)			
IMP		12 (48)	*14* (50)	
HPPR-MP		340 (48)	*37* (50)	
Formycin B--MP		26 (51)	*140* (51)	
Adenylosuccinate				
Succino-APPR-MP				
XMP			*14* (50)	
TPPR-MP		*81* (68)	*34* (50)	
GMP			*17* (50)	
HPPR				4600 (14)
TPPR				3400 (68)
Formycin B				1600[a]

[a]personal observation

nucleotide pool (53), which, when scavenged by the parasite, does not require GMP reductase for its conversion to either adenylate or guanylate nucleotides.

When promastigotes of *L. donovani* are exposed to micromolar concentrations of HPP, RNA levels drop and protein synthesis decreases after 6 hours (54). HPP exposure stimulates RNA degradation, all three major types of RNA being degraded (54). Nucleotide pools, however, are not augmented by the HPP induced concomitant increase in RNA degradation and decrease in RNA content (38). The mechanism by which HPP metabolites promote this RNA catabolism remains unknown.

Effects of allopurinol on Leishmania amastigotes

Since the pathogenic form of *Leishmania* in humans is the intracellular amastigote stage, Berens et al. examined the metabolism of HPP in *Leishmania donovani* amastigotes (55). They found that exposure of *L. donovani* amastigotes to [^{14}C]HPP results in the accumulation, although at lower concentrations, of the same metabolites found in promastigotes. Minced infected hamster spleens incubated with 37 μM [^{14}C]HPP for 6 hours show a 9-fold greater accumulation of HPPR-MP than control uninfected spleens (55). Experiments with *L. donovani* infected P388D$_1$ murine macrophages show a similar metabolic labeling pattern with [^{14}C]HPP. In both the hamster spleens and P388D$_1$ macrophages, APP ribonucleotides are formed only in the parasite infected cells (55). In the macrophage system, HPP at 37 μM is effective in eliminating 80–90 % of the intracellular parasitemia (55). Moreover, addition of 5 μM HPP to isolated *L. donovani* amastigotes inhibits but does not prevent their back-transformation to the promastigote form (14).

Effects of allopurinol on Trypanosoma cruzi

HPP in the 20–80 μM range also inhibits the growth of the epimastigote culture form of most isolates of *T. cruzi* (54, 56, 57). The concentrations of HPP which are growth inhibitory to *T. cruzi* are considerably higher than those found for *Leishmania*, probably reflecting differences in cell culture conditions (36, 56, 57, Figure 2). Marr et al. demonstrated that the growth inhibitory effects of HPP on the Costa Rica strain of *T. cruzi* can be specifically reversed by adenine, hypoxanthine, and inosine (56). These same workers demonstrated that the Costa Rica strain of *T. cruzi* can accumulate HPPR-MP in millimolar amounts and sequentially convert HPPR-MP to APPR-MP, APPR-DP and APPR-TP, and incorporate the last into RNA, a pathway virtually identical to that in *Leishmania*, Figure 3 (56). No specific depletion of cellular nucleotide pools was observed in the *T. cruzi* epimastigotes incubated with 18 μM HPP for 24 hours, although a similar incubation with 180 μM HPP reduced most cellular nucleotide pools approximately 50 % (56).

The biochemical machinery responsible for the sequential metabolic conversions of HPP in *T. cruzi* has not been studied in as great detail as that in *Leishmania*. The HGPRTase described by Gutteridge and Davies (11) is the likely candidate responsible for the phosphoribosylation of HPP to HPPR-MP in *T. cruzi*. The kinetic properties of the adenylosuccinate synthetase-lyase system from *T. cruzi* indicates that the substrate specificity and kinetic parameters for the adenylosuccinate synthetase-lyase system are virtually identical to those described in *Leishmania* and clearly distinguishable from those of the mammalian enzyme (58, 59). Thus, the specificity of the amination process in *T. cruzi* appears to be in the unique substrate specificity of the adenylosuccinate synthetase enzyme, which can convert HPPR-MP to succino-APPR-MP (59). The adenylosuccinate lyase enzyme from *T. cruzi* has the same broad substrate specificity characteristic of this enzyme from most organisms (59).

The pathogenic forms of *T. cruzi* are the intracellular amastigote and bloodstream trypomastigote stages, and thus, the metabolism of HPP was also investigated in these forms. Using [^{14}C]HPP, Berens et al. demonstrated that the bloodstream and intracellular forms of *T. cruzi* can synthesize the same metabolites demonstrated in the epimastigotes (60). Trypomastigotes isolated from *T. cruzi*-infected chinchillas are capable of producing significant amounts of HPPR-MP and moderate quantities of the APP nucleotides. APPR-TP is also incorporated in RNA. Spleen cells from these same chinchillas infected with *T. cruzi* amastigotes also form the same compounds. In an *in vitro* cell culture system using human lung fibroblasts, infected cells produce large quantities of HPPR-MP and moderate amounts of all three aminated derivatives. Again, uninfected control cells produce only small quantities of HPPR-MP. Thus, the epimastigote, trypomastigote, and amastigote forms of *T. cruzi* carry out the same metabolic transformations of HPP as the promastigote and amastigote forms of *Leishmania*. Encouragingly, Berens et al. demonstrated that prolonged multiple treatments with 180 uM HPP can eradicate the *T. cruzi* infection from the human lung cells in culture (60). Moreover, through HPP administration, Avila and coworkers were able to significantly decrease parasitemia and prolong the life span of mice infected with some, but not all, strains of *T. cruzi* compared with infected untreated controls (57, 61).

Effects of allopurinol on the African trypanosomes

Berens et al. also investigated the metabolism of HPP in several strains of African trypanosomes (62). Although neither the metabolism nor the enzymology have been extensively studied, it is clear that HPP has a growth inhibitory effect on the trypomastigote form of these organisms, although at much higher concentrations and to a lesser degree than necessary for growth inhibition of *T. cruzi* or *Leishmania*. When African trypanosomes are incubated with [^{14}C]HPP, they convert the exogenous radiolabeled pyrazolopyrimidine to the same intracellular metabolites as described for the other pathogenic hemoflagellates, Figure 3. Thus, the biological and biochemical responses of African trypanosomes, *T. cruzi*, and *Leishmania* are qualitatively, but not necessarily quantitatively, similar.

Allopurinol ribonucleoside

Effects of allopurinol riboside on Leishmania

The ribonucleoside of HPP is also a potent antileishmanial agent (14). The initial report on HPPR by Nelson and coworkers indicated that HPPR is several hundred fold more effective than HPP toward either *L. donovani* or *L. braziliensis*, although HPP and HPPR are equally efficaceous toward *L. mexicana* promastigotes (14). In our laboratory, HPPR and HPP have variable relative effects toward all four species of *Leishmania* promastigotes, Figure 4. The differences in sensitivities obtained by the two laboratory groups again probably reflect strain differences as well as differences in culture conditions. HPPR is not substantially metabolized (63–65) in humans, although 10 % of administered HPP in humans is converted to HPPR (24, 63, 66).

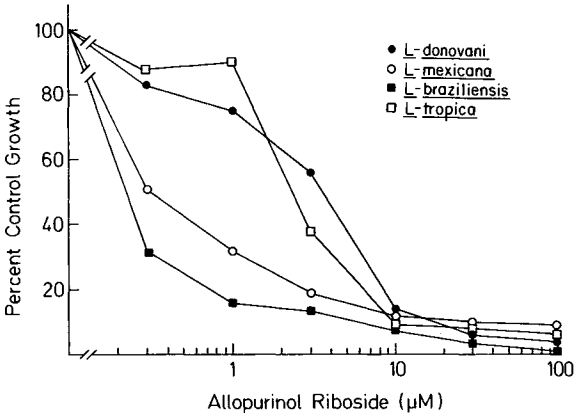

Fig. 4 Growth inhibitory and cytotoxic effects of HPPR on all four species of *Leishmania*. The sensitivity of all four species of *Leishmania* in DME-L medium (39) toward HPPR was performed as described in the legend to Fig. 1.

Metabolic studies with radiolabelled HPPR indicate that it is converted to the same intracellular metabolites in *Leishmania donovani* promastigotes as HPP, Figure 3 (14). Large amounts of HPPR-MP are formed from extracellular [^{14}C]HPPR, and the HPPR-MP is converted to the APP ribonucleotides and incorporated into RNA (14). Little perturbation of endogenous nucleotide pools is observed in *L. mexicana* promastigotes incubated with 18 μM HPPR (14). Experiments using HPPR radiolabelled in the ribose moiety indicate that the specific radioactivity of both HPPR-MP and the aminated metabolites are the same as that of the starting material. Thus, it appears that HPPR is not cleaved to HPP (14), unlike its isomer, inosine, which is rapidly converted to hypoxanthine (6) by two inosine hydrolase activities (12). [^{14}C]HPPR is also effectively metabolized to HPPR-MP in murine macrophages infected with *L. donovani* (55).

HPPR is phosphorylated in cell-free extracts of *L. donovani* by a nucleoside phosphotransferase activity, Figure 3 (14). The product of this reaction is HPPR-MP, Figure 3. HPPR is not phosphorylated by human cells or tissues, which accounts for its biological inertness in humans (14). The leishmanial nucleoside phosphotransferase utilize a variety of phosphate donors, including UMP, TMP and p-nitrophenylphosphate. No ATP dependent phosphorylation of HPPR is observed. However, it must be noted that the nucleoside phosphotransferase activity in other species of *Leishmania* and from HPPR-sensitive strains of *T. cruzi* are at least 20-fold lower than those levels found for *L. donovani* (personal observation). Yet, *L. donovani,* which has the most nucleoside phosphotransferase activity, is the least sensitive of the four *Leishmania* species to HPPR mediated growth inhibition, Figure 4. Moreover, the apparent K_m value of the *L. donovani* nucleoside phosphotransferase for HPPR is 4.6 mM, Table II (19). This apparent K_m value is 1000-fold greater than the concentration of HPPR which inhibits leishmanial growth, creating some doubt as to the physiological relevance of the nucleoside phosphotransferase in HPPR metabolism by *L. donovani.* Nevertheless the selectivity of the HPPR growth inhibitory response toward pathogenic species of *Leishmania* reflects the unique ability of these organisms to convert HPPR to the nucleotide level.

HPPR, like HPP, is also capable of inhibiting the ability of *L. donovani* amastigotes to transform back to the promastigote stage (14) and can markedly inhibit the intracellular growth of these parasites inside P388D$_1$ macrophages (55).

Effects of allopurinol riboside on T. cruzi

Whereas the metabolism and physiological effects of HPP are similar in *T. cruzi* and *L. donovani,* the two organisms handle HPPR quite differently. There are considerable strain differences within the *T. cruzi* population. The growth of both the Costa Rican and CL strains of *T. cruzi* is unaffected by HPPR, and neither strain converts HPPR to the monophosphate derivative in substantial amounts (54, 56). However, other *T. cruzi* strains, such as the Peru and Y strains, are quite sensitive to the cytotoxic effects of HPPR (54). Incubation of Peru strain epimastigotes with [^{14}C]HPPR indicates that they metabolize the nucleoside analog to HPPR-MP and APP ribonucleotides. Clearly, the differential sensitivity of various *Trypanosoma cruzi* strains to HPPR appears to be correlated with their ability to metabolize HPPR to the monophosphate level. However, the role of the nucleoside phosphotransferase in the metabolism of HPPR is questionable, since strains which are sensitive to growth inhibition by HPPR have extremely low levels of the nucleoside phosphotransferase enzyme (Ullman, personal observation).

Effects of allopurinol riboside on African trypanosomes

T. brucei gambiense and *T. brucei rhodesiense* are both sensitive to HPPR (62, 67). *T. b. rhodesiense* is more sensitive to HPPR than HPP, while *T. b. gambiense* is equally sensitive to both (54). Studies with the bloodstream forms of *T. b. gambiense* and *T. b. rhodesiense* indicate that both species convert [^{14}C]HPPR via the same metabolic reaction sequence as the other pathogenic hemoflagellates, Figure 3 (54).

Thiopurinol and thiopurinol riboside

Effects of thiopurinol and thiopurinol riboside on Leishmania

The growth inhibitory and cytotoxic effects of HPP and HPPR toward *Leishmania* and *Trypanosoma* initiated an investigation into the efficacies of other pyrazolopyrimidine analogs against these parasitic protozoa. Of the pyrazolopyrimidines tested, only 4-thiopurinol (TPP) and 4-thiopurinol riboside (TPPR) are effective (68). In humans, TPP which has replaced HPP in certain areas in the treatment of hyperuricemia (69, 70), appears to be metabolized in a pattern analogous to that of HPP except no riboside is formed (68). Moreover, TPP is approximately as effective as HPP in this laboratory toward all species of *Leishmania* except *L. mexicana* (Figure 5). Marr et al. have also found the two nucleobase analogs to be equally effective in their culture system (68). TPPR is more toxic than TPP toward promastigotes from all 4 *Leishmania* species, Figures 5 and 6. Treatment of amastigote infected P388 D$_1$ macrophages with either 65 μM TPP or 68 μM TPPR drastically reduces the percentage of infected cells, and the presence of 25 μM TPP or 50 μM TPPR also inhibits amastigote to promastigote transformation (68).

Using [^{35}S]TPP, [^{35}S]TPPR, and [U-^{14}C]ribose TPPR, it was observed that promastigotes of *L. donovani* accumulate millimolar concentrations (39 pmol/10^{6}cells) of 4-thiopurinol riboside monophosphate (TPPR-MP), a concentration comparable to that of the ATP pool (48 pmol/10^{6}cells) (68), Figure 7. However, unlike the case with HPP, no aminated derivatives are formed, and no desulfuration to HPPR-MP is observed, Figure 7. TPPR-MP is also not further phosphorylated to the di- and triphosphate levels (68). Similarly, radiolabelled TPPR is converted only to TPPR-MP. One other

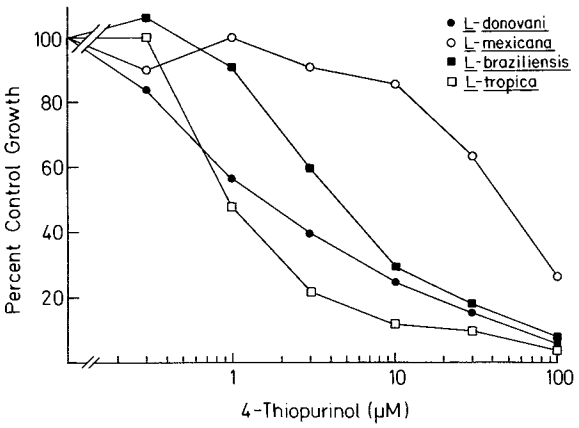

Fig. 5 Thiopurinol cytotoxicity. The growth inhibitory and cytotoxic effects of increasing doses of TPP toward all species of *Leishmania* were performed as described in the legend to Fig. 1.

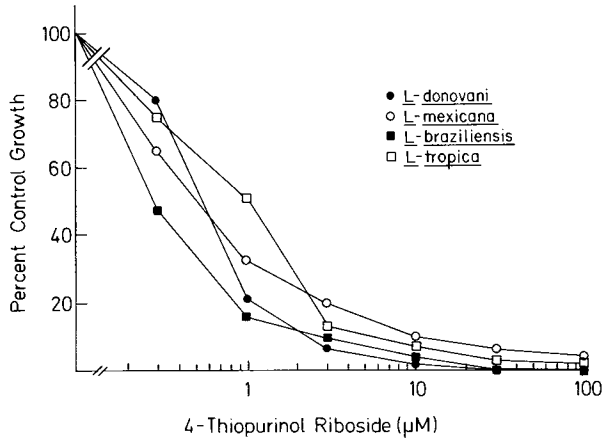

Fig. 6 Effects of thiopurinol riboside toward all four species of *Leishmania*. The cytotoxic effects of TPPR toward promastigotes from all four *Leishmania* species were performed as described in the legend to Fig. 1.

unidentified phosphorylated metabolite was observed, a compound which contained both sulfur and the ribose ring (68). Furthermore, TPPR cannot be cleaved by the cellular nucleosidase activities, since the specific activity of intracellular [U-^{14}C]ribose-TPPR-MP is identical to that of the exogenous precursor [U-^{14}C]ribose-TPPR, and no radiolabel can be detected in the naturally occurring nucleotides (68). Thus, TPPR-MP appears to be the major intracellular metabolite of *L. donovani* exposed to TPP or TPPR.

Metabolism of TPP to the nucleotide level, as with HPP, appears to be catalyzed by the leishmanial HGPRTase activity, Figure 7 (10). The apparent K_m value of the partially purified HGPRTase from *L. donovani* is 440 μM for TPP, Table II. Studies with the purified adenylosuccinate synthetase from *L. donovani* indicated that TPPR-MP is not a substrate for the enzyme and corroborate the lack of APPR-MP formation by *Leishmania* promastigotes treated with either TPP or TPPR. Although, TPPR-MP is not a substrate for the leishmanial adenylosuccinate synthetase, it is an effective inhibitor of the enzyme with a K_i value of 81 μM (Table II), considerably lower than the accumulated intracellular concentration of TPPR-

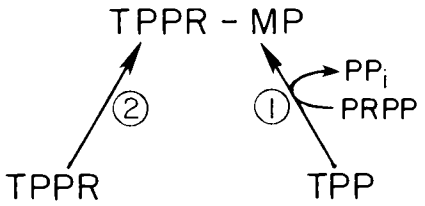

Fig. 7 Metabolism of TPP and TPPR in parasitic protozoa. The metabolic pathways for TPP and TPPR metabolism in *Leishmania* and trypanosomes are described. The relevant enzymes are listed as follows: 1) HGPRTase and 2) nucleoside phosphotransferase.

MP. Coupled with the observation that extracellular TPP can inhibit the formation of aminated derivatives from [^{14}C]HPP, it is quite possible that TPPR-MP might interfere with adenylate nucleotide synthesis. This hypothesis is difficult to test because reversal of TPP toxicity by adenine or adenosine in *Leishmania* is due to the specific inhibition of TPP phosphoribosylation by generated hypoxanthine. Additionally, TPPR-MP also inhibits the GMP reductase activity of *L. donovani* with a K_i value of 34 μM (Table II), again the physiological relevance being unclear (50).

TPPR is also a substrate *in vitro* for the nucleoside phosphotransferase of *Leishmania donovani* (68). As for HPPR, the K_m value for TPPR is high (Table II), around 3.4 mM, 1000-fold higher than the cytotoxic concentrations, and thus the physiological role of the nucleoside phosphotransferase in TPPR phosphorylation still remains open to question.

Effects of thiopurinol and thiopurinol riboside on T. cruzi

T. cruzi also responds somewhat differently than *Leishmania* to the 4-thiopyrazolopyrimidines. Four strains of *T. cruzi* are not affected by TPP or TPPR, yet the Peru strain is capable of accumulating TPPR-MP in the presence of 10% fetal calf serum (54). Adenine, but not hypoxanthine, protects Peru strain *T. cruzi* from TPP and TPPR (54). TPPR-MP is a potent inhibitor of the *T. cruzi* adenylosuccinate synthetase (59) with a $K_i = 33$ μM, suggesting that TPP and TPPR might induce an adenylate starvation in cells deprived of exogenous adenine or adenosine. Since *T. cruzi* lacks adenase, adenine may protect the Peru strain by circumventing a block in the IMP to AMP pathway by TPPR-MP.

Effects of thiopurinol and thiopurinol riboside on African trypanosomes.

Unlike *T. cruzi* or *Leishmania*, the procyclic forms of both *T. b. gambiense* and *T. b. rhodesiense* respond quite poorly to either TPP or TPPR (54).

Formycin B

Effects of formycin B on Leishmania

The formycin compounds differ from the other pyrazolopyrimidines in that there is a rearrangement between the nitrogen and carbon atoms at the 8 and 9 position, Figure 1. Thus, formycin B is a C-nucleoside and an isomer of inosine. Formycin B also has potent antileishmanial activity (71-76 and Figure 8) and is not extensively phosphorylated or cleaved in mammalian cells (77, 78), although the C-nucleoside is a weak competitive inhibitor of purine nucleoside phosphorylase (78) and polyadenosine diphosphoribose synthesis (79) in mamma-

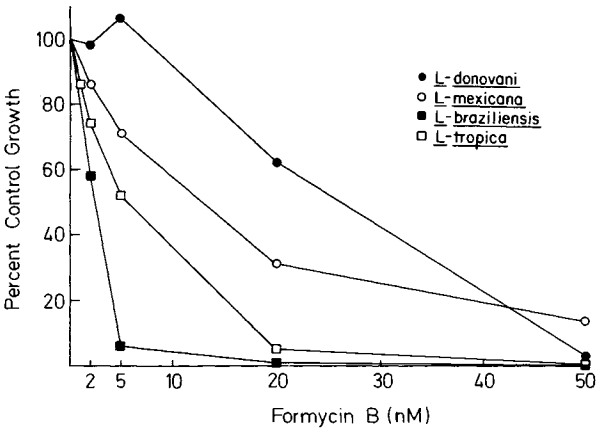

Fig. 8 Effects of formycin B on growth of all four species of *Leishmania*. The growth inhibitory and cytotoxic effects of formycin B on all four species of *Leishmania* were carried out in the same fashion as described for HPP in the legend to Fig. 1.

lian cells. The antileishmanial effects of formycin B on promastigotes were initially reported by Carson and Chang in *L. donovani* and *L. mexicana* (71). Carson and Chang showed that formycin B at a concentration of 1 μM inhibits the growth of *L. donovani* and *L. mexicana* promastigotes, while similar concentrations of formycin B inhibit the growth of *L. mexicana* amastigotes in the J774/G8 macrophage system by 50% (71). When the concentration of formycin B was increased to 10 μM, the inhibition of amastigote growth within the murine macrophages is over 90%, although macrophage growth is also inhibited slightly. Carson and Chang also reported that formycin B administration for 5 successive days diminishes the number of amastigotes in hamster livers infected with *L. donovani* (71).

Subsequent studies by several groups confirmed that formycin B is an effective antileishmanial agent and demonstrated that formycin B monophosphate, like HPPR-MP, is aminated by the adenylosuccinate synthetase-lyase system of *Leishmania* (72, 73, 76). Formycin B monophosphate is thus converted to formycin A monophosphate and subsequently phosphorylated to the di- and triphosphate levels and eventually incorporated into RNA, Figure 9. The metabolic pathways in *Leishmania* for formycin B and HPPR are similar, although a greater proportion of aminated derivates are formed from formycin B (14, 72, 73, 76). These data suggest that the mechanism of action of formycin B might be mediated via conversion to formycin A nucleotides and incorporation into RNA. However, mutant *Leishmania* resistant to both formycin B and formycin A metabolize formycin A normally suggesting another mechanism (76).

The nucleoside phosphotransferase of *L. donovani* is also capable of phosphorylating formycin B to the monophosphate derivative *in vitro*. The apparent K_m value for formycin B is about one millimolar, a 100,000-fold greater value than the $E.C._{50}$ value for formycin B (Table II). Formycin B monophosphate serves both as a substrate and inhibitor for the adenylosuccinate synthetase activity of *L. donovani* (51, 71, 72) and as an inhibitor of the GMP reductase enzyme (51). At present, incorporation of formycin A metabolites into RNA, inhibition of adenylosuccinate synthetase or GMP reductase, or some unknown mechanism must all be considered viable hypotheses for the mechanism of action of formycin B in *Leishmania*.

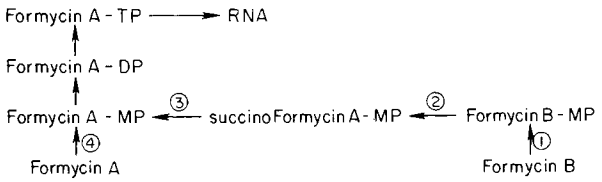

Fig. 9 Metabolic pathways of formycin B in *Leishmania* and *T. cruzi*. The metabolic pathways for formycin B in *Leishmania* are described. The relevant enzymes are enumerated as follows: 1) nucleoside phosphotransferase, 2) adenylosuccinate synthetase, 3) adenylosuccinate lyase, and 4) adenosine kinase.

Effects of formycin B on T. cruzi

Some strains of *T. cruzi* are also sensitive to formycin B (54, 80). In general, those *T. cruzi* strains which are sensitive to HPPR are also sensitive to formycin B, whereas those strains which are resistant to HPPR appear to be insensitive to formycin B (54). Sensitive strains of *T. cruzi*, like *L. donovani*, convert formycin B to the monophosphate level. As in *Leishmania*, formycin B monophosphate is converted to formycin A monophosphate and to the di- and triphosphate level and incorporated into RNA, Figure 9.

The enzymatic machinery responsible for the conversion of formycin B into RNA in *T. cruzi* has not been studied in great detail but is probably similar but not necessarily identical to that in *Leishmania donovani*. The major difference between the two organisms is that extracts of sensitive and resistant strains of *T. cruzi* have no detectable nucleoside (formycin B) phosphotransferase activity using p-nitrophenylphosphate as a phosphate donor (personal observation).

Effects of formycin B on African trypanosomes

African trypanosomes have been shown to be sensitive to growth inhibition by formycin B and can convert formycin B to formycin B monophosphate and the aminated formycin A derivatives and eventually incorporate the adenosine analog into RNA (54).

Summary

The sensitivities of the pathogenic hemoflagellates to the pyrazolopyrimidines offer a rational approach to the treatment of leishmaniasis and trypanosomiasis. The response of these parasitic protozoa to pyrazolopyrimidines results from the selective capacity of these organisms to accumulate the purine analogs to the nucleotide level. The unique purine salvage enzymes, particularly the hypoxanthine-guanine phosphoribosyltransferase, the adenylosuccinate synthetase, and the nucleoside phosphotransferase, appear to play important roles in the selective metabolism of the pyrazolopyrimidines in parasites. HPP has proved to be efficaceous in the eradication of leishmaniasis in mice and monkeys, and in the treatment of Chagas disease in mice. As well, HPP was useful in the treatment of antimony-resistant visceral leishmanasis in 4 of 6 patients. Other pyrazolopyrimidine analogs are currently undergoing toxicological and phase I clinical studies. The overall chemotherapeutic potential of the pyrazolopyrimidine analogs of purine nucleobases and nucleosides remains an untested yet promising approach to the treatment of these devastating parasitic diseases.

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RESEARCH ARTICLES

Pharmacokinetics and Metabolic Fate of Two Nitroxides Potentially Useful as Contrast Agents for Magnetic Resonance Imaging[5]

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Abstract: Paramagnetic nitroxyl-containing compounds have been useful as contrast agents in magnetic resonance imaging (MRI) experiments in animals. Preliminary information on the metabolic fate, pharmacokinetic behavior, stability in tissues, and chemical reduction of two prototypic nitroxides, PCA and TES, is presented. In the dog TES was eliminated more rapidly than PCA. More than 80 % of the dose of both nitroxides was recovered in urine within 6 hours. Nitroxides were reduced *in vivo* to their corresponding hydroxylamines. No other metabolite was observed. Measured reducing activity in tissue homogenates was greater in liver or kidney than in brain, lung or heart. In each tissue PCA was more stable than TES. PCA was also more resistant to reduction by ascorbic acid at physiologic pH. These preliminary results favor the use of PCA, a pyrrolidinyl nitroxide, over TES, a piperidinyl nitroxide, for MRI contrast enhancement.

Nitroxide spin labels have been shown, in animal studies, to have a potential diagnostic applicability as contrast enhancing agents for magnetic resonance imaging (MRI) (1). Applications include the identification of renal disease (2), demonstration of blood-brain-barrier anatomical defects (3), and enhancement of infarcted myocardium (4). Because of their paramagnetic properties nitroxides decrease the relaxation times (T_1 and T_2) of the hydrogen nuclei in the tissues into which they distribute. The contrast between various tissues depends on the relative distribution of nitroxides in these tissues. Knowledge of the metabolic fate and pharmacokinetics of these nitroxides is important in assessing their potential advantages and in optimizing the timing of imaging. Nitroxides are potentially reduced *in vivo* to diamagnetic molecules. However, there is limited information on the pharmacokinetic behavior of this class of compounds (2, 5). In this report, we present preliminary data on the metabolic fate and pharmacokinetics of the two water soluble nitroxides previously tested in animals as *in vivo* MRI contrast agents (2–4). Stability both *in vivo* and *in vitro* is considered.

Materials and Methods

Chemicals

2,2,5,5-Tetramethylpyrrolidine-1-oxyl-3-carboxylic acid (PCA) was obtained from Eastman Kodak Company (lot A10, Rochester, NY). *N*-Succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TES) was synthesized and purified by thin-layer chromatography in our laboratory as previously described (6). PCA and TES (structures given below) were stored as dry powders or as aqueous solutions buffered to pH 7.4 with 0.067 M phosphate buffer. Solutions were passed through a 0.2 μm filter (Acrodisc®, Gelman Sciences, Inc.) prior to intravenous administration. L-Ascorbic acid was obtained from Sigma Chemical Co.

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