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MONOPHOSPHATES OF FORMYCIN B AND ALLOPURINOL RIBOSIDE

INTERACTIONS WITH LEISHMANIAL AND MAMMALIAN SUCCINO-AMP SYNTHETASE AND GMP REDUCTASE*

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Abstract—Formycin B 5′-monophosphate (Form B-MP) and allopurinol riboside 5′-monophosphate (HPPR-MP) are isomers of IMP that are metabolically produced when *Leishmania* spp. are incubated with the antileishmanial agents formycin B and allopurinol or allopurinol riboside. The interactions of Form B-MP with succino-AMP synthetase and GMP reductase from both leishmanial and mammalian sources were compared with the data of earlier studies with HPPR-MP. Both analogs could substitute for IMP as a substrate for succino-AMP synthetase isolated from *Leishmania donovani*. The V'_{max} values of Form B-MP and HPPR-MP were about 1% of the V'_{max} of IMP. Only Form B-MP (and not HPPR-MP) could serve as an alternative substrate for mammalian succino-AMP synthetase. The V'_{max} of Form B-MP was 40% that of IMP. The corresponding analogs of AMP, ADP and ATP were produced when Formycin B was incubated with mouse L cells. The Formycin A residue was incorporated into the cellular RNA. The amount of Formycin A-TP produced (relative to ATP) in mouse L cells was considerably less than that produced in *Leishmania* spp. Both Form B-MP and HPPR-MP were inhibitors of partially purified GMP reductase from *L. donovani*. The binding of Form B-MP and HPPR-MP to human GMP reductase was 40- and 100-fold weaker, respectively, than the binding to leishmanial GMP reductase. Pretreatment of promastigotes of *L. donovani* with either allopurinol or Formycin B resulted in >95% reduction of the incorporation of the radiolabel from [^{14}C]xanthine into ATP and >80% reduction of the incorporation of the label into GTP. The HPPR-MP and Form B-MP present in these cells may have inhibited the leishmanial succino-AMP synthetase and GMP reductase. The analogs had little or no effect on the pool sizes of ATP and GTP of either mouse L cells or *L. donovani*.

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‡ Abbreviations used: HPP, allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine); HPPR, allopurinol riboside; Form B, formycin B (4-hydroxypyrazolo[4,3-*d*]-pyrimidine); Form A, formycin A (4-aminopyrazolo[4,3-*d*]-pyrimidine); MP, 5′-monophosphate; DP, 5′-diphosphate; TP, 5′-triphosphate; succino-AMP, adenylosuccinate; APPR, 4-aminopyrazolo[3,4-*d*]pyrimidine riboside; and HPLC, high performance liquid chromatography.

§ Succino-AMP synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the following reaction: IMP + GTP + L-aspartate $\underset{Mg^{2+}}{\rightleftharpoons}$ succino-AMP + GDP + P_i.

‖ Succino-AMP lyase (EC 4.3.2.2) catalyzes the following reaction: succino-AMP $\rightleftharpoons$ AMP + fumarate.

Allopurinol (HPP)‡, allopurinol riboside (HPPR) and formycin b (Form B) are potent growth inhibitors of *Leishmania donovani* [1–7], a protozoan that causes visceral disease in humans. The metabolism of HPP and HPPR in *Leishmania* spp. has been studied extensively. The parasites convert both of these agents to copious amounts of HPPR 5′-monophosphate (HPPR-MP) [3–8]. The structure of this IMP analog is shown in Fig. 1. HPPR-MP is then converted to APPR-MP, an isomer of AMP [3, 8], via consecutive reactions that are catalyzed by succino-AMP synthetase§ and succino-AMP lyase‖ [9]. Although trace amounts of HPPR-MP are generated from HPP in mammalian tissues [10, 11], APPR-MP is not found in these uninfected tissues [10, 12] because HPPR-MP is not a substrate for mammalian succino-AMP synthetase [13]. In *Leishmania* spp., APPR-MP is further metabolized to a 5′-triphosphate and is incorporated into leishmanial RNA [3, 8].

In addition to its alternative substrate interactions with succino-AMP synthetase, HPPR-MP was found

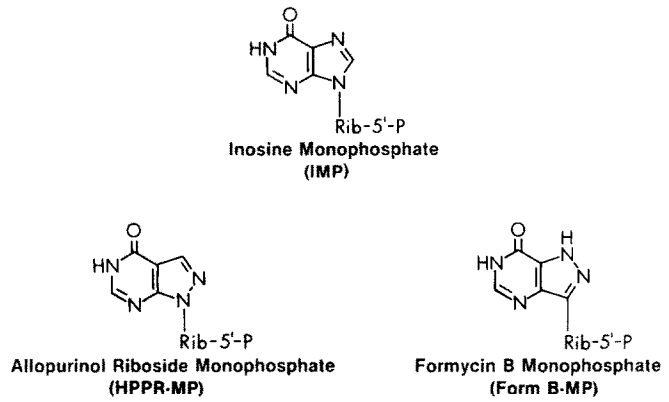

Fig. 1. IMP and relevant isomers.

to have a second locus for potential selective metabolic interference. HPPR-MP proved to be a potent inhibitor of GMP reductase* from *L. donovani* [14] and a very weak inhibitor of the counterpart enzyme from human erythrocytes [15].

The metabolism of Form B in *L. donovani* is similar to that of HPPR. Although Form B was originally reported to be metabolized only to the IMP analog, Form B-MP [4], recent studies have demonstrated that the Form B-MP is converted into analogs of AMP, ADP and ATP (Form A-MP, Form A-DP and Form A-TP) and is incorporated into leishmanial RNA as Form A-MP [6, 7].

In the present study, the interactions of Form B-MP with succino-AMP synthetase and GMP reductase from *L. donovani* and mammalian tissues were investigated and were compared with the earlier studies with HPPR-MP. Enzymatic and metabolic data indicate that there are significant differences between these pyrazolo-(3,4-*d*)pyrimidine and pyrazolo(4,3-*d*)pyrimidine analogs of IMP.

MATERIALS AND METHODS

Materials. [^{3}H]Form B was enzymatically synthesized according to a published procedure [7]. HPLC analysis revealed it to be >99% radiochemically pure. Sources for the purchase or synthesis of all other chemicals can be obtained in Refs. 3, 6, 9, 14 and 15.

Methods. Succino-AMP synthetase was purified from promastigotes of *L. donovani* [9] and from rabbit muscle [13] as previously described. The assays for these enzymes are also described therein. The kinetic constants for HPPR-MP and Form B-MP with the protozoal enzyme were determined as described elsewhere [16]. The purification and assays of GMP reductase from promastigotes of *L. donovani* and human erythrocytes were performed according to the methods previously described [14, 15]. HPLC analysis of cellular nucleotides and analog metabolites was performed as previously described [6, 8, 17].

RESULTS

Amination reactions. Both HPPR-MP and Form B-MP could serve as substrates of succino-AMP synthetase partially purified from *L. donovani* [6, 9]. The V'_{max} values with these analogs were about 1% of the V'_{max} of IMP. In contrast to its low efficiency with the protozoal enzyme, Form B-MP was a highly efficient substrate of partially purified mammalian succino-AMP synthetase with a V'_{max} approaching 40% of the V'_{max} of IMP (Fig. 2). HPPR-MP had no detectable substrate activity with the mammalian enzyme [13]. The kinetic constants are summarized for comparative purposes in Table 1.

The finding that Form B-MP was an efficient substrate of mammalian succino-AMP synthetase suggested that Form A-MP could be generated in tissues which had the capacity to convert Form B to Form

* GMP reductase (reduced NADP:GMP oxidoreductase (deaminating), EC 1.6.6.8) catalyzes the following reaction: GMP + NADPH + H^+ $\rightleftharpoons$ IMP + $NADP^+$ + NH_3.

Table 1. Substrates of succino-AMP synthetase*

	IMP		HPPR-MP		Form B-MP	
Enzyme source	K'_m (μM)	V'_{max} (relative)	K'_m (μM)	V'_{max} (relative)	K'_m (μM)	V'_{max} (relative)
Leishmania donovani	12	100	340	*ca.* 1	26	*ca.* 1
Rabbit muscle	110	100	1200†	<0.05	145	37

* Data from Refs. 6, 9 and 11 and this study.
† K_i value.

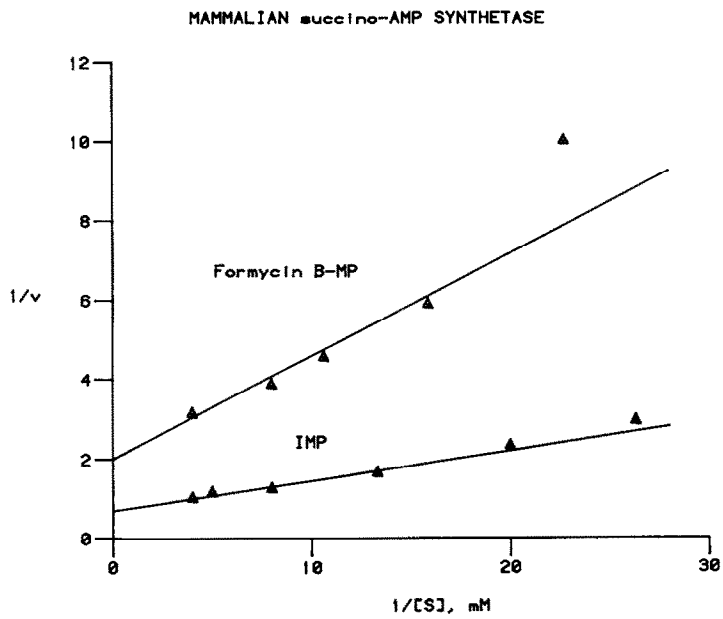

Fig. 2. Substrate activity of IMP and Form B-MP with mammalian succino-AMP synthetase. The results of the spectrophotometric assay are presented here. These data were confirmed with the radiochemical assay. Kinetic constants are presented in Table 1. HPPR-MP was not a substrate of this enzyme.

Table 2. Metabolism of formycin B and allopurinol riboside by mouse L cells*

	Pool size (pmoles 10^6 cells)				
	Addition				
Dose	None	[^{3}H]Form B		[^{3}H]HPPR	
μg/ml (μCi/mmole)		1 (940)	50 (4.5)	1 (270)	50 (4.5)
Nucleotide					
CTP	940	1000	790	1100	900
UTP	2400	3000	2000	3000	2300
ATP	5700	9500	6700	6500	7300
GTP	1300	1500	1400	1200	1600
Metabolite					
Form B-MP		0.05	2.0		
Form A-MP			1.5		
Form A-DP		0.14	5.1		
Form A-TP		0.52	19.4		
Form A in RNA†		ND‡	6.5		
HPPR-MP				0.04	5.5
APPR-MP				<0.007	<0.13
APPR-DP				<0.007	<0.13
APPR-TP				<0.007	<0.13
APPR in RNA†				ND	<0.37
RNA (μg/10^6 cells)	3.1	3.5	3.6	3.6	3.9
Cell density (10^6/ml)	1.24	0.55	1.21	0.55	1.21

* L cells were grown to log phase in Eagle's minimum essential medium supplemented with Earl's salts and 10% horse serum. Incubations were then carried out for 24 hr with the indicated addition. The cellular contents were analyzed as previously described [8].

† Positive identification of the nucleoside analog in hydrolyzed RNA was performed as previously described [6].

‡ Not determined.

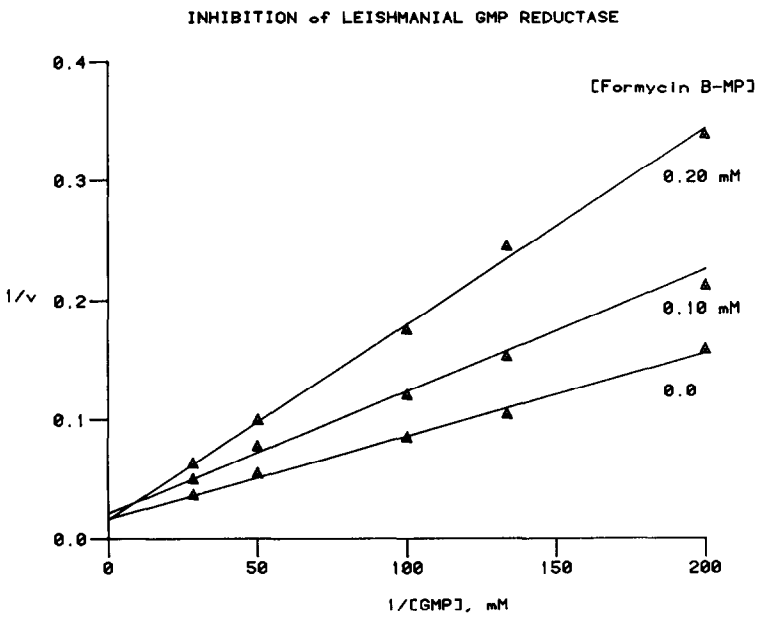

Fig. 3. Competitive inhibition of GMP reductase from *L. donovani* by Form B-MP. The K_i value is presented in Table 3.

B-MP. To test this possibility, mouse L cells were incubated with [^{3}H]Form B, and the production of metabolites was investigated. The results are summarized in Table 2. These cells readily converted Form B to Form B-MP and Form A-MP. The latter was further converted to its diphosphate and triphosphate and was incorporated into cellular RNA. Radiolabel was also incorporated into cellular DNA. However, because the DNA was not hydrolyzed and analyzed as was the RNA, it is not certain that this radioactivity was [^{3}H]Form A. This metabolic pattern is qualitatively similar to that previously observed for Form B in *Leishmania* spp. [6, 7]. In comparable studies with [^{3}H]HPPR in mouse L cells, only trace amounts of HPPR-MP and no analogs of adenine nucleotides were observed. In general, neither analog produced significant changes in the nucleotide pool sizes (Table 2).

Inhibition of GMP reductase. An investigation of the interaction of Form B-MP with GMP reductase that had been purified from promastigotes of *L. donovani* revealed that it was a competitive (vs GMP) inhibitor with a K_i of 140 μM (Fig. 3). Its affinity was somewhat weaker than that of HPPR-MP with this enzyme [14] but considerably stronger than that of either Form B-MP or HPPR-MP with GMP reductase purified from human erythrocytes. The K_i values with these GMP reductases are reviewed in Table 3.

Metabolic effects. It was of interest to determine whether these enzyme data correlated with intracellular effects. To metabolically produce HPPR-MP or Form B-MP, cultured promastigotes of *L. donovani* were preincubated with either 40 μM HPP or Form B for 24 hr. This was followed by a 2-hr pulse exposure of 2 μM [^{14}C]xanthine. It should be noted that since *L. donovani* rapidly convert guanine to xanthine [17], the metabolism was simplified by directly using labeled xanthine. The incorporation of the radiolabel into the purine ribonucleotide pools

Table 3. Inhibitors of GMP reductase*

Inhibitor	Competitive K_i† (μM)		K_i ratio (human/*L. donovani*)
	Enzyme source		
	Leishmania donovani	Human erythrocytes	
IMP	14	150	10.7
HPPR-MP	37	3600	97
Formycin B-MP	140	5700	41

* The constants obtained with IMP and HPPR-MP have been reported previously [14, 15] and are presented here for comparative purposes.

† Competitive inhibition vs GMP. The K'_m for GMP is 20–30 μM with GMP reductase from *L. donovani* and 7.5 μM with GMP reductase from human erythrocytes [Refs. 14 and 15 and this study].

Table 4. Effects of allopurinol and formycin B on [^{14}C]xanthine metabolism in *L. donovani* promastigotes*

	Pool size (pmoles/10^6 cells)		
	None	Addition HPP (40 μM)	Form B (40 μM)
Nucleotide			
NAD and AMP	4.2 (0.21)†	8.4 (0.01)	
NADP	2.6 (0.0)	6.1 (0.0)	2.1 (0.002)
IMP	<0.5	<0.5	<0.5
XMP	<0.5	<0.5	<0.5
ADP	18 (0.16)	16 (0.006)	13 (0.003)
ATP	82 (0.16)	66 (0.003)	75 (0.004)
GMP	<0.5	<0.5	<0.5
GDP	12 (0.66)	12 (0.17)	11 (0.04)
GTP	28 (1.20)	21 (0.32)	30 (0.06)
CTP	1.2	1.4	1.6
UTP	32	30	32
Form B-MP			4.5
Form A-TP			3.6
HPPR-MP		63	
APPR-TP		1.2	

* Approximately 1.5×10^8 promastigotes/ml were incubated for 24 hr at 37° with HPP or Form B. Incubations were then continued with 2 μM [^{14}C]xanthine (54 μCi/μmole) for 2 hr.

† Numbers in parentheses are the percent of the pool derived from [^{14}C] xanthine.

was then measured. The data presented in Table 4 show that these agents produced a drastic reduction of the incorporation of the ^{14}C-label into adenine and guanine ribonucleotide pools. However, there was very little change in the pool sizes of the nucleotides. The amount of analog nucleotides formed is also shown in Table 4.

DISCUSSION

The pyrazolo[3,4-*d*]pyrimidine, HPPR-MP, and the pyrazolo[4,3-*d*]pyrimidine, Form B-MP, isomers of IMP had qualitatively similar metabolism and metabolic effects in the protozoal cells, but very dissimilar metabolism in the mammalian cells. In *Leishmania* spp., both of these IMP analogs were converted to analogs of adenine nucleotides and were incorporated into leishmanial RNA [3, 6–8]. The rate-limiting step for the amination appears to be the condensation reaction that is catalyzed by succino-AMP synthetase [9]. Although, with leishmanial succino-AMP synthetase, Form B-MP and HPPR-MP had similar V'_{max} values (about 1% the V'_{max} of IMP), the K'_m for Form B-MP was 1/12 the K'_m for HPPR-MP. Superficially, this would appear to account for the findings that the leishmanial cells produce Form A nucleotides at concentrations that are 3- to 20-fold higher than the concentrations of APP nucleotides [3, 6–8]. However, the K'_m advantage of Form B-MP is offset in intact cells because the concentration of HPPR-MP is >10-fold higher than that of Form B-MP in *Leishmania* spp. [Table 4 and Refs. 3 and 6–8].

It is possible that the larger accumulation of Form A nucleotides may have been the result of less deamination of Form A-MP by leishmanial AMP deaminase. Although the existence of this enzyme in *Leishmania* spp. has not been confirmed, there are metabolic data that suggest this may be the route for deaminating these AMP analogs. Namely, Form B-MP was detected in promastigotes that were incubated with Form A and 1 μM deoxycoformycin [6]. Since the latter completely inhibited contaminating adenosine deaminase, it was deduced that the Form B-MP could be produced by the deamination of Form A-MP. When APP was incubated with promastigotes in the presence of deoxycoformycin, HPPR-MP was produced.* However, the amount of HPPR-MP produced from APPR was at least 10-fold the amount of Form B-MP produced from Form A. This indicates that APPR-MP may be a more efficient substrate for the AMP deaminase.

The metabolic data of Table 4 demonstrate that both analogs severely reduced the ability of *L. donovani* promastigotes to incorporate the radiolabel from [^{14}C]xanthine into adenine and guanine nucleotides. Because these measurements are not measurements of flux and because the metabolic pathways for GMP and IMP are multi-branched, it is not possible to discern the particular enzyme(s) that is inhibited. However, the data showing decreased incorporation into adenine nucleotides are consistent with the possibility that Form B-MP and HPPR-MP are inhibiting succino-AMP synthetase and GMP reductase. The approximate intracellular concentrations of Form B-MP and HPPR-MP can be calculated† to be 80 and 1000 μM, respectively, and are well above the K'_m values for these analogs with

* Unpublished data of D. J. Nelson, S. W. LaFon, R. L. Berens and J. J. Marr.

† The calculation assumes that there are 10^{10} promastigotes/ml of packed cells [3] and that the contents of the cells are about 60% aqueous phase.

succino-AMP synthetase (Table 1). Therefore, in view of the minuscule pool of IMP (<10 μM), either analog should be an effective alternate-substrate inhibitor of this enzyme. Because the intracellular concentration of GMP is also <10 μM (nondetectable), the same argument applies for the inhibition of GMP reductase.

Although the pathways from xanthine to ATP and GTP were dramatically inhibited, the analogs had little or no effect on the actual pool sizes of the normal nucleotides. This finding has been observed previously in *Leishmania* spp. treated with HPP or HPPR and is addressed in a recent review [18]. The authors advanced a hypothesis (based on unpublished data) that these analogs induce increased breakdown of leishmanial RNA, which thereby maintains the nucleotide pools. RNA breakdown may be related to the antileishmanial activity of these analogs. In this context, it is not certain that amination and incorporation of these IMP analogs are crucial. It is relevant that the 4-thiol congener of HPP, 4-thiopyrazolo[3,4-*d*]pyrimidine, is converted to a thioIMP analog in *L. donovani* [19]. This analog is a strong inhibitor of succino-AMP synthetase [19] and GMP reductase [14], but it is neither converted to an analog of AMP nor is it incorporated into RNA [19]. The identification of the crucial locus or loci of action could be aided by the generation of strains of the parasite that are resistant to these growth inhibitors.

The fact that analogs of adenine nucleotides were produced when the mammalian cells were incubated with Form B clearly reflects the highly efficient substrate activity of Form B-MP with mammalian succino-AMP synthetase. As previously pointed out, the mammalian and leishmanial succino-AMP synthetases have subtle, rather than broad, differences in their catalytic abilities [9]. Although the cleavage of succino-Form A-MP by succino-AMP lyase was not studied, it was shown previously that leishmanial, trypanasomal and mammalian succino-AMP lyase have broad substrate specificities and efficiently catalyze the cleavage of many succino-AMP analogs [9, 16, 20]. It is therefore assumed that this enzyme is not rate limiting in the formation of Form A-MP.

A recent study has shown that 5-iodotubercidin, a specific inhibitor of adenosine kinase, blocks the phosphorylation of Form B in mouse L cells [21]. However, another study [22] has found that a Chinese hamster ovary cell line which lacks adenosine kinase is almost as sensitive as the wild-type line to Form B. Therefore, it is uncertain which mammalian enzyme is responsible for converting Form B to Form B-MP and thereby providing the substrate for succino-AMP synthetase.

The amount of Form A-TP produced per 10^6 mouse L cells from incubations with Form B was similar to the amount produced per 10^6 leishmanial cells [this report and Refs. 6 and 7]. However, because mouse L cells are about 100-fold larger in volume than the protozoal cells, a correction is required to make valid comparisons. If the concentration of Form A-TP relative to that of the ATP is considered, it is apparent that the concentration of Form A-TP is much higher in the parasites (2–30% of the [ATP]) than in the L cells (0.005–0.2% of the [ATP]). The relative toxicity of Form B to *Leishmania* spp. [4, 6, 7] and to L cells [21] correlates with this ratio.

Before Form B can be considered for the possible treatment of protozoal infections in humans, the question of its selective toxicity must be addressed. Form B is toxic to mouse L cells (ED_{50} = 200 μM) [21] and to white blood cells of dogs treated with 50 mg/kg [23] and is lethal to mice injected with 400 mg/kg daily for 10 days [23]. Moreover, it will be essential to examine the long-term toxicity of Form B since it did give rise to metabolites that were incorporated into mammalian RNA and possibly DNA. A recent study has shown that the incorporation of [^{3}H]Form A into the DNA of cultured human colon carcinoma cells correlates with lethality [24].

HPPR caused no toxicity to either mouse L cells at concentrations up to 2 mM [21] or to dogs* dosed with 300 mg/kg per day for 90 days. Since HPPR-MP is not a substrate for mammalian succino-AMP synthetase [13] nor mammalian IMP dehydrogenase [25], it is not metabolized to analogs of AMP or GMP [Refs. 10 and 12 and this study]. Therefore, HPPR may have a selectivity advantage.

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* Unpublished data of K. Ayers of Wellcome Laboratories.

Note added in proof—Recently, J. D. Berman, P. Rainey and D. V. Santi have reported [*J. exp. Med.* **128**, 252 (1983)] that the metabolism of Form B in both uninfected human macrophages and macrophages infected with *Leishmania* amastigotes is similar to the metabolism reported here.

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