Biochemical Pharmacology, Vol. 29, pp. 2397–2398.

0006-2952/80/0901-2397 $02.00/0

SHORT COMMUNICATION

Antileishmanial effect of allopurinol and allopurinol ribonucleoside on intracellular forms of *Leishmania donovani**

(*Received* 2 *February* 1980; *accepted* 10 *March* 1980)

Previous investigations from this laboratory have shown that allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine, HPP) is biologically active against the cultured forms of both the pathogenic leishmania and *Trypanosoma cruzi* [1, 2]. Biochemical investigation of the metabolism of HPP by these organisms [2, 3] has shown that this compound is metabolized by a sequential conversion to allopurinol mononucleotide and 4-aminopyrazolo[3,4-*d*]pyrimidine (APP) mono-, di- and trinucleotides (Fig. 1). The latter are incorporated into RNA. Further investigations have shown that allopurinol ribonucleoside (HPPR) is metabolized in a similar sequence of reactions in the leishmania [4]. Since the intracellular, or amastigote, state is the pathogenic form in man, it is imperative to demonstrate that HPP and its ribonucleoside have similar biological effects and undergo the same biochemical transformation in the intracellular form as has been demonstrated for the extracellular promastigote form. Similarities in HPP metabolism between amastigotes and promastigotes were suggested from the results of transformation experiments [4] in which it was found that both HPP and HPPR significantly reduce the percentage of an amastigote population capable of transforming to the promastigote.

The chemotherapeutic effectiveness of HPP and HPPR was tested using an *in vitro* model system in which the murine macrophage P388D$_1$ cell line serves as the host cell [5]. Intracellular forms of *Leishmania donovani* (strain S1) were isolated from infected hamster spleens as described previously [6] and used to infect cultures of P388D$_1$ cells growing in Leighton tubes. Both HPP and HPPR (Fig. 2) were effective in reducing infection in the cell population over a period of 3–10 days. The percentage of infected cells in the untreated control cultures increased, as did the average number of amastigotes per infected cell. Drug-treated and non-treated infected macrophages showed identical growth kinetics.

Experiments designed to compare HPP metabolism in the promastigote and the intracellular forms included : (1) exposure of free amastigotes to [6-^{14}C]HPP; (2) incubation of minced, infected hamster spleen preparations with [6-^{14}C]HPP; and (3) incubation of infected P388D$_1$ cultures with [6-^{14}C]HPP. The amastigotes of *L. donovani* produced HPPR monophosphate as well as three ribonucleotides of APP (Table 1). While the concentrations were low compared to the promastigotes [3], conversion of HPP to its ribonucleotide and subsequent conversion to APP ribonucleotides did occur. Under these conditions there was no morphological transformation of amastigotes to promastigotes [6]; however, since these organisms were not in their usual intracellular environment, these results might not reflect normal metabolism of an amastigote. To eliminate this possibility the following experiments were done.

A minced, infected, hamster spleen that was incubated for 24 hr in medium containing [6-^{14}C]HPP also formed radiolabeled HPPR monophosphate and APP ribonucleotides (Table 1). Under identical conditions uninfected spleen yielded radiolabeled HPPR monophosphate at one-tenth the concentration found in the infected spleen. Uninfected spleen did not form APP ribonucleotides.

Incubation of an infected P388D$_1$ cell culture for 24 hr in medium containing [6-^{14}C]HPP resulted in a labeling pattern similar to that for minced spleen in that HPPR monophosphate and APP ribonucleoside mono-, di- and triphosphates were formed (Table 1). Uninfected macrophages produced a small amount of HPPR monophosphate but no APP ribonucleotides. The appearance of HPPR monophosphate in P388D$_1$ cells is not unexpected and is in agreement with earlier investigations which showed that mammalian cells were capable of forming small amounts of this nucleotide but were unable to form APP ribonucleotides from HPPR monophosphate [7]. None of the APP ribonucleotide was excreted into the incubation medium in any of the above experiments, indicating that the inability to detect these compounds in the uninfected spleen and tissue culture was not due to rapid elimination of these nucleotides from the host cells.

In these experiments (Table 1), HPP was converted to HPPR, which was found in the incubation medium. Since the latter is active against leishmania promastigotes [4] and amastigotes (Fig. 2), its metabolism in infected macrophages was investigated. Using HPPR (U[^{14}C]ribose) at 2.5 μg/ml, we demonstrated that, in 24 hr, HPPR mono-

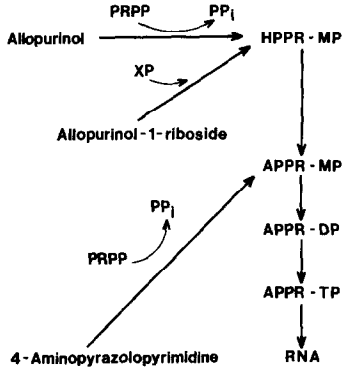

Fig. 1. Metabolic transformations of allopurinol and allopurinol ribonucleoside in *Leishmania* and in *Trypanosoma cruzi*. The major metabolic pathway of allopurinol in these protozoans is to allopurinol ribonucleotide (HPPR-MP), mediated through a phosphoribosyltransferase. Allopurinol ribonucleoside is converted, through a single-step phosphorylation, to HPPR-MP; this is mediated by a nucleoside phosphotransferase. The HPPR-MP is converted subsequently by the adenylosuccinate synthetase to APP ribonucleotide (APPR-MP). This compound is converted subsequently to the diphosphate (DP) and triphosphate (TP) and incorporated into RNA. Aminopyrazolopyrimidine (APP) can be converted directly to APPR-MP by a phosphoribosyltransferase, probably the adenine phosphoribosyltransferase. Abbreviations: PP$_i$, inorganic pyrophosphate; PRPP, phosphoribosylpyrophosphate; and XP, unknown phosphate donor.

* This study was supported by the World Health Organization [(TRY)T16/181/L3/29], the National Institutes of Health (AI15663) and a grant from the Burroughs Wellcome Co.

Table 1. Metabolism of allopurinol by amastigotes of *L. donovani**

Allopurinol metabolites	Amastigote suspension (pmoles/10^6 cells)	Intracellular amastigotes: Hamster spleen Infected (pmoles/mg)	Hamster spleen Noninfected (pmoles/mg)	$P388D_1$ macrophages Infected (pmoles/10^6 cells)	$P388D_1$ macrophages Noninfected (pmoles/10^6 cells)
HPP	1.7	210	334	100	100
HRRP-MP	3.8	6.2	0.7	2.1	1.3
APPR-MP	0.4	ND	ND	0.4	ND
APPR-DP	0.2	1	ND	0.08	ND
APPR-TP	0.1	1.8	ND	0.11	ND

* The amastigote suspension (1.7×10^8 organisms/ml) was exposed to [6-^{14}C]HPP (37 μM; 1.1 μCi/μmole) for 12 hr. The amastigotes were prepared from infected hamster spleen as described previously [6]. A minced, infected, hamster spleen preparation was exposed to [6-^{14}C]HPP (37 μM; 1.1 μCi/μmole) for 6 hr. The weight of the infected hamster spleen was 804 mg. The spleens from three noninfected hamsters (total weight 354 mg) were combined, minced, and exposed to [6-^{14}C]HPP. A culture of $P388D_1$ macrophages (1×10^6 cells/cm^2) was infected as described in Fig. 2 and exposed for 24 hr to [6-^{14}C]HPP (16 μM; 52.4 μCi/μmole). Perchloric acid extracts of cells were analyzed by high pressure liquid chromatography on Partisil 10/SAX (Whatman) with a 0.015–1.0 M gradient of KH_2PO_4, pH 3.5. The metabolic products were identified as described previously [8]. Nondetectable levels (ND) are <0.1 pmoles/mg for hamster spleen and > 0.001 pmoles/10^6 for the macrophages.

phosphate and APP ribonucleoside triphosphate were formed at 0.4 and 0.02 pmoles/10^6 cells, respectively, in the infected $P388D_1$ macrophages.

Our previous investigations demonstrated that allopurinol and allopurinol ribonucleoside are antileishmanial and undergo a series of metabolic conversions unique to these pathogens [1, 3, 4]. Irrespective of the experimental conditions under which the amastigotes are placed—in suspension, within the phagocytic cells of the hamster spleen, or within a murine macrophage tissue culture—the metabolism of HPP and HPPR is the same as in the extracellular forms [3, 4].

Both compounds are effective in eliminating the intracellular parasites from these macrophages. This investigation supports the hypothesis that pyrazolopyrimidine metabolism is identical in the extracellular and intracellular forms of *L. donovani* and suggests that data obtained with extracellular forms will be useful in predicting the chemotherapeutic response of the amastigotes.

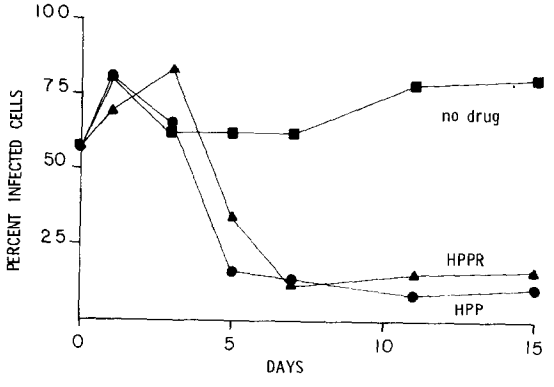

Fig. 2. Inhibition of intracellular growth of *L. donovani* by HPP and HPPR. Intracellular forms of *L. donovani* (strain S1) were isolated from infected hamster spleens. The $P388D_1$ murine macrophages at mid-log growth, in Leighton tubes, were infected for 2 hr with the amastigotes obtained from the hamster spleens at an infection ratio of 10 parasites per host cell. At the end of this exposure, the cultures were washed to remove the non-phagocytized parasites, and cover slips were removed as a zero time control. Fresh media containing 37 μM of either HPP or HPPR (10 μg/ml and 20 μg/ml, respectively) were added to the remaining tubes and changed every 24 hr. Non-treated, infected cells and uninfected, treated cells were run in parallel as controls. Cover slips were removed every 24 hr from both experimental and control cultures, fixed in methanol, stained with Giemsa stain, and a minimum of 200 cells was counted to determine the number of amastigotes per cell and the percentage of infected cells.

Acknowledgements—We thank Ms. M. Bhattacharyya and Ms. Rita Hendricksen for technical assistance and Mrs. Kathleen Lee for preparation of the manuscript. We thank Dr. Gertrude B. Elion for critical evaluation of the manuscript.

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