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Effect of Allopurinol on *Trypanosoma cruzi*: Metabolism and Biological Activity in Intracellular and Bloodstream Forms

RANDOLPH L. BERENS,[1]†* J. JOSEPH MARR,[1]† FERNANDO STEELE DA CRUZ,[2] AND DONALD J. NELSON[3]

Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63104[1]; Instituto de Microbiologia URFJ, 21910 Rio de Janeiro, Brazil[2]; and Burroughs Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709[3]

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Allopurinol (4-hydroxypyrazolo [3,4-*d*]pyrimidine) is an effective agent in vitro against *Trypanosoma cruzi*. The important forms of this parasite, with respect to the pathogenesis of Chagas' disease in man, are the bloodstream (trypomastigote) and the intracellular forms. Experiments with radiolabeled allopurinol and analysis of the metabolic products of this compound by high-performance liquid chromatography showed that both the bloodstream and the intracellular forms of *T. cruzi* metabolize allopurinol in the same manner as has been shown for the epimastigotes in vitro. The metabolic pathways for pyrazolopyrimidines in the pathogenic forms were demonstrated with organisms isolated from infected animals and a tissue culture system infected with *T. cruzi*. Treatment of infected tissue culture with allopurinol eradicated the infection. This investigation implies that allopurinol may be useful in chemotherapy of *T. cruzi* infections, a supposition which has been borne out in one animal study.

Previous investigations from this laboratory have shown that allopurinol (4-hydroxypyrazolo [3,4-*d*]pyrimidine) (HPP) is biologically active against the culture forms of *Trypanosoma cruzi* (8). A biochemical investigation of the metabolism of HPP by this organism has shown that the compound is metabolized by sequential conversion to HPP ribonucleoside monophosphate (HPPR-MP) and 4-aminopyrazolo [3,4-*d*]pyrimidine (aminopurinol) (APP) ribonucleoside mono-, di-, and triphosphate (APPR-MP, APPR-DP, and APPR-TP) (Fig. 1). The latter are incorporated into RNA. This sequence is identical to that in various pathogenic leishmania, both the extracellular and intracellular forms (5, 11), and in the culture (3) and bloodstream forms of the African trypanosomes (W. R. Fish, D. L. Looker, J. J. Marr, and R. L. Berens, manuscript in preparation). Other investigators subsequently reported that HPP undergoes the same metabolic conversions in several *T. cruzi* strains and that this drug, when given intraperitoneally, results in clinical cures of *T. cruzi*-infected mice (1, 2). In the present study we demonstrated that the metabolism of HPP in both the intracellular and bloodstream forms of *T. cruzi* is identical to that previously reported for the culture forms and that this drug will eradicate *T. cruzi* infections from cultured cells.

† Present address: Division of Infectious Diseases, University of Colorado Medical Center, Denver, CO 80262.

MATERIALS AND METHODS

Culture techniques. *T. cruzi* (Peru strain; obtained from S. M. Krassner, University of California, Irvine) was grown in a tissue culture system as described by Sanderson et al. (13) except that the primary human diploid cell line (WI-38, ATCC CCL 75) was replaced with a Simian virus 40-transformed derivative (WI-38, strain VA-13 subline 2RA, ATCC CCL 75.1). These cultures will be referred to as VA-13 cultures.

For drug treatment studies a confluent, infected (~1% infection) culture in a 75-cm^2 plastic flask was split by trypsin digestion into six cultures which were placed in replicate 25-cm^2 flasks. Cells were cultured for 24 h at 37°C in Eagle minimal essential medium (MEM) containing 10% heat-inactivated fetal calf serum (FCS). The percent infection was then determined for each flask by counting the number of infected and noninfected cells found in 20 fields (at 200× magnification with an Olympus IMT inverted phase-contrast microscope). A confluent culture normally had an average of 127 ± 15 cells per field. After the percent infection was determined, the six culture flasks were divided randomly into two sets of three. The medium of one set (drug set) was replaced with MEM containing 2% FCS plus 187 μM HPP (25 μg/ml). The medium of the other set (control set) was replaced with the same medium without HPP. Both sets of flasks were then placed at 31°C and incubated for 1 week. During this period, the medium was replaced every 48 h. At the end of this period, the average percent infection was determined for the treated and control sets. One-half of the cells from each control flask, after trypsinization, were then transferred to a new flask containing MEM plus 10% FCS and incubated at 37°C. The

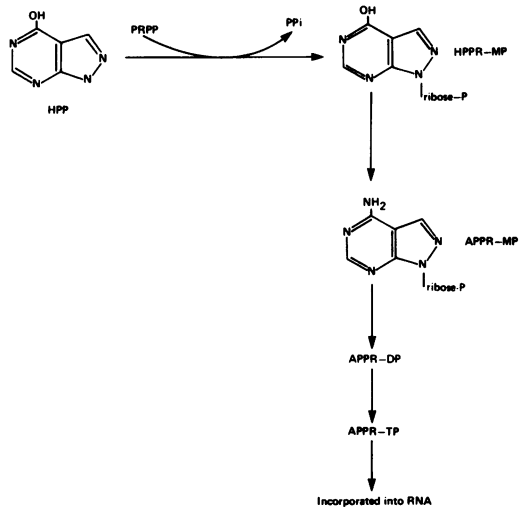

FIG. 1. Metabolism of HPP by *T. cruzi*. PRPP, phosphoribosylpyrophosphate.

remaining cells were discarded. After 48 h at 37°C, the medium was replaced with MEM plus 2% FCS, and the cultures were placed at 31°C for 5 days; the medium was changed on days 2 and 4. This subculture procedure was repeated for the control set until the study was terminated. The drug-treated cultures were manipulated identically except that at each subculture the contents of the drug flask were split into two flasks. One continued with medium containing HPP; the other was cultured in drug-free medium. If, after 7 days, the cultures transferred from HPP-containing medium to drug-free medium showed no sign of infection, they were subcultured as described above for the controls. If any one of the flasks of this set contained infected cells, the average percent infection was determined for the set and they were discarded. Cultures were considered infection free if they could be subcultured three times without detection of parasites in the absence of the drug. These procedures are summarized in Fig. 2. As a further test the culture cells then were trypsinized, collected by centrifugation (3,000 × *g* for 20 min), and suspended in THOSMEM, a semidefined medium used previously (4). Our studies have shown that these conditions result in multiplication and high recovery of *T. cruzi* from infected cells.

HPP metabolism in infected tissue cultures. Incorporation of radiolabeled HPP by infected tissue culture cells was done as follows. A plastic tissue flask (150 cm^2) of VA-13 cells, about 40% confluent, was infected by adding trypsinized cells from a culture in which approximately 50% of the cells contained *T. cruzi*. The

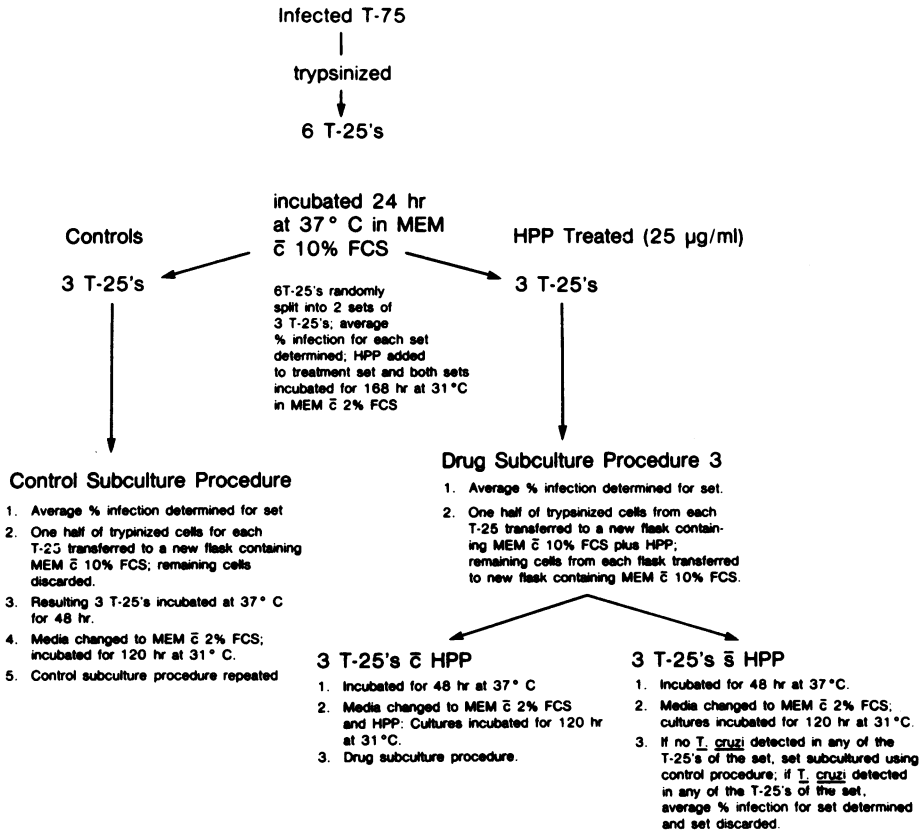

FIG. 2. Flow diagram for drug treatment of infected tissue culture. T-75, 75-cm^2 plastic flask; T-25, 25-cm^2 plastic flask.

TABLE 1. Conditions used for determination of metabolism of HPP by *T. cruzi*

T. cruzi form	Cell density ($\times 10^6$)	[6-^{14}C]HPP (μg/ml)	Sp act (μCi/mmol)	Incubation (h)
Epimastigote	308	5	1.1	6
Trypomastigote				
Infected blood	50 + 12.5% erythrocytes	4.33	52.4	6
Uninfected blood	4.8 (erythrocytes)	23.8	6.4	6
Amastigote				
Infected spleen	35 (mg/ml)	4.33	52.4	6
Uninfected spleen	7.8 (mg/ml)	23.8	6.4	6
Tissue culture				
Infected VA-13	30	1.4 (10.5 μM)	47.5	24
Uninfected VA-13	30	1.4 (10.5 μM)	47.5	24

majority of the parasites were intracellular trypanosomes. The resulting mixed culture was incubated for 24 h at 37°C in MEM containing 10% FCS, and then the monolayer was washed once with Hanks balanced salt solution. The medium was replaced with MEM containing 2% FCS, and the cultures were incubated at 31°C for 48 h. This resulted in a culture in which approximately 23% of the cells were infected with *T. cruzi* (primarily amastigotes). The infected monolayer then was washed twice with Hanks balanced salt solution and MEM containing 2% FCS, and 10.5 μM (1.4 μg/ml) [^{14}C]HPP (specific activity, 47.5 μCi/μmol) was added (Table 1). After the culture was incubated for 24 h at 31°C, it was extracted with perchloric acid and processed for high-pressure liquid chromatography (HPLC) analysis as previously described (11). A control culture was manipulated identically except that trypsinized cells from an uninfected 25-cm^2 culture flask were added to the control 150-cm^2 flask.

HPP metabolism by bloodstream forms. Bloodstream trypanosomes (Peru strain) were grown in and isolated from chinchillas by the procedures described by Gutteridge et al. (6). The trypanosomes, contaminated with approximately 10% erythrocytes, were suspended in a purine-free medium (4) containing 32 μM (4.3 μg/ml) [^{14}C]HPP (specific activity, 52.4 μCi/μmol) and incubated for 6 hours at 31°C (Table 1). At the end of the incubation, the trypanosomes were processed for HPLC analysis. As a control a comparable number of chinchilla erythrocytes, obtained from an uninfected animal, were incubated and processed in the same manner. For this control as well as the control for the amastigotes described below, the concentration of the HPP was increased approximately fivefold to 23.8 μg/ml so that any metabolites that might be formed could be more easily detected.

HPP metabolism by amastigotes. Previous workers (7) have shown that spleens of *T. cruzi*-infected mice are a good source of amastigotes. On the basis of this finding, we investigated spleens from *T. cruzi*-infected chinchillas; they also contained large numbers of amastigotes. Chinchilla hind-limb muscle tissue also is an excellent source of amastigotes, but the spleen cells are easier to adapt to in situ amastigote metabolic studies. For HPP metabolic studies, spleens were removed aseptically from infected chinchillas and teased apart. The resulting dispersed cells were collected by centrifugation (800 × *g* for 10 min) and suspended in MEM containing 8% FCS and 32 μM (4.3 μg/ml) [^{14}C]HPP (specific activity, 52.4 μCi/μmol) (Table 1). After incubation at 37°C for 6 h, this suspension was collected and washed thrice with cold Hanks balanced salt solution by centrifugation, and the final pellet was extracted with cold 0.8 N perchloric acid for HPLC analysis. As a control, spleen from an uninfected chinchilla was subjected to the same procedure.

HPP metabolism by epimastigotes. Epimastigotes were grown in THOSMEM, exposed to HPP (5 μg/ml) (Table 1), and processed for HPLC analysis as previously described (4, 8).

HPLC analysis. Cells were processed and their extracts were analyzed for pyrazolopyrimidine metabolites as previously described (11).

Materials. Plastic cultureware was obtained from Corning Glass Works, Corning, N.Y.; [6-^{14}C]HPP was obtained from RPI, Elk Grove, Ill.; and culture media supplies were obtained from KC Biological, Lenexa, Kans. Biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. All chemicals were of analytical grade or better.

RESULTS

Figure 3 shows that continuous exposure of *T. cruzi*-infected VA-13 cells to HPP eradicated the infection. The need for at least three subcultures from the original infection in the presence of the drug is in agreement with previous findings that HPP exerts only an inhibitory effect on hemoflagellates (10). Addition of the drug to uninfected control cultures had no effect on their growth.

Experiments with [^{14}C]HPP showed that both infected and uninfected VA-13 cultures were capable of converting HPP to HPPR-MP (Table 2). However, only the infected VA-13 culture was capable of further converting the HPPR-MP to APPR-MP, APPR-DP, and APPR-TP). Identical results were found for infected and uninfected murine L-929 cells (unpublished data).

Additional metabolic experiments were performed on both bloodstream trypanosomes and

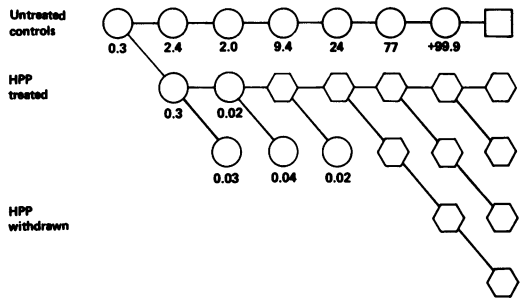

FIG. 3. Effect of HPP on *T. cruzi*-infected VA-13 cells. The numbers represent the average percent infection for three replicate cultures and were determined once a week before subculturing. Symbols: ○, VA-13 cells infected with *T. cruzi*; □, VA-13 culture lost due to *T. cruzi* infection; ○, no *T. cruzi*-infected VA-13 cells found in a minimum of 6,000 cells examined. The latter were considered to be cured after four passages in HPP since subsequent subculturing in the absence of HPP yielded no *T. cruzi*. For details of the culture method, see Fig. 2.

amastigotes in situ in spleen cells. Both forms were isolated from an infected chinchilla. Both the infected and uninfected control chinchilla blood preparations were able to convert HPP to HPPR-MP (Table 2). The appearance of HPPR-MP in tissue and spleen cultures is not unexpected. An earlier investigation showed that mammalian cells are capable of forming small amounts of this ribonucleotide (9). The bloodstream trypanosome preparation formed significant amounts of APPR-MP, APPR-DP, and APPR-TP; the blood from an uninfected chinchilla contained no APP ribonucleotides. Although not shown in the table, both preparations contained HPPR and the oxidation products of HPP and HPPR, i.e., oxipurinol and oxipurinol-1-ribonucleoside. These are the normal HPP metabolites in mammalian cells (12). The percentage of conversion of HPP to these other metabolites was the same irrespective of whether the blood was infected or not. As with the bloodstream preparations, both uninfected and infected chinchilla spleen cells were capable of converting HPP to HPPR-MP (Table 2). However, only the spleen suspension infected with the amastigotes of *T. cruzi* was capable of further converting HPPR-MP to the ribonucleotides of APP. The other metabolic products of HPP described above were present in both the infected and uninfected preparations, indicating that in both preparations the isolated spleen cells were living and capable of metabolizing HPP. No trypomastigotes were observed in the incubation medium during the time of the experiment.

DISCUSSION

This investigation demonstrated that HPP, which is known to undergo a series of metabolic conversions in the epimastigotes of *T. cruzi*, is converted to the same metabolic products by the bloodstream and intracellular forms of these parasites. The intracellular forms are capable of carrying out this metabolic conversion whether they are located within the cells of the spleen, a situation more akin to the clinical disease, or within a continuous cell culture system. This demonstration of the identity of the metabolic pathways in all of the three major forms of this parasite has both biochemical and clinical implications. Of more clinical importance is the ability of HPP to eradicate a *T. cruzi* infection from a cell culture system in which the host cells are of human origin. This therapeutic effectiveness of HPP cannot be a result of dilution and loss of infected cells through subculture, since both treated and untreated cultures were subcultured in the same manner. It cannot be due to an inhibitory effect of HPP with subsequent loss of infected cells by dilution, since only three subcultures were required to effect a cure and both subcultures were saved and observed for the duration of the experiment. This therapeutic activity supports the observations of Avila and Avila (1) who have shown that mice infected with *T. cruzi* and treated by the intraperitoneal administration of HPP undergo a clinical cure of the acute infection. Untreated mice in those experiments died within 14 days, whereas ani-

TABLE 2. Metabolism of HPP by extracellular and intracellular forms of *T. cruzi*

Cellular nucleotide	Amt[a] produced by conversion of HPP by:						
	Epimastigotes	Trypomastigotes		Amastigotes		Tissue culture	
		Infected blood	Uninfected blood	Infected spleen	Uninfected spleen	Infected VA-13	Uninfected VA-13
HPPR-MP	14.7	4.8	16.8	2.3	5.5	174.40	18.58
APPR-MP	0.6	0.1		0.4		10.68	
APPR-DP	1.1	0.3		0.3		7.68	
APPR-TP	0.5	1.1		0.3		10.47	

[a] Expressed as picomoles per 10^6 cells (epimastigotes and trypomastigotes), picomoles per milligram of spleen (amastigotes), or picomoles per 10^6 host cells (tissue culture).

mals treated with HPP survived for more than 270 days. There was no evidence of infection in the animals that were treated, although subculture of blood occasionally revealed the presence of *T. cruzi*. Retreatment of those animals resulted in an apparent cure of the acute disease.

The findings described above indicate that the enzymatic composition of *T. cruzi*, with respect to its ability to metabolize pyrazolopyrimidines as purine analogs, is the same in all three forms (Fig. 1 and Table 2). Specifically, they indicate that the specificity of the hypoxanthine-guanine phosphoribosyltransferase and of the adenylosuccinate synthetase and lyase, with respect to pyrazolopyrimidines, is the same in epimastigotes, trypomastigotes, and amastigotes. The presence of this phosphoribosyltransferase activity in these three morphological forms of *T. cruzi* has been reported previously (7). The adenylosuccinate synthetase and lyase have been studied only in the epimastigote form (14). The mechanisms for concentrating HPP within the parasites must be present as well. These common metabolic pathways and the evidence that a therapeutic effect can be achieved in tissue culture and in mice with HPP suggest that this or another pyrazolopyrimidine may be useful in the treatment of Chagas' disease.

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