Biochemical Pharmacology, Vol. 32, No. 4, pp. 749-752, 1983.
Printed in Great Britain.

0006-2952/83/040749-04$03.00/0

PRELIMINARY COMMUNICATIONS

THE METABOLISM AND CYTOTOXIC EFFECTS OF FORMYCIN B IN *TRYPANOSOMA CRUZI*

Petrie Rainey[1,3], Charles E. Garrett[2] and Daniel V. Santi[1,2*]

Departments of [1]Pharmaceutical Chemistry, [2]Biochemistry and Biophysics, and the [3]Division of Clinical Pharmacology, Department of Medicine, University of California, San Francisco, California, 94143

(Received 2 December 1982; accepted 6 December 1982)

Formycin B (FoB), an inosine analog with a stable carbon-carbon glycosidic bond, is a potent inhibitor of promastigote and amastigote forms of *Leishmania* (1-4) but is remarkably non-toxic to animals (5). As with the related analog, allopurinol riboside (6), the selectivity of FoB is believed to result from its conversion in the parasite to the 5'-monophosphate, FoB-MP, by a nucleoside phosphotransferase (2,3); in mammalian cells, FoB is apparently not converted to FoB-MP (7). In *Leishmania*, FoB-MP is subsequently aminated to give 5'-nucleotides of the cytotoxic Formycin A (FoA), which is incorporated into RNA (2,3).

Trypanosoma cruzi, an organism closely related to *Leishmania*, is the causative agent of Chagas' disease, an illness for which there is no effective cure. We report here that FoB is a potent inhibitor of the growth of *T. cruzi* epimastigotes in culture, and that it is metabolized in a manner directly analogous to that in *Leishmania*.

Materials and Methods

T. cruzi epimastigotes (Tulahuen strain, ATCC No. 30266) were grown in THOSMEM medium (8) at 26°. EC_{50} refers to the concentration of drug necessary to inhibit the control growth rate of organisms by 50%. Cells were seeded at *ca.* 10^6 cells/ml in the presence of varying concentrations of the drug, and were counted at 72 hr using a Coulter Counter ZBI.

[^{3}H]FoB was prepared from [^{3}H]FoA (5 Ci/mmol; Moravek Biochemicals) by treatment with 1.1 units/ml of adenosine deaminase (Sigma) for 1 hr at 25° and pH 7.4. The product was purified by HPLC using a Lichrosorb C_{18} column (4.6 x 250 mm), with a 50 ml gradient of 0 to 25% MeOH/H_2O at 2 ml/min. FoA and FoB had RV's of 20 and 40 ml, respectively. FoB, FoA-MP and FoA-TP were obtained from Calbiochem; FoA-DP was a minor contaminant of the FoA-TP preparation. The FoB nucleotides were obtained by nitrous acid deamination of the corresponding FoA nucleotides (9). Compounds were characterized by HPLC mobility (System 1), uv spectra and HPLC (System 2) of the corresponding nucleoside after digestion with *Crotalus atrox* venom (Sigma). Conversion of nucleotides to nucleosides was performed by treatment with 1 mg/ml *Crotalus atrox* venom in 50 m*M* Tris-HCl (pH 7.4) at 37° for 18 hr.

Acid-soluble cell extracts were prepared and analyzed on HPLC System 1 as previously described (10). The acid-insoluble fraction was treated with 0.3 N KOH for 18 hr at 25° to

---

Abbreviations: FoA, Formycin A; FoB, Formycin B. The respective mono-, di-, and triphosphates are abbreviated FoA-MP, FoA-DP, FoA-TP and FoB-MP, FoB-DP, FoB-TP. RV, retention volume. TCA, trichloroacetic acid.

*To whom reprint requests should be addressed.

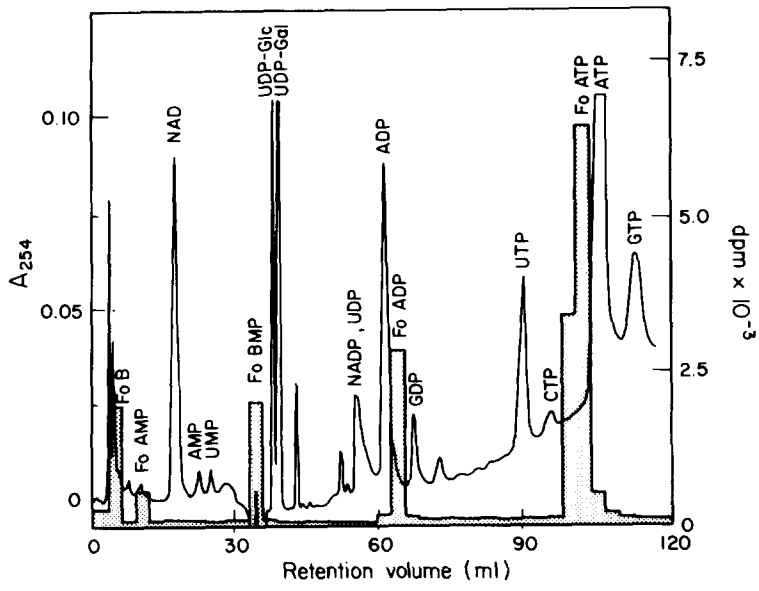

Fig. 1. HPLC (System 1) of the acid-soluble extract from 1.0 x 10^7 *T. cruzi* epimastigotes treated 8 hr with 3.0 μ*M* [^{3}H]FoB. The histogram indicates the radioactivity in each 3.0 ml fraction.

hydrolyze RNA. The DNA was precipitated by addition of 0.15 vol 6M TCA. The supernatant was neutralized with 0.5 M tri-n-octylamine in Freon 113 (11), adjusted to pH 8.5 with 1 M Tris and incubated with 1 unit/ml each of alkaline phosphatase and snake venom phosphodiesterase (Worthington) for 24 hr at 25° to complete digestion of RNA; the resulting ribonucleosides were analyzed by HPLC using System 2.

The HPLC systems used were as follows: System 1 used a Partisil SAX column (4.6 x 250 mm) as previously described (10). Retention volumes were FoA, 3 ml; FoB, 4 ml; FoA-MP, 12 ml; FoB-MP, 34 ml; FoB-DP, 63 ml; FoA-DP, 65 ml; FoA-TP, 104 ml; FoB-TP, 108 ml. System 2 used a Lichrosorb C_{18} column (4.6 x 250 mm) with 50 ml of a 0 to 25% MeOH linear gradient containing 5 m*M* sodium hexanesulfonate and 5 m*M* HOAc as eluant; flow rate was 2 ml/min. Relevant RV's are FoB, 20 ml; adenosine, 32 ml; and FoA, 40 ml.

Results

The EC_{50} of FoB for *T. cruzi* epimastigotes was 7.5 μ*M*. When *T. cruzi* epimastigotes were incubated with 3.0 μ*M* [^{3}H]FoB (450 mCi/mmol), HPLC analysis of the acid-soluble fraction gave five radioactive peaks (Figure 1). The radioactive peak eluting earliest was identified as FoB by co-chromatography on HPLC Systems 1 and 2. The other radioactive peaks were identified as FoA-MP, FoB-MP, FoA-DP and FoA-TP by the following criteria: (a) The radioactive metabolites co-chromatograph with authentic standards on HPLC System 1. (b) Each radioactive peak was collected and converted with *Crotalus atrox* venom to the corresponding nucleoside, which was identified as either FoB or FoA using HPLC System 2.

Table 1 shows the kinetics of accumulation of FoB metabolites in *T. cruzi* over an 8 hr period. Throughout the period there was an increase in cell metabolites, with FoA-TP predominating. There was also a progressive incorporation of radioactivity into RNA, which by 8 hr reached levels of 1.5 pmol/10^7 cells. Degradation of the RNA to its component nucleosides and HPLC on System 2 demonstrated that the radioactivity was exclusively associated with FoA, and that at 8 hr, the molar ratio of adenosine to FoA was 4000.

Table 1. Accumulation of Metabolites of FoB (pmol/10^7 cells) in *Trypanosoma cruzi*[a]

	FoB-MP	FoA-MP	FoA-DP	FoA-TP	FoA-RNA
2 hr	4.8	0.2	2.5	5.1	0.2
4 hr	3.8	0.3	7.4	10.2	0.5
8 hr	5.1	1.2	7.4	27.0	1.5

[a]Experiments were performed as described in the legend to Figure 1. The incorporation of FoA into RNA was determined as described in the Materials and Methods.

Incorporation of radioactivity into DNA was below the limits of our detection (<0.1 pmol/10^7 cells). Over the 8 hr period of incubation we observed no major changes in the pool sizes of the naturally occurring ribonucleotides labelled in Figure 1.

Discussion

The results described here show that FoB is a good inhibitor of growth of *T. cruzi* epimastigotes. FoB is less potent towards *T. cruzi* than it is towards *Leishmania* (1-4), but significantly more cytotoxic than it is towards mammalian cells (5,12). From the metabolites identified in organisms treated with [^{3}H]FoB, we may reasonably conclude that the metabolism is as depicted in Figure 2. The pathway is identical to that proposed for FoB (2,3) and allopurinol riboside (6) in *Leishmania*. As with *Leishmania*, we presume that the initial conversion of FoB to FoB-MP is catalyzed by a nucleoside phosphotransferase. The conversion of FoB-MP to FoA-TP and subsequent incorporation into RNA likely utilize the enzymes that catalyze analogous conversions of the natural substrate IMP.

It is of interest to compare the effects of FoB on *T. cruzi* epimastigotes to those of allopurinol riboside and allopurinol. The reported EC_{50} of allopurinol (13) is similar to that reported here for FoB. Allopurinol is converted to allopurinol riboside-5'-monophosphate by a phosphoribosyl transferase, which then undergoes transformations analogous to those depicted in Figure 2. Indeed, the extensive work reported on the metabolism of allopurinol in *T. cruzi* provides additional credence to the pathway proposed for FoB metabolism. As with FoB, allopurinol does not cause changes in nucleotide pool sizes and its cytotoxicity is believed to result from incorporation of the corresponding adenosine analog,

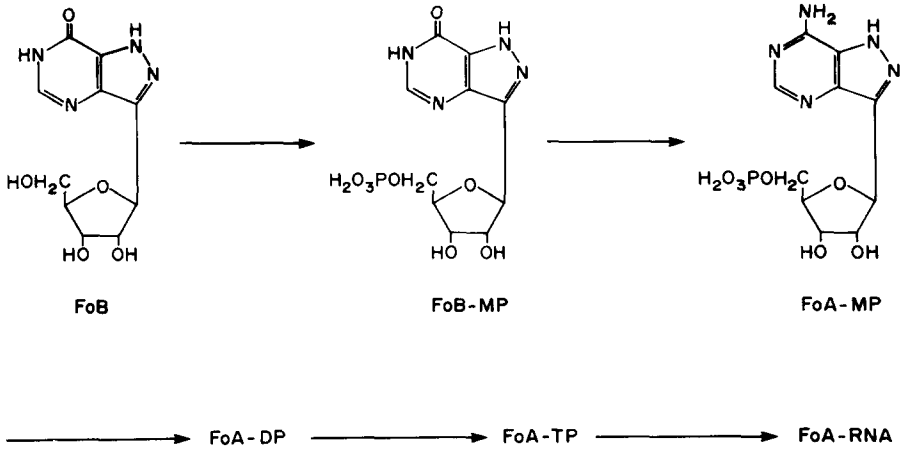

Fig. 2. Proposed metabolism of Formycin B in *T. cruzi* epimastigotes. (Reproduced from Ref. 2)

aminopurinol, into RNA (13,14). The major intracellular metabolite of allopurinol in *T. cruzi* epimastigotes is allopurinol riboside-5'-phosphate, which greatly exceeds the aminopurinol metabolites (13). In contrast, the major metabolites of FoB in *T. cruzi* are nucleotides of FoA, which are believed to be responsible for the cytotoxic effects. Interestingly, although allopurinol riboside is a good anti-leishmanial agent it has no effect on *T. cruzi* growth at concentrations as high as 100 μM (13). Thus, phosphorylation of FoB in *T. cruzi* appears to be much more efficient than phosphorylation of allopurinol riboside.

The mechanism of action of FoB in *T. cruzi* has not been rigorously established. However, in view of the well established cytotoxicity of FoA (6,15), analogy to the effects of FoB in *Leishmania* (2,3) and the aforementioned mechanism of allopurinol action in *T. cruzi*, it is reasonable to conclude that the inhibitory effects of FoB towards *T. cruzi* reside in its ability to serve as a precursor to nucleotides of FoA and the subsequent incorporation of FoA into RNA. As with *Leishmania* (2,3), the selectivity of FoB for *T. cruzi* versus mammalian cells probably resides in the ability of the parasite to convert it efficiently to FoB-MP.

Although the work described here utilized *T. cruzi* epimastigotes, the enzymes believed to be responsible for metabolism of FoB-MP have been shown to be present in all forms of *T. cruzi* (14). Thus, with the proviso that FoB is converted to FoB-MP in the bloodstream (trypomastigote) and intracellular (amastigote) forms, the drug may be effective in the treatment of Chagas' disease. Further, as with *Leishmania* (2,4), FoB may be even more effective towards the intracellular form of the parasite than the tissue culture form used in the present study. Since there are no effective curative agents for Chagas' disease, further investigation of the effects of FoB and related analogs towards *T. cruzi* is clearly warranted.

Acknowledgements. This investigation received financial support from the Rockefeller Foundation and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. P.R. received partial support from USPHS training grant GM 07546.

References

1. D.A. Carson and K.-P. Chang, *Biochem. Biophys. Res. Comm.* **100**, 1377-1383 (1981).
2. P. Rainey and D.V. Santi, *Proc. Nat. Acad. Sci.* (in press).
3. D.J. Nelson, S.W. Lafon, T.E. Jones, T. Spector, R.L. Berens and J.J. Marr, *Biochem. Biophys. Res. Comm.* **108**, 349-354 (1982).
4. J.D. Berman, *J. Inf. Dis.* **145**, 279 (1982).
5. M. Ishizuka, T. Sawa, S. Hori, H. Takazama, T. Takeuchi and H. Umezawa, *J. Antibiotics (Tokyo)* **21**, 5-12 (1968).
6. D.J. Nelson, S.W. Lafon, J.V. Tuttle, W.H. Miller, R.L. Miller, T.A. Krenitsky, G.B. Elion, R.L. Berens and J.J. Marr, *J. Biol. Chem.* **254**, 11544-11549 (1979).
7. H. Umezawa, T. Sawa, Y. Fukagawa, I. Hommo, M. Ishizuka and T. Takeuchi, *J. Antibiotics (Tokyo)* **20**, 308-316 (1967).
8. R.L. Berens, J.J. Marr, S.W. Lafon and D.J. Nelson, *Mol. Biochem. Parasitol.* **3**, 187-196 (1981).
9. S. Suzuki and S. Marumo, *J. Antibiotics (Tokyo)* **14**, 34-38 (1961).
10. A.L. Pogolotti, Jr. and D.V. Santi, *Anal. Biochem.* **126**, 335-345 (1982).
11. J.X. Khym, *Clin. Chem.* **21**, 1245-1252 (1975).
12. T. Sawa, Y. Fukagawa, Y. Shimauchi, K. Ito, M. Hamada, T. Takeuchi and H. Umezawa, *J. Antibiotics (Tokyo)* **18**, 259-266 (1965).
13. J.J. Marr, R.L. Berens and D.J. Nelson, *Science* **201**, 1018-1020 (1978).
14. R.L. Berens, J.J. Marr, F.S. da Cruz and D.J. Nelson, *Antimicrob. Agents Chemother.* **22**, 657-661 (1982).
15. R.J. Suhadolnik, *Nucleotides as Biological Probes*, pp. 169-183, Wiley, New York (1979).