Vol. 108, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1982 Pages 349-354

THE METABOLISM OF FORMYCIN B IN LEISHMANIA DONOVANI

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Received August 2, 1982

Formycin B, a pyrazolo(4,3-d)pyrimidine C-nucleoside, inhibited the growth of Leishmania donovani promastigotes in culture with an ED_{90} of 0.2 μg/ml. Promastigotes incubated for 24 hrs with Formycin B at 10 μg/ml were found to convert it to the ribonucleotide, formycin B 5'-monophosphate. The parasites were also capable of aminating formycin B 5'-monophosphate as evidenced by the appearance of formycin A di- and triphosphate. The RNA contained the formycin A moiety in 3',5'-polynucleotide linkage. Succino-AMP synthetase from these parasites was able to use formycin B 5'-monophosphate as an alternate-substrate with a K'm of 26 μM and a V'm of about 1% the V'm IMP. Formycin B 5'-monophosphate was also a substrate for mammalian succino-AMP synthetase with a V'_m of 40% the V'_m of IMP.

INTRODUCTION

Allopurinol and allopurinol ribonucleoside (HPPR) have significant anti-leishmanial activity. The selective toxicity of these agents for leishmania is thought to possibly depend upon their conversion to high levels of allopurinol ribonucleoside 5'-monophosphate (HPPR-MP) followed by the unique amination to 4-aminopyrazolo(3,4-d)pyrimidine 5'-ribonucleotide (APPR-MP), which is converted to a triphosphate and is incorporated into RNA (1,2,3). The amination reaction does not occur to any extent in the host mammalian cells (4,5,6). A

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Abbreviations used are: HPPR, allopurinol ribonucleoside; HPPR-MP, allopurinol ribonucleoside 5'-monophosphate; APPR-MP, 4-aminopyrazolo(3,4-d)pyrimidine 5'-ribonucleotide; FormB-MP, formycin B 5'-monophosphate; FormA-MP, formycin A 5'-monophosphate; FormA-DP, formycin A 5'-diphosphate; FormA-TP, formycin A 5'-triphosphate; HPLC, high performance liquid chromatography; dCF, 2'-deoxycoformycin; succino-AMP, adenylosuccinate.

0006-291X/82/170349-06$01.00/0

closely related pyrazolo(4,3-d)pyrimidine C-nucleoside, formycin B, was shown by Carson and Chang (7) to block the growth of L. donovani promastigotes as well as reduce infections in hamsters. These authors reported that formycin B was converted to formycin B 5'-monophosphate (FormB-MP) and that this compound inhibited the conversion of IMP to succino-AMP as catalyzed in vitro by succino-AMP synthetase. The amination of FormB-MP by L. donovani, which was not detected at that time, is the subject of this report.

MATERIALS AND METHODS

Promastigotes of L. donovani were grown in HOSMEM medium and the drug sensitivity testing was conducted as previously described (8).

Formycin A, formycin B, FormA-MP and FormA-TP were purchased from Sigma Chemical Co. FormB-MP was enzymatically synthesized as described (7). Protein was removed by ultrafiltration. The product was >99% homogeneous as analyzed by HPLC. HPLC chromatography was performed as described earlier (9). Succino-AMP synthetase was purified from promastigotes of L. donovani (3) or from rabbit muscle (4) as previously described. The inhibition assays of succino-AMP synthetase utilized the spectral assay that couples the formation of GDP to the oxidation of NADH (3,4) and the alternate substrate assays used purified [^{14}C]aspartic acid. The latter was clearly separated from any potential products by high-voltage electrophoresis (10).

RESULTS AND DISCUSSION

Effect of formycin B on the growth of L. donovani promastigotes - Formycin B inhibited the growth of L. donovani promastigotes in culture, with an ED_{90} of 0.2 μg/ml as shown in Table 1. This confirms the observation of Carson and Chang who found similar growth inhibition with formycin B (7). HPPR, a related pyrazolo[3,4-d]pyrimidine ribonucleoside, has an ED_{90} of 0.1 μg/ml (2) against L. donovani promastigotes. Leishmania amastigotes also are sensitive to both inhibitors (11).

Table 1

Growth of L. donovani Promastigotes in the Presence of Formycin B.

Formycin B (μg/ml)	Cell Density (million/ml)†	Growth (% control)
0	25.0	100
0.1	16.2	65
0.5	1.57	6.3
1.0	0.7	2.8
5.0	0.13	0
10.0	0.26	0

†Determined after 6 days of growth. Initial cell density was 0.15.

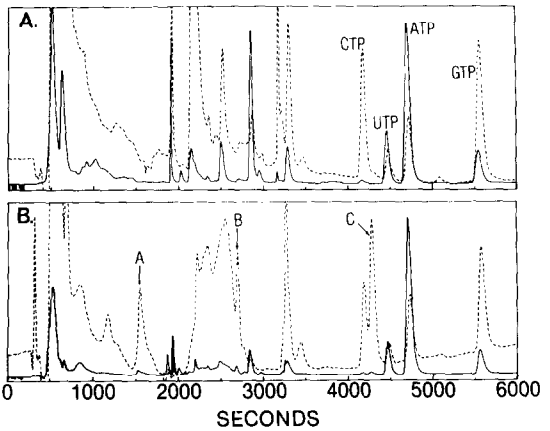

Fig. 1 - L. donovani promastigotes at a density of 1.3 x 10^7 cells/ml were incubated with 10 µg/ml of formycin B for 24 hrs. at 27°C. The cell extract was analyzed for ribonucleotides and formycin B metabolites by anion exchange HPLC. The eluting peaks were monitored at 254 nm (solid lines) and at 292 nm (broken line). A, control; B, 10 µg/ml formycin B

Metabolism of formycin A and formycin B in L. donovani promastigotes. Promastigotes of L. donovani were incubated with formycin B at 10 µg/ml and with formycin A, 10 µg/ml, for 24 hours and the perchloric acid extracts of the cells were analyzed by HPLC. Several new peaks appeared in the mono- di- and triphosphate regions of the chromatogram (Figure 1). The component designated as peak C (Figure 1, B) co-eluted with authentic FormA-TP in this HPLC system. Peak C was collected and found to have a u.v. absorption maximum at 292 nm (pH 3.5) which exactly corresponded to that of authentic FormA-TP. When isolated from the HPLC effluent and treated with E. coli alkaline phosphatase (Boehringer Mannheim), or with 5'-nucleotidase, peak C yielded the riboside, formycin A.

Examination of the cell free medium by reverse phase HPLC (2), after 2 hours of incubation of 1.4 x 10^7 promastigotes per ml with formycin B at 10 µg/ml, showed no formycin A to be present. Incubation of formycin A at 10 µg/ml and 100 µg/ml with the promastigotes under the same conditions led to rapid conversion to formycin B (Table II) by the serum adenosine deaminase present in the HOSMEM medium. In the presence of 1 µM 2'-deoxycoformycin (dCF), a potent adenosine deaminase inhibitor, formycin A was completely spared from deamination. In this experiment dCF stimulated the formation of FormA-TP by three-fold, probably as a result of the increased availability of formycin A for phosphorylation by adenosine kinase. FormB-MP was observed in

Table II

Metabolism of Formycin B and Formycin A in Promastigotes† of *Leishmania donovani*

Expt.	1	2	3	4	5	6
Additions	None	Form-B	Form-A	Form-A (+ 1 μM dCF)	Form-B	Form-A
μg/ml		10	100	100	10	10
Inc. time (hr)	2	2	2	2	24	24
Intracellular Nucleotide				(pmole/10^6 cells)		
CTP	3	2	1	1	2	1
UTP	28	17	16	13	23	13
ATP	75	46	39	43	96	59
GTP	28	19	22	22	17	11
FormB-MP		5	7	5	3	10
FormA-DP		1	3	11	5	5
FormA-TP		1	9	27	15	16

†0.5-2.5 x 10^7/ml in 100 ml total volume.

Form-B, formycin B. Form-A, formycin A.

the experiments with formycin A, both with and without dCF, suggesting that FormA-MP was possibly deaminated by AMP deaminase.

Peak B is believed to be the 5'-diphosphate of formycin A by inference from its HPLC elution position and the characteristic 292 nm/254 nm u.v. absorption ratio.

Authentic FormA-MP eluted from the HPLC at about 700 seconds (see Fig. 1) and was not clearly detectable in the cell extracts because of its coelution with a large amount of cellular material at that position.

Peak A was shown to be FormB-MP by its 292/254 U.V. absorption ratio and HPLC retention time which coincided with that of authentic FormB-MP.

RNA was hydrolyzed from the perchloric acid insoluble pellet (Experiment 5, Table II) with KOH 0.3 N, for 24 hours and the mixture of 2'3'-ribonucleotides was treated with 3'-nucleotidase (rye grass, Sigma Chemical Co.). This specifically yielded ribonucleosides which had been in polynucleotide linkage, whereas 5'-nucleotides which might have carried over from the cytosolic pool were unaffected. The mixture of RNA-derived ribonucleosides was separated by

HPLC on an Whatman ODS-3 reversed phase column (1). The ratio of adenosine to formycin A was 87:1, indicating that a significant incorporation of FormA-TP had occurred, probably by replacement of adenylate in the RNA.

FormB-MP as a substrate for adenylosuccinate (succino-AMP) synthetase.

In contrast to the results of Carson and Chang (7), the present study revealed that FormB-MP was a substrate for partially purified succino-AMP synthetase in vitro. The ability to detect the formation of succino-FormA-MP was probably due to the extreme sensitivity (10) of the assay and the purification of the enzyme (3). The V'_m with FormB-MP was only about 1% of the V'_m of IMP. The K'_m for FormB-MP was 26 μM as determined by alternate-substrate inhibition vs. IMP in which the K_{is} is equal to the K'_m (12). The K'_m for IMP was 12 μM. In a parallel control reaction, the V'_m for HPPR-MP also was determined to be about 1% the V'_m of IMP. Its K'_m was previously shown to be 340 μM (3), which is considerably higher than the K'_m of either IMP or FormB-MP. The intracellular concentration of both FormB-MP (Table II) and HPPR-MP (2) significantly exceed their respective K'_m values for this enzyme.

Although the cleavage of succino-FormA-MP to FormA-MP, as catalyzed by succino-AMP lyase, was not studied, it has previously been shown that this enzyme has a broad substrate specificity and catalyzes the efficient cleavage of many succino-AMP analogs (3). Preliminary studies with succino-AMP synthetase from rabbit muscle revealed that FormB-MP was a very good substrate for this mammalian enzyme. Its V'_m was (relative to V'_m IMP) about 40-fold faster with the mammalian enzyme than with the protozoal enzyme. This finding points to a significant difference between FormB-MP and HPPR-MP as the latter was not a substrate for mammalian succino-AMP synthetase (4). Thus one might expect that mammalian cells capable of phosphorylating formycin B might also form formycin A ribonucleotides.

CONCLUSIONS - The present studies extend the original observations of Carson and Chang (7) on the metabolism of formycin B (Fig.2). In L. donovani the convers of formycin B to FormB-MP was followed by amination through succino-AMP synthe-

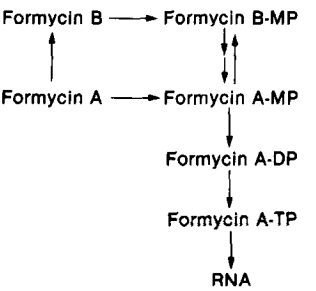

Fig. 2. Metabolism of formycin B in L. donovani

tase/lyase to FormA-MP. The latter was further phosphorylated to the 5'-di- and triphosphates and incorporated into RNA. All of these steps parallel those previously described for the related pyrazolo[3,4-d]pyrimidine ribonucleoside, HPPR (2). Considering the well known toxicity of the amino derivative, formycin A, the observation here that formycin B was transformed to formycin A ribonucleotides is possibly related to the antileishmanial properties of formycin B.

ACKNOWLEDGEMENTS

The authors thank Mrs. Rita Hendricksen and Ms. Sally Tricomi for technical assistance and Dr. Gertrude B. Elion for many helpful discussions.

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