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Pyrazofurin Inhibition of Purine Biosynthesis via 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-Monophosphate Formyltransferase[1]

John F. Worzalla[2] and Martin J. Sweeney

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

ABSTRACT

Pyrazofurin (PF), a C-nucleoside which inhibits pyrimidine biosynthesis, is being tested clinically as an anticancer agent. Pyrazofurin 5′-monophosphate (PF-PO_4), the active metabolite of PF, has a structural resemblance to 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR), a nucleotide intermediate in the biosynthesis of purines. Because of this structural similarity, the effects of PF and PF-PO_4 on AICAR formyltransferase and purine synthesis were studied.

PF-PO_4 inhibited AICAR formyltransferase in rat liver supernatants 46, 69, and 89% at concentrations of 0.2, 0.4, and 1 mM, respectively. The K_i for AICAR formyltransferase by PF-PO_4 was 3×10^{-5} M. AICAR formyltransferase was inhibited 32% by 2 mM PF when ATP (20 mM) and $MgCl_2$ (20 mM) were present, but 2 mM PF alone did not inhibit AICAR formyltransferase.

The inhibition of AICAR formyltransferase *in vivo* should result in a buildup of AICAR and a subsequent increase in the urinary excretion of 5-aminoimidazole-4-carboxamide (AIC), which is the normal urinary degradation product of AICAR. Male Sprague-Dawley rats given single i.p. doses of PF at 7.5, 10, or 30 mg/kg showed increased urinary excretions of AIC as the dose of PF was increased. A single dose of 10 mg/kg resulted in a 64% increase in the amount of urinary AIC (41 μg/day for the treated rats *versus* 25 μg/day for the untreated rats). A higher dose of 30 mg/kg resulted in a 233% increase in the urinary AIC (70 μg/day for the treated rats *versus* 21 μg/day for the untreated rats).

PF has a unique ability to inhibit the *de novo* biosynthesis of both purine and pyrimidine nucleotides.

INTRODUCTION

PF[3] is a nucleoside antibiotic isolated from a fermentation broth of a strain of *Streptomyces candidus* (15). PF shows activity against some viruses (30), fungi (33), and tumors in rodents (31). PF has been tested clinically for cancer (3, 7, 13, 14, 21, 23, 28, 32) and has shown activity against multiple myeloma.[4]

The antiviral effect of PF was reversed by uridine, suggesting that PF inhibited pyrimidine synthesis (30). Subsequent studies showed that PF-PO_4, formed through the phosphorylation of PF by adenosine kinase (9, 18), inhibited orotidylate decarboxylase (8). The inhibition of orotidylate decarboxylase by PF-PO_4 resulted in decreased levels of uracil and cytosine nucleosides and nucleotides (2, 4, 25). This ability of PF-PO_4 to decrease the levels of uracil and cytosine compounds can be exploited by using PF in combination chemotherapy to enhance the activation of other antitumor compounds such as 5-azacytidine and 1-β-D-arabinofuranosylcytosine (5, 6, 16, 26).

Clinical studies show that some patients receiving PF have decreased serum uric acid levels and increased excretion of uric acid (15, 24). This suggests a possible interference of purine metabolism caused by PF or its phosphate. PF-PO_4 has a structural resemblance not only to orotidine 5′-monophosphate (12) but also to AICAR, an intermediate in the *de novo* biosynthesis of IMP (Chart 1). This report describes the inhibition of AICAR formyltransferase (EC 2.1.2.3) *in vitro* by PF-PO_4 and the inhibition of *de novo* purine synthesis in rats given PF.

Materials and Methods

PF and PF-PO_4 are products of Eli Lilly and Company, Indianapolis, Ind. AIC, 5-aminoimidazole-4-carboxamide riboside, and brewer's bottom yeast were purchased from Sigma Chemical Company, St. Louis, Mo. Leucovorin calcium salt and methotrexate were purchased from Lederle Laboratories, Pearl River, N. Y.

AICAR was synthesized from 5-aminoimidazole-4-carboxamide riboside with a brewer's yeast preparation, purified by ion-exchange chromatography, and crystallized (17). AICAR was characterized by UV spectrophotometry, mass spectrometry, and elemental analysis. Later supplies of AICAR were purchased from Sigma. Samples of aqueous AICAR solution were stored at 4°.

N^{10}-Formyltetrahydrofolate was synthesized from leucovorin (27). Oxidation of N^{10}-formyltetrahydrofolate solutions was retarded by mercaptoethanol (0.004%). These tetrahydrofolate solutions were frozen and stored for periods of up to 4 weeks before use.

The assay of Flaks and Lukens (11) for AICAR formyltransferase activity was run at 37° in a total volume of 1.0 ml containing 100 mM Tris-HCl buffer (pH 7.4), 10 mM KCl, 0.8 mM AICAR, 1.6 mM N^{10}-formyltetrahydrofolate, and 0.2 ml of a 25,000 × *g* supernatant fraction prepared from 20% (w/v) rat liver homogenates. The loss of AICAR was determined by analyzing a 0.3-ml sample of the incubation mixture at zero time and after 20 min. The reaction was stopped by adding the 0.3-ml sample to 0.2 ml of 10% (w/v) cold trichloroacetic acid. After centrifugation for 10 min at 1800 rpm, 0.3 ml of the clear supernatants was treated with 0.1 ml of acetic anhydride for 20 min, and then 2 ml of 0.2 N H_2SO_4 were added. The samples

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[2] To whom requests for reprints should be addressed, at Lilly Research Laboratories, MC305, Eli Lilly and Company, 307 East McCarty Street, Indianapolis, Ind. 46285.

[3] The abbreviations used are: PF, pyrazofurin; PF-PO_4, pyrazofurin 5′-monophosphate; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate; AIC, 5-aminoimidazole-4-carboxamide.

[4] R. C. Dyke, Lilly Laboratories for Clinical Research and the Department of Medicine, Indiana University School of Medicine, Indianapolis, Ind., personal communication.

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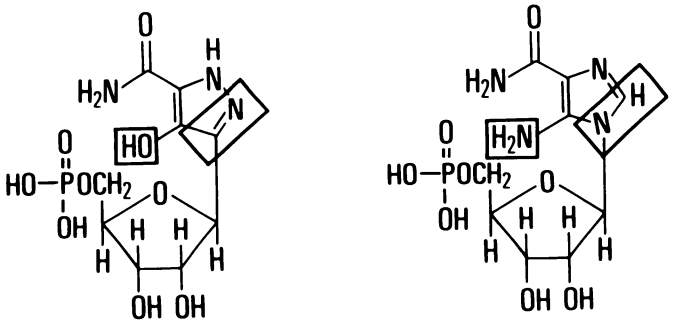

Chart 1. Structural similarity between PF-PO_4 and AICAR. The dissimilar areas of their structures are shown in the *outlined areas*.

were then assayed for AICAR using the Bratton-Marshall procedure (11). The K_i for AICAR formyltransferase was determined by the method of Dixon (10). Male Sprague-Dawley rats (200 g) from Harlan Industries, Cumberland, Ind., were housed in metabolism cages for urine collection. The 24-hr urines were acidified with 0.3 ml of 1 N HCl and frozen until they were assayed.

The method of Skibba *et al.* (29) was used to measure AIC excreted in rat urine with slight modifications to allow for determination of AIC in individual rat urines. After thawing, the urine samples were heated for 1 hr at 80° and then cooled and adjusted to pH 8 with NaOH. These urine samples were then centrifuged for 10 min at 5000 rpm, and the supernatant was filtered using medium-porosity sintered glass funnels. The filtrates were added to 7-x 25-mm AG 1-formate columns (AG 1-X8, 200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.). The effluents were collected together with 2 subsequent 8-ml water rinses. These solutions were acidified with HCl to pH 1.5 and added to 7-x 25-mm AG 50W-H^+ columns (AG 50W-X8, 200 to 400 mesh; Bio-Rad). These effluents and 2 subsequent 8-ml rinses with 0.1 N HCl were discarded. The AG 50W-H^+ columns were treated with an 8-ml and a 16-ml portion of 2.4 N HCl. The effluents were collected together in 25-ml volumetric flasks and diluted to volume with 2.4 N HCl. Duplicate 0.7-ml samples were taken for the Bratton-Marshall assay (29). The assay was run in borosilicate glass tubes at 0°, and the absorbance was read at 520 nm in a Gilford 2400-2 spectrophotometer.

RESULTS AND DISCUSSION

AICAR is a nucleotide intermediate in the *de novo* biosynthesis of purines. The addition of a formyl group to AICAR is catalyzed by the enzyme AICAR formyltransferase, and the formyl-AICAR which is produced is the immediate precursor of IMP. Because of the structural similarity between PF-PO_4 and AICAR, the effect of PF and PF-PO_4 on AICAR formyltransferase and purine metabolism were investigated.

PF-PO_4 inhibited AICAR formyltransferase from rat liver supernatants *in vitro*. Using 0.8 mM AICAR, the AICAR formyltransferase was inhibited 46% by 0.2 mM PF-PO_4, 69% by 0.4 mM PF-PO_4, and 89% by 1.0 mM PF-PO_4 (Table 1). PF-PO_4 was a competitive inhibitor with a K_i of 3×10^{-5} M calculated by the method of Dixon (10).

PF alone, at concentrations up to 2 mM. did not show any inhibition of AICAR formyltransferase (Table 1), but 2 mM PF together with 20 mM ATP and 20 mM $MgCl_2$ showed 32% inhibition. Presumably, this inhibition was caused by PF-PO_4, the active metabolite of PF (9, 35). The inhibition of AICAR formyltransferase *in vitro* by PF-PO_4 but not by PF is similar to the inhibition seen for the pyrimidine-biosynthetic enzyme, orotidylate decarboxylase, which also was inhibited by PF-PO_4 but not by PF (12).

PF was administered to rats to study its effect on purine synthesis *in vivo*. The formation of PF-PO_4 *in vivo* would result in the inhibition of AICAR formyltransferase and an accumulation of AICAR, followed by an increased urinary excretion of AIC, the normal urinary metabolite of AICAR. The daily urinary excretion of AIC is constant and is similar to creatinine excretion in this respect (19).

The amounts of AIC in individual rat urines were measured before and after the administration of PF, using a modified method of Skibba *et al.* (29). AIC standards containing 10 to 50 μg gave a linear response in this assay, with recoveries of the standards ranging from 91 to 96%. The AIC excretion in 61 urine samples from untreated Sprague-Dawley male rats averaged 23.2 ± 4.9 μg/day. This value agrees with the 23 μg/day reported by McGeer *et al.* (20) for male Sprague-Dawley rats. Oace *et al.* (22) reported that AIC excretion in untreated male Sprague-Dawley rats ranged from 15 to 25 μg/day and that the urinary excretion of AIC was unrelated to size or age of the rats. Skibba *et al.* (29) reported an average of 10.6 μg/day in pooled urines from male Sprague-Dawley rats.

Because methotrexate increases the urinary excretion of AIC in rats (19, 29), it was used as a positive control. A single i.p. dose of methotrexate (3 mg/kg) in rats resulted in a 45% increase in the urinary excretion of AIC (Table 2).

When PF was given i.p. to rats, the AIC excretion was increased in proportion to the dose of PF (Table 2). In rats given a therapeutic dose of PF (10 mg/kg), there was an AIC excretion of 41.0 μg/day compared to a pretreatment level of 25.2 μg/day in the same rats (Table 2). Urinary AIC approached the pretreatment levels 24 to 48 hr after the PF dose when the AIC had decreased to 27.1 μg/day. In rats given a second dose of PF (10 mg/kg) on the fourth day, there was an AIC excretion of 48.4 μg/day (192% of pretreatment levels).

A single i.p. dose of PF (30 mg/kg) resulted in an AIC excretion of 70.2 μg/day (331% of the pretreatment level). AIC excretion was still elevated, 33.8 μg/day, 24 to 48 hr after this dose. Administration of a single dose of 7.5 mg/kg resulted in

Table 1

Inhibition of AICAR formyltransferase by PF-PO_4 and by PF with ATP and $MgCl_2$

Assays were done with liver supernatants from male Sprague-Dawley rats as the source of the enzyme. The substrate was 0.8 mM AICAR. ATP and $MgCl_2$ concentrations were 10 times the respective concentrations of PF.

	Concentration (mM)	No. of experiments	% of inhibition
PF-PO_4	1.0	5	89
	0.4	6	69
	0.2	6	46
PF	2.0	7	0
	0.8	7	0
	0.4	7	0
PF + ATP + $MgCl_2$	2.0	6	32
	0.8	6	16
	0.4	6	9

Table 2
Daily urinary excretion of AIC by rats

	No. of animals	Av. amount of AIC in urine (μg)	p (t test)[a]	% of control
24-hr control urines	3	20.6 ± 5.0[b]		
0–24 hr after PF (7.5 mg/kg)	4	24.0 ± 11.6	>0.05	117
24–48 hr after PF (7.5 mg/kg)	4	17.8 ± 11.0	>0.05	86
24-hr control urines	8	25.2 ± 5.2		
0–24 hr after PF (10 mg/kg)	8	41.0 ± 8.2	<0.001	163
24–48 hr after PF (10 mg/kg)	8	27.1 ± 4.9	>0.05	108
0–24 hr after second dose of PF (10 mg/kg) on Day 4	8	48.4 ± 13.7	<0.001	192
24-hr control urines	11	21.2 ± 4.3		
0–24 hr after PF (30 mg/kg)	12	70.2 ± 25.2	<0.001	331
24–48 hr after PF (30 mg/kg)	8	33.8 ± 14.1	<0.05	159
24-hr control urines	8	21.8 ± 5.8		
0–24 hr after methotrexate (3 mg/kg)	8	31.7 ± 6.7	<0.02	145

[a] Mean of treated *versus* mean of respective control group. Statistical significance determined using a 2-tailed Student *t* test.
[b] Mean ± S.D.

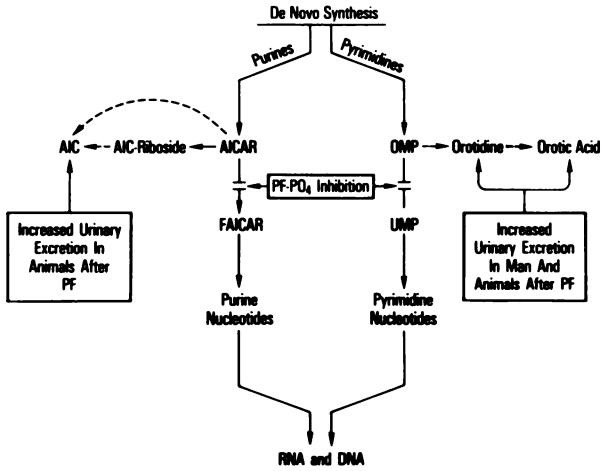

Chart 2. Inhibition of purine and pyrimidine biosynthesis by PF-PO_4. *FAICAR*, formyl-AICAR; *OMP*, orotidine 5′-monophosphate.

a 17% increase in the amount of AIC excreted, but the difference was not statistically significant ($p > 0.05$).

Because the normal excretion of AIC is constant, these increases in the excretion of AIC indicate that PF inhibits AICAR formyltransferase and *de novo* purine biosynthesis in rats. PF-PO_4 might be a useful biochemical tool for the study of purine biosynthesis, especially since there are few known inhibitors of the latter steps in the biosynthesis of IMP, although the 5′-phosphate of virazole has also been shown to inhibit AICAR formyltransferase (1).

The biological effects of PF have been previously attributed to the ability of its monophosphate metabolite, PF-PO_4, to inhibit the synthesis of UMP through its inhibition of orotidylate decarboxylase (15). Although PF-PO_4 is a more potent inhibitor *in vitro* of the pyrimidine enzyme orotidylate decarboxylase ($K_i = 5 \times 10^{-9}$ M) (9) than of the purine enzyme AICAR formyltransferase ($K_i = 3 \times 10^{-5}$ M), this report shows that PF had a significant effect on *de novo* purine metabolism in rats.

PF is a known inhibitor of pyrimidine biosynthesis, and the cytotoxic effect of PF can be completely overcome by uridine (25), suggesting that inhibition of pyrimidine nucleotide synthesis is the primary reason for the action of this compound. The conversion of PF to PF-PO_4, however, results in a nucleotide that *in vitro* is not only an inhibitor of orotidylate decarboxylase in the *de novo* synthesis of pyrimidines but is also an inhibitor of the purine biosynthetic enzyme, AICAR formyltransferase (Chart 2). Evidence that both these enzymes are also inhibited *in vivo* is provided by the fact that elevated urinary levels of AIC, orotidine, and orotic acid are seen after PF is given. Many anticancer drugs are active due to their inhibition of purine or pyrimidine biosynthesis, but PF has the unique ability to inhibit the *de novo* biosynthesis of both pathways (Chart 2).

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