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# FEEDBACK INHIBITION OF PURINE BIOSYNTHESIS IN ASCITES TUMOR CELLS BY PURINE ANALOGUES

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**Abstract**—The ability of 37 purine analogues to inhibit purine biosynthesis *de novo* in Ehrlich ascites tumor cells *in vitro* has been examined in an attempt to define structural requirements for this reaction. Only 8 analogues were active feedback inhibitors. 6-Methylthiopurine ribonucleoside was more active than adenine, while 6-methylpurine was as active as adenine. 2,6-Diaminopurine, 6-benzylthiopurine, psicofuranine, 2-amino-6-benzylthiopurine, purine, and thioguanine ribonucleoside were approximately as active as the less active natural purines. No compound tested interfered with feedback inhibition by adenine. Combinations of adenine with purine or 2,6-diaminopurine, or of purine with diaminopurine, inhibited in a potentiative manner. No correlation was observed between feedback inhibitory activity and nucleotide formation by purine analogues.

FEEDBACK inhibition of purine biosynthesis *de novo* by natural purines in Ehrlich ascites tumor cells *in vitro* has been described.[1] Adenine, guanine, hypoxanthine, and 4-amino-5-imidazolecarboxamide exhibited feedback inhibitory activity, in order of decreasing activity, whereas xanthine and uric acid were inactive. Gots and Gollub[2] studied the ability of purine analogues to inhibit purine biosynthesis *de novo* by a feedback mechanism in a bacterial mutant, and found a rough correlation between feedback inhibition and the ability of a compound to inhibit growth. LePage and Jones,[3] however, observed no such relationship in a series of eight 6-thiopurines in several ascites tumor lines *in vivo*. These authors also found that only those analogues that could be converted to nucleotides by the ascites cells were inhibitory.

The present study tests 37 purine analogues for their ability to inhibit purine biosynthesis *de novo* by a feedback mechanism. Only 8 analogues were active feedback inhibitors. The formation of nucleotides by selected active and inactive purine analogues was measured, but there was no correlation between feedback inhibitory activity and nucleotide formation.

## MATERIALS* AND METHODS

Feedback inhibition was measured, as previously described,[1] in cells pretreated with azaserine to inhibit the enzyme a-(N-formyl) glycinamide ribonucleotide-aminotransferase in the pathway of purine biosynthesis *de novo*. The amount of

* 2-$^{14}$C-glycine of several specific activities was purchased from Volk Radiochemicals, Inc., and 2-$^{14}$C-8-azaguanine, 5·5 mc/mmole, from California Corp. for Biochemical Research. 6-$^{14}$C-amino-pyrazolopyrimidine, 2,000 counts/min/$\mu$g, was a gift from Dr. L. L. Bennett, Jr., of the Southern

Research Institute, Birmingham, Ala. Azaserine, 6-methylthiopurine, 6-benzylthiopurine, 2-amino-6-benzylthiopurine, 1-methyl-2-amino-6-purinethione, 9-butyl-6-thiopurine, 1-methyl-4-aminopyrazolopyrimidine, 4-amino-6-hydroxypyrazolopyrimidine, 6-thioguanine ribonucleoside, 6-mercaptopurine ribonucleoside, 4-aminopyrazolopyrimidine, and 6-methylthiopurine ribonucleoside were obtained from the Cancer Chemotherapy National Service Center; 2-amino-6-methylthiopurine, 1-methyl-6-purinethione, 9-butyl-2-amino-6-thiopurine, 2-(methylamino)-6-thiopurine, and 6-thioguanine, from Dr. R. K. Robins; 6-thioxanthine and 6-phenylaminopurine from Mann Research Laboratories, Inc.; adenine-N-1-oxide, 6-ethoxypurine, and 6-furfurylaminopurine from Aldrich Chemical Co., Inc.; purine, 6-chloropurine, 8-azahypoxanthine, 8-azaadenine, 8-azaguanine, isoguanine, 2,6-diaminopurine, adenine, and L-glutamine from Nutritional Biochemicals Corp.; caffeine, 6-methylpurine, 6-mercaptopurine, and 6-methyl-2-oxypurine, from California Corp. for Biochemical Research; 4-hydroxytrimethylenepyrimidine, 4-aminotrimethylenepyrimidine, 2-amino-4-hydroxyltrimethylenepyrimidine, and 2-amino-4-mercaptotrimethylenepyrimidine were synthesized by Dr. B. R. Baker and furnished by Dr. J. Greenberg; adenine arabinoside from Dr. G. A. LePage, and psicofuranine (adenine psicofuranoside) from Dr. L. Slechta.

FGAR* that accumulates because of this block is a measure of the rate of the pathway, and decreases in the amount of FGAR in the presence of exogenously supplied purines have been shown to be due to feedback inhibition. Incubation conditions and the procedure for the isolation of FGAR have been described elsewhere.[1] Purine analogues were dissolved in 0·1 N NaOH and added immediately in 0·1-ml portions to the buffered medium. Spectrophotometric examination of the medium gave no indication that any of the compounds was altered by this procedure.

Nucleotide formation was tested by a modification of the method of LePage;[4] 120 mg wet weight of tumor cells was incubated in air for 1 hr in calcium-free Krebs-Ringer phosphate medium, pH 7·4, at 38° with 5·5 mM glucose and 1 mM purine analogue. The cells were then inactivated with perchloric acid at a final concentration of 0·2 M, and the precipitate removed by centrifugation. The perchloric acid extract was poured onto a 10 × 30 mm Dowex 50-$H^+$ column, followed by 6 ml of 0·05 M formic acid. It was demonstrated that this column retained all free bases and nucleosides tested and in addition retained possible degradation products such as hypoxanthine, xanthine, and 6-thioxanthine. Under these conditions more than 95% of the adenine ribonucleotides in the cells passed through the column. Because almost all the compounds tested are more acidic than adenine, it was assumed that their nucleotides would also be recovered by this procedure. The quantity of nucleotide formed was measured spectrophotometrically at the wave length of maximum absorption of each free base, after the subtraction of tissue blanks at each wave length. Radioactive forms of 4-aminopyrazolopyrimidine and 8-azaguanine were available, and hence nucleotide formation by these two compounds was not usually measured spectrophotometrically, although preliminary experiments revealed good agreement between measurement of nucleotide formation by the two methods. It must be stated that the compounds called nucleotides were not definitely characterized. The tissue blank was high at the wave length of maximum absorption of certain compounds, thus making the difference measurements less precise, whereas the absorption of the tissue blank was very small at the wavelength of maximum absorption of most of the 6-thiopurines. Because authentic nucleotides of most of the compounds tested were not available, calculations were made on the basis of the extinction coefficient of each free base, which is not necessarily that of the nucleotide. These considerations indicate that the measurements of nucleotide formation presented in this paper cannot be considered to be more than semiquantitative, but they appear to be of sufficient accuracy for comparative purposes.

* FGAR: α-(N-formyl)glycinamide ribonucleotide.

## RESULTS

Purine analogues were chosen to include a wide variety of chemical structures and biological activities. Compounds with well-established chemotherapeutic efficacy were tested, together with those with no carcinostatic activity.[3, 5, 6] Representative

TABLE 1. FEEDBACK INHIBITION BY PURINE ANALOGUES

Ehrlich ascites tumor cells, 24 mg wet weight, were incubated in Krebs-Ringer phosphate medium, pH 7·4, in air for 1 hr at 38° with $2 \times 10^{-3}$ M 2-$^{14}$C-glycine, $10^{-3}$ M glutamine; $5{\cdot}5 \times 10^{-3}$ M glucose; $6{\cdot}4 \times 10^{-6}$ M azaserine, and $10^{-3}$ M purine analogue. Each figure is the average of results from six flasks in three experiments. Average deviations did not exceed ±5%. Average control FGAR radioactivity was 15,600 counts/min. For comparative purposes, antitumor activities (when available) against Ehrlich ascites carcinoma and adenocarcinoma 755 have also been presented.[3, 5, 6]

| Compound | FGAR (% inhibition) | Antitumor activity Ehrlich | Ad. 755 |
|---|---|---|---|
| 6-Methylthiopurine ribonucleoside | 96 | | ± |
| 6-Methylpurine | 92 | | + |
| 6-Benzylthiopurine | 88 | | + |
| Psicofuranine | 85 | | |
| Purine | 75 | | + |
| 2,6-Diaminopurine | 69 | | − |
| 2-Amino-6-benzylthiopurine | 56 | | + |
| Thioguanine ribonucleoside | 55 | | + |
| 4-Aminopyrazolopyrimidine | 39 | + | |
| 6-Mercaptopurine ribonucleoside | 39 | | + |
| Thioguanine | 39 | + | + |
| Adenine-N-1-oxide | 34 | | |
| 1-Methyl-4-aminopyrazolopyrimidine | 31 | + | |
| 8-Azaadenine | 29 | | − |
| 2-Amino-6-methylthiopurine | 28 | − | + |
| 6-Chloropurine | 28 | | + |
| 4-Aminotrimethylenepyrimidine | 27 | | |
| Adenine arabinoside | 26 | | |
| 8-Azahypoxanthine | 25 | | + |
| 6-Furfurylaminopurine | 25 | | |
| 9-Butyl-2-amino-6-thiopurine | 23 | − | |
| 6-Thioxanthine | 21 | | − |
| 6-Phenylaminopurine | 16 | | |
| 9-Butyl-6-thiopurine | 15 | | |
| 2-Methylamino-6-thiopurine | 14 | − | + |
| Isoguanine | 10 | | |
| 4-Hydroxy-6-aminopyrazolopyrimidine | 10 | | ± |
| 2-Amino-4-hydroxytrimethylenepyrimidine | 10 | | |
| 6-Methylthiopurine | 9 | | |
| 6-Dimethylaminopurine | 8 | | + |
| Caffeine | 7 | | |
| 6-Ethoxypurine | 3 | | |
| 4-Hydroxytrimethylenepyrimidine | 2 | | |
| 1-Methyl-6-purinethione | 1 | | |
| 6-Mercaptopurine | 0 | | |
| 8-Azaguanine | 0 | | + |
| 1-Methyl-2-amino-6-purinethione | −5 | + | + |

purine analogues in three unnatural ring systems—pyrazolopyrimidine, triazolopyrimidine (8-azapurine), and trimethylenepyrimidine—were included, as well as normal purine compounds with substituents at the 2 and especially at the 6 positions,

and a few compounds substituted at the 1, 7, 8, and 9 positions. A series of 6-thiopurines related to 6-mercaptopurine and thioguanine was selected for more detailed structure-activity study because these relatively similar chemical structures possess a wide range of biological activities.[3, 5] Five nucleosides included two in which adenine was linked to sugars other than ribose.

The results of this survey are presented in Table 1. For purposes of comparison, the antitumor activities of some of the compounds tested are included. Only 8 purine analogues of the 37 tested inhibited purine biosynthesis *de novo* by more than 50% at $10^{-3}$ M, and only these are classified as "active" in subsequent discussion.

Concentration-activity data were obtained for the 8 active purine analogues, and are presented in Table 2.

TABLE 2. FEEDBACK INHIBITION BY PURINE ANALOGUES*

| Compound | Analogue concentration (M) FGAR (% inhibition) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-3}$ | $5 \times 10^{-4}$ | $10^{-4}$ | $5 \times 10^{-5}$ | $10^{-5}$ | $5 \times 10^{-6}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| 6-Methylthiopurine ribonucleoside | 95 | 88 | 83 | 79 | 75 | | 47 | 26 | 4 |
| 6-Methylpurine | 92 | 90 | 80 | 52 | 32 | 13 | − 4 | | |
| 6-Benzylthiopurine | 88 | 75 | 2 | | | | | | |
| Psicofuranine | 85 | 69 | 6 | | | | | | |
| Purine | 75 | 31 | −6 | | | | | | |
| 2,6-Diaminopurine | 69 | 27 | 6 | | | | | | |
| 2-Amino-6-benzylthiopurine | 59 | 15 | −5 | | | | | | |
| Thioguanine ribonucleoside | 54 | 33 | 7 | | | | | | |

* Experimental conditions, except for concentrations of adenine and purine analogues, are given in Table 1.

Those purine analogues found to inhibit purine biosynthesis *de novo* less than 50% at $10^{-3}$ M, were tested at this concentration for their ability to interfere with the 91·2% feedback inhibition produced by $10^{-3}$ M adenine. None of the compounds tested decreased this inhibition. Although this experiment might not have revealed small amounts of interference because of the low degree of activity of some of these drugs, it is evident that no major effect was produced.

Combinations of adenine with guanine or 4-amino-5-imidazolecarboxamide were found to inhibit *de novo* purine synthesis in a potentiative manner, although the mechanism by which this occurs is not understood. Table 3 shows the results of a similar study in which combinations of weakly inhibitory doses of adenine and purine analogues and of two analogues together were tested. The feedback inhibitory activity of these combinations were not additive, but potentiative. Combinations of individually weakly inhibitory concentrations of purines and purine analogues in some cases exerted stronger effects than even high concentrations of each compound alone.

Experiments were conducted to determine whether the active inhibitors functioned at the level of the free base (or nucleoside) or whether they could be converted to nucleotides under these conditions. In order to facilitate analysis, five times as many cells were used as in the previous experiments. The purine analogues were present at

the highest concentration tested for feedback inhibition—$10^{-3}$ M. These results are presented in Table 4. It is evident that no generalizations can be made concerning the relationship between nucleotide formation and feedback inhibitory activity.

TABLE 3. FEEDBACK INHIBITION BY COMBINATIONS OF ADENINE AND PURINE ANALOGUES*

| Adenine (M) | 2,6-Diaminopurine (M) | Purine (M) | FGAR (% inhibition) |
|---|---|---|---|
| 0 | 0 | 1 | 0 |
| $10^{-5}$ | | | 36 |
| | $5 \times 10^{-4}$ | | 27 |
| | | $5 \times 10^{-4}$ | 29 |
| $10^{-5}$ | $5 \times 10^{-4}$ | | 98 |
| $10^{-5}$ | | $5 \times 10^{-4}$ | 89 |
| | $5 \times 10^{-4}$ | $5 \times 10^{-4}$ | 90 |

* Experimental conditions, except for concentrations of adenine and purine analogues, are given in Table 1.

TABLE 4. FORMATION OF NUCLEOTIDE BY PURINE ANALOGUES

Tumor cells, 120 mg wet weight, were incubated in Krebs–Ringer phosphate medium, pH 7·4, in air for 1 hr at 38° with $5{\cdot}5 \times 10^{-3}$ M glucose and $10^{3-}$ M purine analogue. Each figure is the average of results from four flasks in two experiments. Average deviations did not exceed ±8%.

| Compound | Nucleotide formed (mμmoles) |
|---|---|
| A. Active inhibitors* | |
| 6-Methylthiopurine ribonucleoside | 153 |
| 6-Benzylthiopurine | 0 |
| 2-Amino-6-benzylthiopurine | 0 |
| Thioguanine ribonucleoside | 6 |
| Psicofuranine | 64 |
| Purine | 22 |
| 6-Methylpurine | 35 |
| B. Inactive compounds* | |
| 4-Aminopyrazolopyrimidine | 89 |
| 6-Mercaptopurine ribonucleoside | 25 |
| Thioguanine | 57 |
| 2-Amino-6-methylthiopurine | 0 |
| 2-Methylamino-6-thiopurine | 0 |
| 6-Methylthiopurine | 4 |
| 1-Methyl-2-amino-6-purinethione | 0 |
| 6-Mercaptopurine | 3 |
| 8-Azaguanine | 90 |

* Active inhibitors are those that exerted feedback inhibition of more than 50% at $10^{-3}$ M.

## DISCUSSION

The relatively small number of compounds that could inhibit purine biosynthesis *de novo* in this system suggests that the structural requirements for this reaction are quite strict. However, the comparison of chemical structure with feedback inhibitory activity does not reveal any particular pattern. Compounds substituted at positions

other than 2 and 6 were not active, however, with the exception of the three active nucleosides, whereas a variety of substituents on the 6 position produced active inhibitors.

The feedback inhibitory activities of purine, thioguanine ribonucleoside, psicofuranine, 2-amino-6-benzylthiopurine, 2,6-diaminopurine, and 6-benzylthiopurine declined rapidly with decreasing concentrations, and were less active than the active natural purines at these concentrations. 6-Methylpurine had approximately the same dose-activity relationship as did adenine, whereas 6-methylthiopurine ribonucleoside was much more active than any natural purine and still retained activity at 1/500 the lowest inhibitory concentration of adenine.

Many (but not all) carcinostatic purine analogues are active only in the form of their ribonucleotides, and LePage and Jones[3] found that only those analogues that could be converted to nucleotides exerted feedback inhibition in ascites tumors *in vivo*. The present study, in which a much larger number of compounds was studied, revealed no particular pattern in this regard. Active inhibitors included analogues that were converted to nucleotides together with those that were not, and these two types of metabolism were also included among the compounds with no inhibitory activity in this system. Feedback inhibition in a bacterial mutant by a purine analogue that does not form a ribonucleotide has also recently been reported by Zimmerman and Mandel.[7]

There was no apparent correlation between activity in this system and carcinostatic efficacy, and the order of relative activities was different from that found by Gots and Gollub in bacteria[2] and by LePage and Jones in ascites cells *in vivo*.[3] Most of the active compounds are carcinostatic,[3, 5, 6] but other potent carcinostatic drugs tested had little or no feedback inhibitory activity.

The results of studies of feedback inhibition of purine biosynthesis by purine analogues in bacteria,[2] ascites cells *in vivo*,[3] and ascites cells *in vitro* give somewhat different results, although the same compounds were not used for all these investigations. These differences may result from a possibly different enzyme structure in the case of the bacteria, to differences in the metabolism of these systems at different growth rates, or in the different environments. Within this single system, however, these purine analogues can be ranked quantitatively with respect to feedback inhibitory activity, and compared with that of the natural purines. Most are quite inactive, and only one was more active as a feedback inhibitor than adenine.

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