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FEEDBACK INHIBITION OF PURINE BIOSYNTHESIS IN H. EP. #2 CELLS BY ADENINE ANALOGS*

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Abstract—Fifteen analogs and derivatives of adenine have been evaluated as feedback inhibitors of purine synthesis in H. Ep. # 2 cells in culture. Of these compounds, only four—2-fluoroadenine, 2-fluoroadenosine, 8-aza-adenosine, and 7-deaza-adenosine (tubercidin)—were as active as adenine, which inhibited by 50% or more at a concentration of 3·7 μM. 4-Aminoimidazo (4,5-*d*)pyridazine also caused feedback inhibition at relatively low concentrations. 4-Aminopyrazolo(3, 4-*d*)pyrimidine, although toxic to H.Ep. #2 cells at about the same concentration as fluoroadenine, produced feedback inhibition only at concentrations much in excess of the toxic concentration. Substitution of adenine or fluoroadenine at the 9-position destroyed or markedly reduced the capacity for feedback inhibition. The results suggest that feedback inhibition may be a factor in the growth-inhibiting activity of some of these agents.

NUMEROUS studies have shown that natural purines, after conversion to the nucleotides, inhibit the synthesis of purines *de novo* by a feedback action; [1–6] from results in a cell-free system, the site of feedback inhibition appears to be the first step of the pathway, the synthesis of 5-phosphoribosylamine from glutamine and PRPP.[2, 3] Some purine analogs, notably 6-mercaptopurine and 6-thioguanine, also markedly inhibit purine synthesis *de novo*, apparently as a result of the capacity of their nucleotides to simulate the feedback action of the natural nucleotides.[3, 4, 6–9] The adenine nucleotides were highly effective feedback inhibitors in cell-free systems,[2, 3] and adenine was similarly effective in intact cells.[1, 4–6] It would appear likely, then, that feedback inhibition might account, in whole or in part, for growth inhibition by certain analogs of adenine such as 2-fluoroadenine (F-Ad), 2-fluoroadenosine (F-AdR), 4-aminopyrazolo(3, 4-*d*)pyrimidine (APP), and tubercidin (7-deaza-adenosine)— compounds of high toxicity to mammalian cells and of some interest as inhibitors of the growth of experimental tumors.[10–13] In the present study, adenine analogs of diverse structure have been evaluated for effectiveness as feedback inhibitors of purine biosynthesis in cultured H. Ep. #2 cells. In considering feedback inhibition by natural purines on the one hand and by purine analogs on the other, it should be noted that the two inhibitions presumably are mechanistically the same but qualitatively different in effect, in that feedback inhibition by analogs results in inhibition of overall nucleotide synthesis and of growth, whereas feedback inhibition by natural purines

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is a normal regulatory process that does not inhibit growth. The term "pseudofeedback inhibition" has been used by some[3] to distinguish these two types of inhibition, but in this paper the more general term "feedback inhibition" has been applied to both processes.

MATERIALS AND METHODS

Compounds

F-Ad, F-AdR, 9-butyladenine, 2-fluoro-9-benzyladenine, 2-fluoro-6-dimethylaminopurine, and 9-cyclohexyladenine were synthesized in our laboratories,[14, 15] 1-deaza-adenine and 3-deaza-adenine were synthesized by the methods of Kögl, van der Want and Salemink.[16, 17] APP, APP-deoxyribonucleoside, 8-aza-adenosine, and 2-aza-adenine were obtained from the Cancer Chemotherapy National Service Center through the courtesy of Dr. Burns Ross. Tubercidin was a gift from Dr. C.G Smith of the Upjohn Company and 4-aminoimidazo(4,5-*d*)pyridazine, a gift from Dr. J. A. Carbon of Abbott Laboratories.

Measurement of feedback inhibition

Feedback inhibition was measured by the method of LePage, Jones and Sartorelli[4, 8] and Henderson[5, 6] as modified by Brockman.[9] This method utilizes azaserine to isolate the first few steps of the purine pathway by the specific blockade of the conversion of formylglycinamide ribonucleotide (FGAR) to the corresponding amidine.[18] Compounds that act as feedback inhibitors of purine biosynthesis decrease the amounts of FGAR and its nucleoside (FGAriboside) accumulating in azaserine-treated cells, and the extent of this decrease is a measure of the efficacy of a given agent as a feedback inhibitor.

For these studies, H. Ep. #2 cells were grown in suspension culture, each flask containing at the beginning of the experiment about 4×10^7 cells in 100 ml of SRI-14 medium.[19] To these cells azaserine was added to a final concentration of 10 μg/ml of medium, followed 0·5 hr later by the purine analog and 0·5 hr thereafter by sodium formate-^{14}C (25 μc/flask, sp. act. 3·96 mc/mmole). To control flasks only azaserine and formate were added. Cells were harvested 2hr after addition of formate, washed with isotonic saline, and extracted with boiling 80% ethanol. The extract was lyophilized to dryness and taken up in H_2O, and aliquots were subjected to two-dimensional paper chromatography (in phenol-H_2O and in butanol-propionic acid) as described elsewhere.[18] Radioactive spots were located by radioautography, cut out, and assayed for ^{14}C content in a Tri-Carb liquid scintillation counter

In the cells thus treated with azaserine, essentially all of the ^{14}C on the chromatogram appears in four spots—FGAR and its ribonucleoside, serine, and a spot falling in the polyphosphate area of the chromatogram. The identities of FGAR and FGAriboside were assigned on the basis of agreement of R_F values in the two-dimensional solvent system and in four one-dimensional solvent systems (ethanol-ammonium acetate, phenol-formic acid–water, isobutyric acid–acetic acid–water and isobutyric acid–ammonium hydroxide) with those reported by Anderson and Brockman.[20] For further identification, FGAR was treated with intestinal phosphatase, after which the ^{14}C migrated as FGAriboside in the four one-dimensional systems mentioned above. Serine was identified by its R_F value in the two-dimensional solvent system and by the coincidence of ^{14}C (as detected by radioautography) with the colour developed by spraying the chromatogram with ninhydrin. Brockman *et al.*[21] in similar studies with

H. E. #2 cells have identified the spot falling in the polyphosphate area as higher phosphates of FGAriboside, as evidenced by the fact that, after treatment with alkaline phosphatase, the ^{14}C in this area of the chromatogram migrated like FGAriboside in several solvents; no evidence was obtained for the presence of purine nucleosides. This identification was confirmed in the present study. Both the polyphosphate area and the FGAR spot contained a small amount of another compound which was not identified.

Experiments with *Escherichia coli* were carried out in a similar fashion; the details are given in the footnote to Table 4.

In Table 5 are presented data correlating feedback inhibition and inhibition of growth of H. Ep #2 cells by some of these adenine analogs. Growth-inhibitory concentrations were determined in suspension culture as described elsewhere.[22]

RESULTS AND DISCUSSION

Table 1 presents the results of a typical group of experiments shown in detail in order to illustrate the kind of data obtained and its reproducibility. In this table and the three following, the concentrations of the purine analogs are expressed in μmoles per liter; the odd numbers are the result of the fact that all compounds were referred to adenine as a standard, and in initial experiments adenine was assayed at a concentration of 1·0 μg/ml (7·4 μM). In the controls, most of the ^{14}C was present in FGAR, about half as much was present in the polyphosphates, and an even smaller amount was present in FGAriboside. Adenine or F-Ad at a concentration of 3·7 μM produced marked inhibition of incorporation of ^{14}C into FGAR, FGAriboside, and the polyphosphates. Inhibition might be calculated in terms of counts in either FGAR + FGAriboside, or FGAR + FGAriboside + polyphosphates; both methods gave essentially the same percentage inhibition.

Table 2 presents the results of all the experiments in summary form. Each experiment is based on a detailed analysis as shown in Table 1, and each experiment contained two controls and multiple concentrations of the same inhibitor or of different inhibitors. The results of individual experiments emphasize reproducibility and in some instances point to minor anomalies that are discussed below.

As a feedback inhibitor adenine is highly active,[1–6] and one might therefore take as a criterion of good activity of an analog that it be the same order as that of adenine. By this criterion, four compounds—F-Ad, F-AdR, tubercidin, and 8-aza-adenosine—can be regarded as good feedback inhibitors. Each of these produced almost complete inhibition at concentrations of 7·4 μM and inhibited by 50% or more at a concentration of 3·7 μM. 4-Aminoimidazo(4,5-*d*)pyridazine, although of lower activity, markedly inhibited at 7·4 μM. All the other agents were either inactive or had a much lower order of activity.

Several 9-substituted derivatives were included in this study because of the capacity of certain 9-substituted derivatives of 6-mercaptopurine (6-MP), hypoxanthine, and adenine to inhibit growth of cultured cells.[22] Substitution of an alkyl or cycloalkyl group at the 9-position of adenine or F-Ad destroyed or greatly decreased the capacity for feedback inhibition. Only at the highest concentration assayed was 9-benzyl-2-fluoroadenine active, and then only moderately so. 9-Butyladenine and 9-cyclohexyladenine were essentially inactive at concentrations (ca. 25 μM) that were toxic to H.Ep. #2 cells in growth studies.[22] At a much higher concentration (175 μM),

TABLE 1. EFFECTS OF ADENINE AND 2-FLUOROADENINE ON THE INCORPORATION OF FORMATE-^{14}C INTO SOLUBLE CONSTITUENTS OF AZASERINE-TREATED H.EP. #2 CELLS

Compounds added*	Counts per minute in						Percentage of azaserine controls	
	FGAR	FGAriboside	Polyphosphates	Serine	FGAR + FGAriboside	FGAR + FGAriboside + polyphosphates	FGAR + FGAriboside	FGAR + FGAriboside + polyphosphates
Azaserine	56,290	12,415	37,250	6,593	68,705	105,955		
Azaserine	63,400	13,290	30,100	4,183	76,690	106,970		
Azaserine + adenine (0·74 μM)	52,500	9,190	17,180	3,532	61,690	78,870	85	74
Azaserine + adenine (3·7 μM)	29,600	3,610	7,140	3,430	33,210	40,350	46	38
Azaserine + F-Ad (0·74 μM)	65,960	9,580	19,960	4,522	75,540	95,500	103	90
Azaserine + F-Ad (3·7 μM)	8,031	5,225	3,910	5,225	13,256	17,166	18	16

* Azaserine was present in all cultures at a concentration of 10 μg/ml. Concentrations of adenine and F-Ad are in μmoles/liter.

TABLE 2. EFFECTIVENESS OF ADENINE, ADENOSINE AND ADENINE ANALOGS AS FEEDBACK INHIBITORS IN H. EP. #2 CELLS*

Compound	Counts per minute in FGAR + FGAriboside as percentage of controls† at inhibitor concentrations (μM) of							
	0·74	3·7	7·4	14·8	37	74	175	222
Adenine	85	46	2, 3	2	2			
Adenosine	96	46	4					
2-Fluoroadenine	103	18, 12	2		1			
2-Fluoroadenosine	86	20	2		1			
Tubercidin	98	51	2, 15		2	3		
8-Aza-adenosine		39, 40	11, 14	6				
4-Aminoimidazo (4, 5-*d*) pyridazine			59, 55	30	12, 15	10		
APP	105		97, 125	94	84	56, 63		28, 43
APP-deoxyribonucleoside		96	109		96			
2-Aza-adenine		116	57, 92, 101	62, 82				
8-Aza-adenine		104		88, 90, 145	69	54, 88		
9-Benzyl-2-fluoroadenine			*91*	*91*	*55*, 68			
9-Butyladenine			*106*	*85*	*84*		67	
9-Cyclohexyladenine			*96*	*108*	*97*			
2-Fluoro-6-dimethylaminopurine			*62*	*68*	*60*, 63			
1-Deaza-adenine			113	105	97			
3-Deaza-adenine			104	121	103			

* The italicized values indicate in that particular experiment that the concentration of analog was different from the one shown at the head of the column. Since these corrections apply to several compounds, and since none of the concentrations was markedly inhibitory, in the interest of brevity the correct concentrations are indicated below as a range (column heading followed by correct concentration in parentheses): 7·4 μM (4·5–5·5); 14·8 μM (8·4–11·0); 37 μM (21·0–27·5).

† Percentage of controls containing azaserine alone. All flasks contained azaserine at a concentration of 10μg/ml. Each value given represents a single experiment.

9-butyladenine produced a small degree of feedback inhibition. Certain 9-alkyl derivatives of 6-MP have been shown to produce moderate feedback inhibition at concentrations very high relative to the toxic concentrations;[21] it is possible that the relatively poor inhibition by high concentration of the 9-alkylpurines is not a true feedback effect but a reflection of general growth inhibition resulting from inhibiton of some other metabolic site.

Studies in a cell-free system have shown that nucleotides, but not nucleosides or bases, function as feedback inhibitors;[2] these results were confirmed in intact cells by the demonstration that there was no feedback inhibition by hypoxanthine, 6-MP, or 6-thioguanine in cell lines that had lost the capacity to convert these compounds to the nucleotide.[9] The necessity for nucleotide formation no doubt explains the lack of activity of the 9-substituted derivatives of adenine. It would appear also that F-Ad, F-AdR, 8-aza-adenosine, tubercidin, and 4-aminoimidazo(4,5-*d*)pyridazine must all be readily converted to the nucleotides in H.Ep. #2 cells. No direct information is available on the formation of nucleotides from any of these agents in H.Ep. #2 cells; however, the observation[23] that a mammalian adenylic pyrophosphorylase catalyzed the conversion of F-Ad (as well as APP, 2-aza-adenine, and 8-aza-adenine) to the nucleotide is perhaps pertinent. The implication that in H.Ep. #2 cells tubercidin is converted to the nucleotide is of interest in view of the evidence[24] that in *Streptococcus faecalis* tubercidin inhibits growth as the nucleoside. With regard to nucleotide formation, it is also of interest that 8-aza-adenosine is a more effective inhibitor than is 8-aza-adenine. This result is in accord with the relative toxicity of these agents (Table 5) and would suggest that 8-aza-adenosine is converted to the nucleotide directly by the action of adenosine kinase (an enzyme present in mammalian cells), and that in this cell line this pathway is the better route to the nucleotide, possibly because of the relatively poor rate of reaction of the free base with PRPP. 8-Aza-adenine was also a poor feedback inhibitor in the *in vitro* system of Henderson.[6]

In view of the fact that the toxicity to H.Ep. #2 cells of F-Ad and APP is of the same order (Table 5), it is of particular interest that APP was a relatively poor feedback inhibitor. Significant inhibition appeared only at a concentration of 74 μM (tenfold higher than the concentration of F-Ad that produced 98% inhibition), and even at this concentration, the degree of inhibition produced by APP did not approach that produced by F-Ad or the other potent inhibitors. These results appear to eliminate feedback inhibition as a primary site of action of APP in this cell line. APP-deoxyribonucleoside, which is relatively nontoxic to H.Ep. #2 cells, was without activty as a feedback inhibitor at the highest concentration assayed. It has also been observed that APP produces some degree of feedback inhibition in *E. coli*[7] and in Ehrlich ascites cells *in vitro*,[6] but in neither of these systems was it among the better agents.

The data for several of the other agents which were inactive or slightly active also deserve some comment. 1-Deaza-adenine and 3-deaza-adenine were without activity at the highest concentration (37 μM) studied; it is possible that higher concentrations might have shown activity, but inactivity at 37 μM would indicate that these compounds at best are relatively poor feedback inhibitors. The results with 2-flouro-6-dimethylaminopurine are somewhat puzzling in that some inhibition was produced at a concentration of 7·4 μM, but the degree of inhibition was not increased by concentrations two-and fivefold greater. Several explanations might be advanced to explain these results; however, this compound is not readily soluble and the

aberrant results may simply be a consequence of some decomposition in the process of putting it into solution or of some precipitation when it was added to the cell culture medium. (Similar difficulties with solubility were met with all of the 9-substituted derivatives.) Nevertheless, the results do suggest that substitution of the amino group of F-Ad markedly reduces its activity. The results with 2-aza-adenine also showed some degree of nonreproducibility. Concentrations of 7·4 or 14·8 μM in some experiments produced a moderate inhibition and in others did not. This analog is extensively converted to the nucleotide in mammalian cells,[25] and it is possible that higher concentrations, if assayed, would have produced feedback inhibition.

Because of the high efficacy of the fluoro derivatives as feedback inhibitors, it was desirable to compare more precisely than in Table 2 their effectiveness relative to that of adenine and adenosine. None of the four compounds (Table 3) produced a significant inhibition at a concentration of 0·74 μM and all four compounds inhibited significantly at 3·0 μM. F-Ad appeared to be the most effective of the compounds; at a concentration of 1·5 μM, it alone of the four inhibited significantly, and at a concentration of 3·0 μM it was significantly better than the others. At concentrations above 3·7 μM, however, adenine was as effective as F-Ad. Adenine was, perhaps, superior to adenosine, F-Ad appeared to be slightly superior to F-AdR, and adenosine and F-AdR were about equal in effectiveness. Although these results do not indicate a clear superiority of F-Ad to adenine at all concentrations, they do show that F-Ad is at least equal to adenine in producing feedback inhibition in this system.

TABLE 3. A COMPARISON OF ADENINE AND ADENOSINE AND THEIR 2-FLUORO DERIVATIVES AS FEEDBACK INIBITORS*

	Counts per minute in FGAR + FGAriboside as percentage of controls at inhibitor concentrations (μM) of					
	0·74	1·5	3·0	3·7	4·6	7·4
Adenine	85	88	51	46	5	2, 3
Adenosine	96	92	65	46	27	4
2-Fluoroadenine	103	70	31	18, 12	14	2
2-Fluoroadenosine	86	89	54	26, 15	31	1, 2

* The conditions of the experiment were the same as those for the experiments of Table 2. Each values given represents an independent determination.

The limited number of experiments in *E. coli* (Table 4) show F-AdR to be a potent inhibitor in this system also, and to be as effective as adenosine, or even more so.

If feedback inhibition by these analogs is an important factor in growth inhibition, there should be some correlation between growth-inhibitory concentrations and concentrations giving feedback inhibition. However, the assays for the two types of inhibition are so different (a short-term isotope assay on the one hand and a determination of cell increase during a 48–72 hr period on the other) that a direct correspondence of degree of feedback inhibition with degree of growth inhibition would not be expected. In the correlation of feedback and growth-inhibitory concentrations in Table 5, the growth-inhibitory concentrations are those that produced very marked toxicity to the cells (see footnote to Table 5). For the more potent feedback inhibitors,

TABLE 4. EFFECTIVENESS OF ADENOSINE AND 2-FLUOROADENOSINE AS FEEDBACK INHIBITORS IN *Escherichia coli*

Inhibitors	FGAR + FGAriboside (cpm)	Azaserine control (%)
Exp. 1		
Azaserine*	101,500	
Azaserine + F-AdR (21 μM)	9,930	10
Azaserine + adenosine (21 μM)	48,800	48
Exp. 2		
Azaserine	81,300	
Azaserine + F-AdR (14 μM)	30,700	38
Azaserine + adenosine (14 μM)	49,500	61

* Azaserine was present in all cultures at a concentration of 4 μg/ml; concentrations of adenosine and F-AdR are given in μmoles/liter. Azaserine and adenosine or fluoroadenosine were added simultaneously to rapidly growing cultures; sodium formate-^{14}C (35 μc/flask) was added 20 min thereafter and the cells were harvested 15 min after addition of formate.

TABLE 5. CORRELATION OF FEEDBACK INHIBITION AND GROWTH INHIBITION BY ADENINE ANALOGS

Compound	Concentrations (μmoles/l.) giving inhibitions*	
	Feedback	Growth
2-Fluoroadenine	3·0	1·4
2-Fluoroadenosine	3·7	1·8
Tubercidin	3·7	1·1
8-Aza-adenosine	3·7	7·5
8-Aza-adenine	>74	74
2-Aza-adenine	>14·8	30
4-Aminioimidazo (4, 5-*d*) pyridazine	14·8	7·4
APP	222	3·7
APP-deoxyribonucleoside	>20	>20

* Concentrations giving feedback inhibition were taken from Tables 2 and 3 and represent those concentrations that gave inhibitions of 50% or more. Growth-inhibitory concentrations were based upon the results of a number of experiments and represent those concentrations that reproducibly gave cytostasis or actual reduction of initial cell number during a 48–72 hr period of exposure to the inhibitor.

the growth-inhibitory concentrations, thus defined, were of the same order of magnitude as the concentrations causing pronounced feedback inhibition. The growth-inhibitory concentrations were generally lower than the concentrations causing feedback inhibition, but the correlation may be considered good in view of the qualifications mentioned above.

Because of the great number of biochemical conversions in which adenine nucleotides participate, analogs of adenine could conceivably block metabolism at a number of sites. Therefore, the demonstration that some of these analogs are potent feedback inhibitors is no proof of the importance of feedback inhibition to their growth-inhibitory properties. However, feedback inhibition may be the primary site of action of 6-MP on purine biosynthesis in several mammalian systems,[9,26,27] and a comparison

of the feedback inhibition produced by 6-MP in H.Ep. #2 cells[9] with that produced by the better inhibitors in the present sudy shows that F-Ad, F-AdR, tubercidin, and 8-aza-adenosine are as potent as 6-MP; in addition, as has already been mentioned, they were as potent as adenine. These facts, together with the fact that feedback inhibition and toxicity appeared at about the same concentrations, suggest that feedback inhibition may be a significant factor in the growth-inhibitory action of these analogs in H.Ep. #2 cells.

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