Effects of Certain 5′-Substituted Adenosines on Polyamine Synthesis: Selective Inhibitors of Spermine Synthase†

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ABSTRACT: A number of nucleosides related to *S*-adenosylmethionine were tested for their inhibitory action on three enzymes involved in the biosynthesis of polyamines. The particular objective of the experiments was to determine whether any of the compounds could be used as selective inhibitors of the synthesis of spermine by spermine synthase. None of the nucleosides examined were potent inhibitors of *S*-adenosylmethionine decarboxylase. 5′-[(3-Aminopropyl)amino]-5′-deoxyadenosine dihydrochloride was quite a strong inhibitor of spermidine synthase (I_{50} of 7 μM) but was more than an order of magnitude less active than *S*-adenosyl-1,8-diamino-3-thiooctane, which is a mechanism-based inhibitor of this enzyme. 5′-[(3-Aminopropyl)amino]-5′-deoxyadenosine also inhibited spermine synthase with an I_{50} of 17 μM, but more selective inhibition of spermine synthase was produced by 9-[6(*RS*),8-diamino-5,6,7,8-tetradeoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine (I_{50} of 12 μM) and by dimethyl(5′-adenosyl)sulfonium perchlorate (I_{50} of 8 μM) since these compounds were much less active against spermidine synthase. Both 9-[6(*RS*),8-diamino-5,6,7,8-tetradeoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine and dimethyl(5′-adenosyl)sulfonium perchlorate were able to reduce the synthesis of spermine in SV-3T3 cells, but there was a compensatory increase in the concentration of spermidine, and there was no effect on cell growth. These results and those from experiments in which these spermine synthesis inhibitors were combined with inhibitors of spermidine synthase and ornithine decarboxylase indicated that the cells compensated for the inhibition of the aminopropyltransferases by increasing the production of decarboxylated *S*-adenosylmethionine and putrescine. It appears therefore that it is necessary to limit the synthesis of decarboxylated *S*-adenosylmethionine in order to fully exploit the potential of these inhibitors to block polyamine synthesis.

Polyamines are known to be essential for a normal growth rate in mammalian cells (Pegg & McCann, 1982; Tabor & Tabor, 1984a; Pegg, 1986). The evidence for this comes from two experimental approaches. First, it is known that mutants lacking ornithine decarboxylase, and therefore unable to synthesize any of the polyamines, do not grow unless polyamines are provided (Steglich & Scheffler, 1982; Pohjanpelto et al., 1985). Second, the application of inhibitors of ornithine decarboxylase (Mamont et al., 1980, 1982, 1984; Pegg & McCann, 1982; Porter & Bergeron, 1983; Pegg, 1984a,1986) greatly slows the rate of cell growth when putrescine and spermidine become depleted. Although there are certain important exceptions (Luk et al., 1982; Sunkara et al., 1983; Sano et al., 1984), the general consensus is that the effects of these inhibitors are cytostatic rather than cytotoxic, suggesting that cells can remain in a quiescent but viable state even in the absence of a normal rate of polyamine synthesis. However, it should be noted that these ornithine decarboxylase inhibitors produce little or no effect on the cellular spermine content, and it is possible that the residual spermine is responsible for maintaining cellular viability (Pegg et al., 1982a; Casero et al., 1984; McGovern et al., 1986).

The role of spermine in mammalian cell growth is not well understood. Spermine is not depleted in cells treated with ornithine decarboxylase inhibitors, but the addition of exogenous spermine can restore a normal growth rate to these cells (Mamont et al., 1980; Pegg, 1984a). The availability of a specific inhibitor of spermine synthase would enable the importance of spermine synthesis for the growth and viability of mammalian cells to be examined, and it is possible that the combination of such an inhibitor with an ornithine decarboxylase inhibitor would have useful pharmacological potential.

In this work we have tested a number of nucleosides related to AdoMet[1] for their effects on the three enzymes in the polyamine biosynthetic pathway that use AdoMet or decarboxylated AdoMet as substrates. These are AdoMet decarboxylase (Pegg, 1984b; Tabor & Tabor, 1984b), spermidine

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; AdoDap, 9-[6(*RS*),8-diamino-5,6,7,8-tetradeoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine (as the tetraformate salt trihydrate); AdoDato, *S*-adenosyl-1,8-diamino-3-thiooctane; $AdoS^+(CH_3)_2$, dimethyl(5′-adenosyl)sulfonium (as the perchlorate salt); DFMO, DL-2-(difluoromethyl)ornithine; MGBG, methylglyoxal bis(guanylhydrazone).

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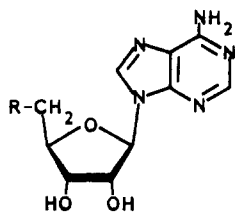

1, R = $^{6}CH(CONH_2)^{7}CH_2^{8}CH_2OH$ [6(R,S)]

2, R = $CH(NH_2)CH_2CH_2NH_2$ [6(R,S)]

3, R = $CH(NH_2)CH_2CH_2OH$ [6(R,S)]

4, R = $CH(NH_2)CH_2CH_2CH_2NH_2$ [6(S)]

5, R = $^{6}CH(NH_2)^{7}CH_2^{8}CH_2^{9}CH(CO_2H)NH_2$ [6(S), 9(S)], sinefungin

6, R = NH_2

7, R = $NHCH_2CH_2CH_2NH_2$

8, R = $N(CH_3)CH_2CH_2CH_2NH_2$

9, R = $NHCH_2CH_2CH_2NHCH_3$

10, R = $NHC(=NH)NH_2$

11, R = $SCH(CH_2CH_2NH_2)(CH_2)_5NH_2$

12, R = $\overset{+}{S}(CH_3)_2$

FIGURE 1: Structures of compounds used as possible inhibitors. The numbering system refers only to the carbohydrate portion of the nucleoside structure.

synthase (Samejima et al., 1983), and spermine synthase (Raina et al., 1983a). Two of the compounds that were identified as potential spermine synthase inhibitors were tested for their ability to be taken up by transformed mouse fibroblasts (SV-3T3 cells) and to influence polyamine metabolism in vivo. It was found that the production of spermine could be substantially depleted in these cells but that the rate of cell growth and of total polyamine production was not reduced.

Materials and Methods

Potential Inhibitors. The compounds tested are shown in Figure 1. They are as follows: **1**, 9-[6(*RS*)-*C*-carboxamido-5,6,7-trideoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine (with 0.5 ethanol and 0.8 H_2O); **2**, 9-[6(*RS*),8-diamino-5,6,7,8-tetradeoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine (as the tetraformate salt trihydrate) (AdoDap); **3**, 9-[6(*RS*)-amino-5,6,7-trideoxy-β-D-*ribo*-octofuranosyl]-9*H*-purin-6-amine (hydrochloride); **4**, 9-[6(*S*),9-diamino-5,6,7,8,9-pentadeoxy-β-D-*ribo*-nonafuranosyl]-9*H*-purin-6-amine (hydrated); **5**, sinefungin, 6(*S*),9(*S*)-diamino-1-(6-amino-9*H*-purin-9-yl)-1,5,6,7,8,9-hexadeoxydecofuranuronic acid; **6**, 5′-amino-5′-deoxyadenosine; **7**, 5′-[(3-aminopropyl)-amino]-5′-deoxyadenosine dihydrochloride; **8**, 5′-[(3-aminopropyl)methylamino]-5′-deoxyadenosine dihydrochloride; **9**, 5′-[[3-(methylamino)propyl]amino]-5′-deoxyadenosine trihydrochloride dihydrate; **10**, 5′-[(aminoiminomethyl)-amino]-5′-deoxyadenosine dihydrogen sulfate; **11**, *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDato); **12**, dimethyl(5′-adenosyl)sulfonium [$AdoS^+(CH_3)_2$] (as the perchlorate salt).

Compounds **1–4** related to sinefungin were synthesized by J. A. Secrist III et al. (unpublished results). Sinefungin (**5**) was obtained from Lilly Research Laboratories. Compounds **6–10** containing a nitrogen atom in place of the sulfur were synthesized by Kolb et al. (1982) and were generous gifts from the Centre de Recherche Merrell International, Strasbourg, France. Compound **11** (AdoDato) and compound **12** [$AdoS^+(CH_3)_2$] were synthesized as described by Tang et al. (1980; 1981).

Other Materials. DFMO was generously provided by Merrell Dow Research Institute, Cincinnati, OH. Radiochemicals were obtained from New England Nuclear, Boston, MA. Biochemical reagents and polyamines were purchased from Sigma Chemical Co., St. Louis, MO. *Escherichia coli* strain HT383/pSPD1 was kindly provided by Drs. C. W. Tabor and H. Tabor, National Institutes of Health, Bethesda, MD. Purified *E. coli* AdoMet synthetase was a generous gift from Dr. G. D. Markham, Institute for Cancer Research, Fox Chase, PA.

Assay of AdoMet Decarboxylase. The assay was carried out by measuring the release of $^{14}CO_2$ from [*carboxyl*-^{14}C]-AdoMet (Pösö & Pegg, 1982; Pegg & Jacobs, 1983). Several different enzyme preparations were used. AdoMet decarboxylases from *E. coli* strain HT383/pSPD1 (Markham et al., 1983a) and from rat prostate were isolated as described by Shirahata et al. (1985) and those from rat liver and rat psoas muscle as described by Pösö and Pegg (1982).

Assay of Aminopropyltransferases. The reaction was assayed by following the production of labeled 5′-methylthioadenosine from labeled decarboxylated AdoMet in the presence of the appropriate amine acceptor (Pegg, 1983a). The product was separated on small columns of cellulose phosphate as described by Raina et al. (1983b). The standard assay medium contained 5 μM labeled decarboxylated AdoMet (0.1–0.25 μCi), 0.5 mM spermidine (for spermine synthase), or 0.5 mM putrescine (for spermidine synthase), 50 mM sodium phosphate buffer, pH 7.2, and 0.1 mM dithiothreitol in a total volume of 0.2 mL. Sufficient enzyme was added to ensure that about 7000 cpm was incorporated into the product in a 30-min incubation at 37 °C in the absence of any inhibitors. Some of the experiments were carried out with decarboxylated [*methyl*-^{3}H]AdoMet and some with decarboxylated [^{35}S]-AdoMet. Both labeled substrates and unlabeled decarboxylated AdoMet were prepared with the bacterial AdoMet decarboxylase (Pegg, 1983a). The [^{35}S]AdoMet was prepared with the use of AdoMet synthetase from *E. coli* and [^{35}S]-methionine (Markham et al., 1983b). Spermidine synthase was purified from rat prostate and spermine synthase from rat brain (Pegg et al., 1981; Samejima et al., 1983).

Cell Culture Experiments. SV-3T3 cells were maintained and transferred as described by Pegg (1984a). They were grown in Dulbecco's modified Eagle's medium with 3% horse serum/2% fetal calf serum. Polyamine content was determined by harvesting the cells, extracting and deproteinizing with 10% (w/v) trichloroacetic acid, and analyzing aliquots of the supernatant fraction with an amino acid analyzer equipped with fluorescence detection (Pegg et al., 1982a; Pegg, 1984a). The cell number, protein content, and viability were determined by standard methods (Pegg, 1984a; McGovern et al., 1986).

Results

The AdoMet analogues tested consisted of three groups: (a) those containing a nitrogen atom in place of the sulfur (compounds **6–10**), (b) those related to sinefungin and having a carbon atom in place of the sulfur (compounds **1–5**), and (c) AdoDato (**11**) and $AdoS^+(CH_3)_2$ (**12**), which have a sulfur in this position.

The effects of the nucleosides considered as potential aminopropyltransferase inhibitors on AdoMet decarboxylase from both *E. coli* and rat prostate are shown in Table I. None of the compounds related to sinefungin were inhibitory toward

Table I: Effect of Potential Inhibitors on AdoMet Decarboxylase[a]

inhibitor structure	no.	effect (% inhibition) at concn shown on AdoMet decarboxylase from *E. coli* 0.1 mM	*E. coli* 1 mM	rat prostate 0.1 mM	rat prostate 1 mM
Ado—CH(CONH$_2$)—(CH$_2$)$_2$OH	**1**	4	15	6	3
Ado—CH(NH$_2$)—(CH$_2$)$_2$NH$_2$	**2**	59	97	0	4
Ado—CH(NH$_2$)—(CH$_2$)$_2$OH	**3**	3	9	3	5
Ado—CH(NH$_2$)—(CH$_2$)$_3$NH$_2$	**4**	12	73	0	1
Ado—CH(NH$_2$)—(CH$_2$)$_2$CH(COOH)NH$_2$	**5**	0	0	0	0
Ado—NH$_2$	**6**	0	2	20	65
Ado—NH—(CH$_2$)$_3$NH$_2$	**7**	4	35	32	82
Ado—N(CH$_3$)—(CH$_2$)$_3$NH$_2$	**8**	8	37	35	84
Ado—NH—(CH$_2$)$_3$NHCH$_3$	**9**	0	0	12	40
Ado—NH—C(=NH)—NH$_2$	**10**	12	64	25	77
Ado—S$^+$(CH$_3$)$_2$	**12**	61	99	74	100

[a] The assay medium contained 0.2 mM AdoMet.

the mammalian enzyme and only **2** (AdoDap) and **4** had any activity against the bacterial AdoMet decarboxylase. The compounds containing a nitrogen atom instead of the sulfur of AdoMet were weak inhibitors of the mammalian AdoMet decarboxylase as previously reported by Pankaskie and Abdel-Monem (1980) and Kolb et al. (1982) and were even less active against the bacterial AdoMet decarboxylase. The most potent nucleoside inhibitor of AdoMet decarboxylase was AdoS$^+$(CH$_3$)$_2$ (**12**), which is in agreement with the reports of Kolb et al. (1982) and Pegg and Jacobs (1983). It is noteworthy that the results in Table I show clearly that the ability of nucleosides to inhibit mammalian putrescine-activated AdoMet decarboxylase cannot always be predicted from the findings with the Mg^{2+}-activated bacterial AdoMet decarboxylase [c.f. Pegg & Jacobs (1983)].

It is known that two different forms of mammalian AdoMet decarboxylase exist with slightly different K_ms for AdoMet and K_is for MGBG and related inhibitors (Pösö and Pegg, 1982; Pegg, 1983b). These two forms of the enzyme are represented by the enzyme from psoas muscle and from liver (Pösö & Pegg, 1982). Both forms were tested for inhibition by all of the compounds shown in Table I which gave more than 50% inhibition at 1 mM. The psoas enzyme was slightly more sensitive to inhibition by all of the derivatives tested, but there was no difference in their relative potency. The slightly greater activity of these competitive inhibitors toward the psoas enzyme is not unexpected since this enzyme has a higher K_m for AdoMet (Pösö & Pegg, 1982; Pegg, 1983b; Williams-Ashman & Seidenfield, 1986).

The effects of these nucleosides on spermidine synthase from rat prostate are shown in Table II. None of the compounds was nearly as inhibitory toward this reaction as AdoDato (**11**), a mechanism-based inhibitor (Tang et al., 1980, 1981; Pegg et al., 1982a). All of the compounds containing a nitrogen atom in place of the sulphur were inhibitory toward spermidine synthase, and **7** was the most potent. The compounds related to sinefungin were in general less active, and even **2** (AdoDap), which was the most inhibitory, required 0.1 mM concentrations to give 50% inhibition. [It should be noted that **2** and **4**, which contain an amine moiety, could be potential acceptor substrates for spermidine synthase. When putrescine was replaced in the assay by **4**, there was about 10% of the normal amount of 5′-methylthioadenosine production, suggesting that this is indeed the case. When putrescine was replaced by **2** (AdoDap), there was only 1% of the normal amount of 5′-methylthioadenosine production, indicating that this nucleoside is at best an extremely weak substrate.]

Table II: Inhibition of Spermidine Synthase[a]

inhibitor structure	no.	% inhibition of spermidine synthase by concn shown 1 mM	0.1 mM	0.02 mM	0.005 mM
Ado—CH(CONH$_2$)—(CH$_2$)$_2$OH	**1**	0	0	0	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_2$NH$_2$	**2**	100	48	16	0
Ado—CH(NH$_2$)—(CH$_2$)$_2$OH	**3**	55	3	0	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_3$NH$_2$	**4**	80	25	12	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_2$CH(COOH)NH$_2$	**5**	20	0	0	*b*
Ado—NH$_2$	**6**	82	18	*b*	*b*
Ado—NH—(CH$_2$)$_3$NH$_2$	**7**	100	96	76	46
Ado—N(CH$_3$)—(CH$_2$)$_3$NH$_2$	**8**	69	34	*b*	*b*
Ado—NH—(CH$_2$)$_3$NHCH$_3$	**9**	100	43	7	*b*
Ado—NH—C(=NH)—NH$_2$	**10**	75	3	*b*	*b*
AdoDATO	**11**	*b*	*b*	100	*b*
Ado—S$^+$(CH$_3$)$_2$	**12**	36	0	*b*	*b*

[a] Assayed in the presence of 0.5 mM putrescine and 0.005 mM decarboxylated AdoMet. [b] Not assayed.

Table III: Inhibition of Spermine Synthase[a]

inhibitor structure	no.	% inhibition of spermine synthase by concn shown 1 mM	0.1 mM	0.02 mM	0.005 mM
Ado—CH(CONH$_2$)—(CH$_2$)$_2$OH	**1**	3	0	0	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_2$NH$_2$	**2**	100	93	65	22
Ado—CH(NH$_2$)—(CH$_2$)$_2$OH	**3**	76	30	20	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_3$NH$_2$	**4**	81	56	25	*b*
Ado—CH(NH$_2$)—(CH$_2$)$_2$CH(COOH)NH$_2$	**5**	82	48	27	*b*
Ado—NH$_2$	**6**	70	23	*b*	*b*
Ado—NH—(CH$_2$)$_3$NH$_2$	**7**	100	84	54	20
Ado—N(CH$_3$)—(CH$_2$)$_3$NH$_2$	**8**	89	22	*b*	*b*
Ado—NH—(CH$_2$)$_3$NHCH$_3$	**9**	100	85	46	*b*
Ado—NH—C(=NH)—NH$_2$	**10**	100	80	38	*b*
Ado—S$^+$(CH$_3$)$_2$	**12**	100	93	60	32

[a] Assayed in the presence of 0.5 mM spermidine and 0.005 mM decarboxylated AdoMet. [b] Not assayed.

Table IV: Summary of Effects of Potential Inhibitors of Spermidine and Spermine Production

compd	approximate I_{50} at substrate concn shown: assayed with 5 μM decarboxylated AdoMet: spermine synthase	assayed with 5 μM decarboxylated AdoMet: spermidine synthase	assayed with 0.2 mM AdoMet: AdoMet decarboxylase
Ado-S^+-$(CH_3)_2$ (**12**)	8 μM	>1 mM	60 μM
AdoDap (**2**)	12 μM	120 μM	>1 mM
Ado-NH-$(CH_2)_3NH_2$ (**7**)	17 μM	7μM	>0.2 mM
AdoDato (**11**)	>0.2 mM	0.1 μM	>1 mM

Table V: Effect of AdoDap (**2**) on Polyamine Content of SV-3T3 Cells[a]

concn of AdoDap (**2**) added	cell no. (×10^{-6})	polyamine content of culture (nmol): putrescine	spermidine	spermine	total[b]
none	11.0 ± 1.8	6.0 ± 0.4	36.7 ± 0.2	16.2 ± 0.5	58.9
50 μM	9.7 ± 1.5	5.8 ± 0.2	56.8 ± 1.2	10.5 ± 0.8	73.1
100 μM	11.2 ± 2.2	4.8 ± 0.5	48.9 ± 2.6	7.1 ± 0.4	60.8
200 μM	9.7 ± 1.3	4.2 ± 0.4	46.2 ± 1.3	5.0 ± 0.7	55.4
400 μM	9.5 ± 0.5	4.4 ± 1.2	46.0 ± 2.0	4.6 ± 1.3	55.0
none + 5 mM DFMO	1.4 ± 0.3	*d*	*d*	2.0 ± 0.4	2.0
100 μM + 5 mM DFMO	1.3 ± 0.3	*d*	*d*	1.9 ± 0.3	1.9
initial values[c]	0.4	0.2	1.4	0.6	2.1

[a] Results are shown as the mean ± SEM for four to six estimations. [b] The sum of putrescine, spermidine, and spermine. [c] This assumes 100% plating efficiency and is a maximal estimate. [d] Below limit of detection.

Inhibition of spermine synthase by these nucleosides is shown in Table III. All were active inhibitors except for **1**. The most potent compounds were AdoDap (**2**) and **7**, both only slightly less active than $AdoS^+(CH_3)_2$ (**12**), which has recently been reported to be a good inhibitor of mammalian spermine synthase (Pegg & Coward, 1985).

The most active compounds were examined in more detail, and a summary is shown in Table IV. AdoDato (**11**), which is included for comparison, is known to be a specific and very potent inhibitor of spermidine synthase (Tang et al., 1980; 1981). $AdoS^+(CH_3)_2$ (**12**) is clearly a potent inhibitor of spermine synthase and a weaker inhibitor of AdoMet decarboxylase but has no significant effect on spermidine synthase. AdoDap (**2**) is also a potent inhibitor of spermine synthase, and it is an order of magnitude less active on spermidine synthase and has no significant effect on AdoMet decarboxylase. Compound **7** is an inhibitor of both spermidine and spermine synthase and is a weaker inhibitor of AdoMet decarboxylase. These findings suggested that AdoDap (**2**) and $AdoS^+(CH_3)_2$ (**12**) might be useful compounds to interfere selectively with spermine synthesis in mammalian cells.

This possibility was examined by growing SV-3T3 cells in the presence of these nucleosides. (Tables V and VI). Both compounds led to a dose-dependent decrease in the cellular content of spermine and an increase in spermidine, which is consistent with a block at the spermine synthase step. Exposure to 0.4 mM $AdoS^+(CH_3)_2$ (**12**) led to a decrease in total polyamines, which as previously reported (Pegg & Coward, 1985) is probably due to its ability to inhibit the production of decarboxylated AdoMet by AdoMet decarboxylase (see Table IV). However, although AdoDap (**2**) and $AdoS^+(CH_3)_2$ (**12**) at concentrations below 0.4 mM inhibited the production of spermine, they did not reduce total polyamine content of the culture or cell growth (Tables V and VI). In fact, there was a slight increase in the total polyamine content since spermidine levels increased by more than the decline in spermine. These results indicate that AdoDap (**2**) and $AdoS^+(CH_3)_2$ (**12**) do enter these cells and can exert effects in vivo on spermine synthesis. Cell growth was not affected significantly by the reduction in spermine content but it should be emphasized that there was a compensatory increase in spermidine.

Table VI: Effect of **12** [$AdoS^+(CH_3)_2$] on Polyamine Content of SV-3T3 Cells[a]

concn of $AdoS^+$-$(CH_3)_2$ (**12**) added	cell no. (×10^{-6})	polyamine content of culture (nmol): putrescine	spermidine	spermine	total[b]
none	10.8 ± 1.5	5.9 ± 0.6	35.6 ± 2.3	15.7 ± 1.1	57.2
50 μM	10.8 ± 0.3	3.2 ± 0.4	53.2 ± 3.3	4.0 ± 0.3	60.4
100 μM	10.4 ± 1.6	4.2 ± 1.4	55.6 ± 5.2	3.3 ± 0.4	63.1
200 μM	10.4 ± 1.8	4.9 ± 1.5	47.8 ± 4.5	2.3 ± 0.7	55.0
400 μM	9.6 ± 2.1	4.1 ± 1.6	31.9 ± 5.1	1.9 ± 0.2	39.9
none + 5 mM DFMO	1.3 ± 0.2	*d*	*d*	2.1 ± 0.3	2.1
100 μM + 5 mM DFMO	1.2 ± 0.2	*d*	*d*	2.2 ± 0.1	2.2
initial values[c]	0.4	0.2	1.4	0.6	2.1

[a] Results are shown as mean ± SEM for five or six estimations. [b] The sum of putrescine, spermidine, and spermine. [c] This assumes 100% plating efficiency and is a maximal estimate. [d] Below limit of detection.

The presence of the ornithine decarboxylase inhibitor DFMO greatly reduced the synthesis of polyamines and growth in these cell cultures (Mamont et al., 1980, 1982; Pegg & McCann, 1982; Pegg et al., 1982b; Pegg, 1984a). The levels of spermidine and putrescine were below the limit of detection in DFMO-treated cells, but the content of spermine did not decrease if the results in Tables V and VI are expressed on a per cell basis. The combination of DFMO with either 100 μM AdoDap (**2**) or $AdoS^+(CH_3)_2$ (**12**) did not produce any further reduction in spermine.

A possible explanation for the lack of effect of these spermine synthase inhibitors when added in the presence of DFMO is that DFMO leads to a several hundred fold increase in the content of decarboxylated AdoMet (Pegg et al., 1982b; Mamont et al., 1982; Pegg, 1984a,b). A full kinetic study of the nature of the inhibition of spermine synthase by AdoDap (**2**) or $AdoS^+(CH_3)_2$ (**12**) has not yet been carried out, but as shown in Figure 2, the inhibition is much less profound when the decarboxylated AdoMet concentration is increased, and it is likely that the inhibition is competitive with respect to decarboxylated AdoMet. The ability of the cell to respond to polyamine synthesis inhibition by increasing the supply of decarboxylated AdoMet (Pegg & McCann, 1982; Pegg, 1984b; 1986) may limit the usefulness of the aminopropyltransferase inhibitors unless an inhibitor of AdoMet decarboxylase can also be provided.

These experiments, along with our earlier studies on the effects of AdoDato (**11**) on polyamine metabolism (Pegg et al., 1982a), are in agreement with the concept that the two aminopropyltransferases present in mammalian cells compete for the available decarboxylated AdoMet substrate. When either of the spermine synthase inhibitors AdoDap (**2**) or $AdoS^+(CH_3)_2$ (**12**) is added, spermidine synthesis predomi-

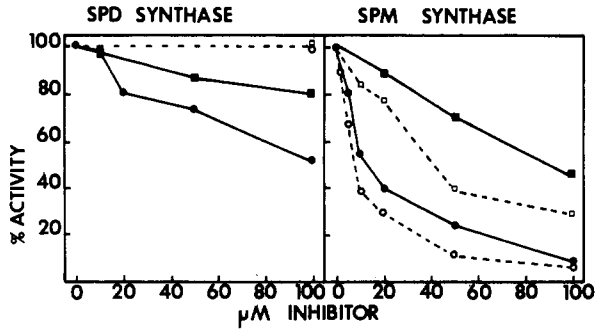

FIGURE 2: Effect of concentration of decarboxylated AdoMet on inhibition of spermidine and spermine synthase by **2** (AdoDap) or **12** [$AdoS^+(CH_3)_2$]. Results are expressed as the percentage of activity in the absence of any inhibitor for spermidine synthase (spd synthase) in the left panel and for spermine synthase (spm synthase) in the right panel. Results are shown for assays carried out with 20 μM decarboxylated AdoMet (■ and □) and for assays carried out with 5 μM decarboxylated AdoMet (● and ○) with AdoDap (**2**) as inhibitor (● or ■) or **12** [$AdoS^+(CH_3)_2$] as inhibitor (○ or □).

Table VII: Effect of $AdoS^+(CH_3)_2$ (**12**) plus AdoDato (**11**) on Growth and Polyamine Content in SV-3T3 Cells

treatment	cell no. ($\times 10^{-6}$)	polyamine content of culture (nmol) putrescine	spermidine	spermine	total[a]
control	12.5 ± 0.4	7.8 ± 0.9	38.2 ± 2.2	13.1 ± 2.2	59.1
AdoDato, 50 μM	12.0 ± 0.8	18.3 ± 3.2	6.9 ± 1.1	24.8 ± 3.5	50.0
$AdoS^+(CH_3)_2$, 200 μM	11.3 ± 1.4	5.5 ± 1.4	51.2 ± 6.8	2.2 ± 0.3	58.9
$AdoS^+(CH_3)_2$, 400 μM	11.1 ± 1.5	6.9 ± 2.2	40.2 ± 3.6	1.8 ± 0.5	48.9
AdoDato, 50 μM, + $AdoS^+(CH_3)_2$, 200 μM	9.6 ± 2.0	16.4 ± 1.8	32.4 ± 3.2	12.0 ± 1.4	60.8
AdoDato, 50 μM, + $AdoS^+(CH_3)_2$, 400 μM	12.0 ± 2.1	17.3 ± 1.4	38.6 ± 2.2	8.6 ± 1.0	64.5

[a] The sum of putrescine, spermidine, and spermine.

nates; but when AdoDato (**11**) is administered to reduce spermidine synthase activity, the production of spermine is favored (Pegg et al., 1982a).

This concept is further illustrated by the experiment shown in Table VII in which the combined effects of AdoDato (**11**) and $AdoS^+(CH_3)_2$ (**12**) were examined. AdoDato (**11**) led to a marked reduction of spermidine content in SV-3T3 cells but an increase in putrescine and spermine as previously reported (Pegg et al., 1982a). $AdoS^+(CH_3)_2$ (**12**) gave rise to an increase in spermidine and a decrease in spermine. When $AdoS^+(CH_3)_2$ (**12**) and AdoDato (**11**) were added together, the spermidine and spermine content of the cells was restored to values quite similar to those in control cells to which no inhibitors were added. The cells with the combined inhibitors contained elevated levels of putrescine. The total polyamine content per cell was not decreased at all by the presence of the two aminopropyltransferase inhibitors (Table VII). These results suggest that the cell can compensate for the inhibition of aminopropyltransferases by increasing the production of putrescine and decarboxylated AdoMet, the substrates required for the synthesis of the higher polyamines.

The long-term exposure to a spermine synthase inhibitor was tested by growing cells in the presence of 200 μM $AdoS^+(CH_3)_2$ (**12**) for up to 20 days (Figure 3). The plating

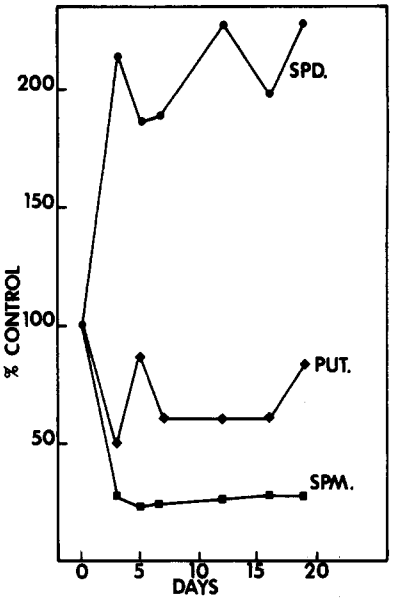

FIGURE 3: Effect of $AdoS^+(CH_3)_2$ (**12**) on the polyamine content of SV-3T3 cells grown for up to 20 days. The cells were plated and grown in the presence of 200 μM **12** or no addition. Approximately every 3 days they were reseeded into dishes containing fresh medium of the same composition. At the time points shown, aliquots of the cell cultures were used for polyamine analysis, and the results were expressed as the percentage of the polyamine content of the control cells not exposed to the drug and harvested at the same time. Results are shown for spermine (SPM., ■), spermidine (SPD., ●), and putrescine (PUT., ▲). The results shown are the mean of triplicate estimations (each estimation representing a separate culture dish), which agreed within ±10%.

efficiency and the rate of growth were not significantly affected in the presence of this concentration of the nucleoside. The cells grew with about 25% of their normal spermine content and about twice the normal content of spermidine. Putrescine levels were somewhat reduced. The total polyamine per cell was about 5.3 fmol in the control cells and 8.4 fmol in the cells treated with $AdoS^+(CH_3)_2$ (**12**) for 12–20 days. This result is in agreement with the conclusion discussed above that the cellular capacity to synthesize polyamines is actually somewhat increased in response to the spermine synthase inhibitors.

Discussion

Mammalian cells differ from bacteria and some lower eukaryotes in that they contain a separate spermine synthase enzyme that converts spermidine into spermine (Pegg & McCann, 1982; Tabor & Tabor, 1984a). The function of spermine is unknown and it is clearly not essential for life since many organisms (for example, *E. coli* or *Trypanosoma brucei brucei*) do not normally contain spermine although their spermidine synthase can produce it under some exceptional circumstances (Pegg & McCann, 1982; Tabor & Tabor, 1984a; Pegg, 1986). A selective inhibitor of spermine synthase would be of value in the design of experiments to examine the role of spermine in mammalian cell growth and differentiation.

It is noteworthy that the AdoMet analogues containing a nitrogen atom instead of the sulfur that were conceived as potential AdoMet decarboxylase inhibitors (Pankaskie & Abdel-Monem, 1980; Kolb et al., 1982) are in fact better inhibitors of the aminopropyltransferases. These compounds may have some value as general blockers of polyamine biosynthesis, but as shown in Tables I–IV, they are not sufficiently selective against any single one of the enzymes in the pathway to be used to evaluate the roles of the individual polyamines. In contrast, both $AdoS^+(CH_3)_2$ (**12**) and AdoDap (**2**), which were the most active inhibitors of spermine synthase found in

the present work, were only weakly active toward the other polyamine biosynthetic enzymes. Furthermore, the comparison of the effects of $AdoS^+(CH_3)_2$ (**12**) and AdoDap (**2**) on cells has the advantage that while they are comparably active against spermine synthase, they differ in their effects on the other enzymes with AdoDap (**2**) having no significant effect on AdoMet decarboxylase and $AdoS^+(CH_3)_2$ (**12**) having no significant effect on spermidine synthase.

Both $AdoS^+(CH_3)_2$ (**12**) and AdoDap (**2**) were found to selectively suppress the synthesis of spermine in SV-3T3 cells (Tables V and VI), but the reduction in spermine synthesis did not affect cell growth. The lack of effect on cell growth may be due to the corresponding increase in cellular spermidine content. $AdoS^+(CH_3)_2$ (**12**) is only slightly more active as an inhibitor of spermine synthase than AdoDap (**2**) in vitro (Table I and Figure 2), but it was considerably more potent in the cell cultures. This may be due to the better uptake or resistance to metabolic decomposition of $AdoS^+(CH_3)_2$ (**12**). However, it is clear that both compounds do enter mammalian cells relatively well and exert effects over a considerable period of time. Therefore, syntheses of adenosyl derivatives either as sulfonium salts or as sinefungin analogues are reasonable approaches to the design of potential pharmacological agents. Further evidence that these compounds do enter mammalian cells is that two of the sinefungin analogues, compounds **2** (AdoDap) and **4**, have modest antiviral activity against vaccinia virus in L cells (J. A. Secrist III, unpublished results).

It has been argued that the limiting factor in the synthesis of spermidine and spermine in mammalian cells is the supply of decarboxylated AdoMet (Pegg & Hibasami, 1979; Pegg et al., 1982a; Pegg, 1984b). The activities of the aminopropyltransferases are in excess of those of both ornithine decarboxylase and AdoMet decarboxylase, and the rate of synthesis of the polyamines by the aminopropyltransferases is limited by the availability of their substrate, decarboxylated AdoMet. There is competition between spermidine synthase and spermine synthase for the use of the available decarboxylated AdoMet. In the presence of either $AdoS^+(CH_3)_2$ (**12**) or AdoDap (**2**), which inhibit spermine synthase, the spermidine synthase reaction is favored. Therefore, the cellular content of spermidine is increased, and that of spermine declines as shown in Tables V–VII. In the presence of the spermidine synthase inhibitors such as AdoDato (**11**) (Pegg et al., 1982a; see also Table VII) or cyclohexylamine (Mitchell et al., 1985), spermine synthase is favored, and the content of spermine increases while that of spermidine declines.

These aminopropyltransferase inhibitors produce little net inhibition of total polyamine (spermidine + spermine) content although they drastically alter the balance between them. The lack of effect on total polyamine levels is due to the regulation of the polyamine biosynthetic pathway by means of changes in the level of ornithine and AdoMet decarboxylases. It is well established that both of these enzymes are repressed by spermidine or spermine and that their activities increase in response to the fall in cellular polyamine levels (Pegg et al., 1982a; Pegg & McCann, 1982; Pegg, 1984a,b; Mamont et al., 1982, 1984; Tabor & Tabor, 1984a, Mitchell et al., 1985). The ability of the cell to increase the supply of decarboxylated AdoMet by increasing AdoMet decarboxylase activity probably provides enough of this nucleoside to overcome any inhibition of total polyamine production by these inhibitors. This point is well illustrated by the results in Table VII in which the SV-3T3 cells grown in the presence of AdoDato (**11**) and $AdoS^+(CH_3)_2$ (**12**) had a cellular content of spermidine and spermine that was close to control values.

Similarly, the total polyamine content of cells grown for an extended period in $AdoS^+(CH_3)_2$ (**12**) was actually slightly increased because the increase in spermidine levels exceeded the decrease in spermine (Figure 3). These results suggest that spermine may be somewhat more effective than spermidine at bringing about the repression of ornithine and AdoMet decarboxylase activities and that inhibitors such as $AdoS^+(CH_3)_2$ (**12**) and AdoDap (**2**) will be useful tools to investigate the regulation of these key enzymes.

The inability of $AdoS^+(CH_3)_2$ (**12**) or AdoDap (**2**) to deplete spermine in cells exposed to DFMO also suggests that these inhibitors are ineffective when the cellular content of decarboxylated AdoMet is greatly increased. It therefore appears that these inhibitors will only be of value in depletion of total polyamines (which may be needed for antineoplastic and other therapeutic effects) if they can be combined with a potent inhibitor of AdoMet decarboxylase. At present, the only good inhibitors of this enzyme that have been reported are MGBG and its derivatives (Pegg, 1983b; Pegg & Jacobs, 1983; Tabor & Tabor, 1984a). Unfortunately, these compounds have a variety of other effects on cells that are unrelated to the inhibition of polyamine synthesis. Since our results show that compounds related to AdoMet do enter mammalian cells, the synthesis of specific AdoMet decarboxylase inhibitors by means of substrate analogues appears to be feasible.

Registry No. **1**, 102537-60-0; **2**·$4HCO_2H$, 102537-62-2; **3**, 102537-63-3; **4**, 102537-64-4; **5**, 58944-73-3; **6**, 14365-44-7; **7**, 81162-85-8; **8**, 81162-86-9; **9**, 81162-87-0; **10**, 81162-89-2; **11**, 76426-40-9; **12**·ClO_4^-, 58936-08-6; spermine, 71-44-3; spermine synthase, 74812-43-4; AdoMet decarboxylase, 9036-20-8; spermidine synthase, 37277-82-0; aminopropyltransferase, 67016-02-8; spermidine, 124-20-9; putrescine, 110-60-1; decarboxylated *S*-adenosylmethionine, 22365-13-5.

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Human Class 1 Heparin-Binding Growth Factor: Structure and Homology to Bovine Acidic Brain Fibroblast Growth Factor†

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ABSTRACT: The structure of the major class 1 heparin-binding growth factor from human brain has been analyzed. Edman degradation performed on the native mitogen and on fragments generated by chemical and enzymatic cleavage allows the sequence to be described by four nonoverlapping segments. The sum of the amino acids of the four segments is in excellent agreement with the experimentally determined amino acid composition of the mitogen itself, suggesting that, jointly, they account for the entire molecule. The four segments can be aligned into a presumptive complete sequence that shows 92% identity with that of bovine acidic brain fibroblast growth factor. The data indicate that the human mitogen has the following sequence: Phe[1]-Asn-Leu-Pro-Pro-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr[15]-Cys-Ser-Asn-Gly-Gly-His-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly-Thr[30]-Val-Asp-Gly-Thr-Arg-Asp-Arg-Ser-Asp-Gln-His-Ile-Gln-Leu-Gln[45]-Leu-Ser-Ala-Glu-Ser-Val-Gly-Glu-Val-Tyr-Ile-Lys-Ser-Thr-Glu[60]-Thr-Gly-Gln-Tyr-Leu-Ala-Met-Asp-Thr-Asp-Gly-Leu-Leu-Tyr-Gly[75]-Ser-Gln-Thr-Pro-Asn-Glu-Glu-Cys-Leu-Phe-Leu-Glu-Arg-Leu-Glu[90]-Glu-Asn-His-Tyr-Asn-Thr-Tyr-Ile-Ser-Lys-Lys-His-Ala-Glu-Lys[105]-Asn-Trp-Phe-Val-Gly-Leu-Lys-Lys-Asn-Gly-Ser-Cys-Lys-Arg-Gly[120]-Pro-Arg-Thr-His-Tyr-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Leu[135]-Pro-Val-Ser-Ser-Asp[140].

Heparin-binding growth factors (HBGF's)[1] are mitogens for a variety of mesoderm-derived cells in vitro and can induce neovascularization in vivo [for review, see Lobb et al. (1986a)]. They are characterized by an unusual affinity for the complex glycosaminoglycan, heparin, and this affinity has been exploited for their purification and characterization from a variety of tissues and species (Lobb et al., 1986a). HBGF's can be grouped into two classes on the basis of a number of criteria (Lobb et al., 1986b). Class 1 HBGF's are found in high levels in neural tissue and are typified by bovine acidic brain FGF (Thomas et al., 1984). Class 2 HBGF's are found in a variety of tissues and are exemplified by bovine pituitary FGF (Gospodarowicz et al., 1984) and cartilage-derived growth factor (Sullivan & Klagsbrun, 1985).

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[1] Abbreviations: HBGF, heparin-binding growth factor; RS-HBGF, reduced and S-sulfopropylated heparin-binding growth factor; FGF, fibroblast growth factor; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Cys-Sp, *S*-(sulfopropyl)cysteine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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