Effect of 5'-deoxy-5'-S-isobutylthioadenosine on putrescine uptake and polyamine biosynthesis by chick embryo fibroblasts

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(Received 1 March 1982/Accepted 18 March 1982)

The effect of 5'-deoxy-5'-S-isobutylthioadenosine (SIBA) on polyamine biosynthesis has been studied by using cultured chick embryo fibroblasts. It has been shown that the drug inhibits the uptake of [^{14}C]putrescine and its conversion into labelled spermidine or spermine. The inhibitory effect is reversed by removing the inhibitor after exposing the cells to the drug for 24 h. SIBA also caused a significant decrease in cellular spermine levels and an accumulation of putrescine. These changes are reversed by removing the inhibitor. SIBA had the same effect on chick embryo fibroblasts transformed by Rous sarcoma virus; a decrease in cellular spermine levels in SIBA-treated cells was observed. In all the experiments SIBA caused a reduction in the spermine/putrescine and spermidine/putrescine ratios. It is suggested that SIBA is not only an inhibitor of transmethylation but also interferes with polyamine biosynthesis, probably by blocking aminopropyltransferase.

SIBA is a nucleoside that resembles both SAH and SAM in its structure (Fig. 1). The latter is an important metabolite that plays a central role in various enzymic reactions. Numerous studies demonstrated the involvement of SAM in the transmethylation reactions, by serving as a methyl donor (Cantoni, 1977). Transmethylation has been implicated in gene expression (Both *et al.*, 1975; Razin & Riggs, 1980; Lindahl, 1981) and in controlling neoplastic growth (Mittelman *et al.*, 1967; Mandel *et al.*, 1969; Borek, 1971; Berquist & Mathews, 1972; Paik *et al.*, 1972).

Transmethylation, however, is not the only metabolic pathway involving SAM. It has been well established that it also serves as an aminopropyl donor in the synthesis of polyamines from putrescine. Polyamines are known as organic cations that regulate growth, development and differentiation (Bachrach, 1973; Cohen, 1971; Jänne *et al.*, 1978).

The similarity in the structure of SIBA and SAH (Fig. 1) led to the suggestion that SIBA might inhibit transmethylation. Studies in our laboratory (Robert-Géro *et al.*, 1975, 1980; Vedel *et al.*, 1978; Enouf *et al.*, 1979) and elsewhere (Jacquemont & Huppert, 1977; Casellas & Jeanteur, 1978) indeed

demonstrated the inhibition of transmethylation by SIBA.

It was reasonable to assume that transmethylation is not the only metabolic pathway affected by SIBA and that polyamine biosynthesis might also be inhibited by this compound, which is also a structural analogue of SAM.

In the present paper we demonstrate that SIBA inhibits polyamine biosynthesis by cultured chick embryo fibroblasts. This nucleoside also inhibits the uptake of radioactive putrescine in these cells.

Experimental

Materials

SIBA was synthesized by Dr. P. Blanchard in our laboratory by the method of Hildesheim *et al.* (1971).

[1,4- ^{14}C]Putrescine (sp. radioactivity 60 Ci/mol, 50 $\mu\text{Ci/ml}$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. X-ray films (X-ray, no screen) and t.l.c. silica-gel plates (Eastman Chromatogram Sheet, 100 μm thick; 13179) were from Kodak.

Methods

Cell cultures. Secondary cultures from 11-day-old lymphomatosis-free Brown Leghorn chick embryos were prepared and cultivated as described previously (Goldé & Vigier, 1961). The virus used was

Abbreviations used: SIBA, 5'-deoxy-5'-S-isobutylthioadenosine; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; MTA, 5'-S-methylthioadenosine.

Fig. 1. Structure of SIBA and SAM

a clonal isolate (SR4) of Schmidt-Ruppin strain of Rous sarcoma virus type D. Infection was carried out with adequate amounts of virus to obtain 80% transformation in 3–4 days at 37°C. Cells were cultivated in Eagle's medium supplemented with glutamine, antibiotics and 5% newborn-calf serum. All experiments were performed in duplicate. At 1 day after seeding, fresh medium, with or without SIBA, was added to all cultures. After different exposure times the medium was aspirated off and cells washed twice with 10 ml of cold phosphate-buffered saline. Cells were then scraped off in 0.5 ml of water. Samples were freeze-dried and kept at –20°C until analysed. For reversal experiments cells were treated with SIBA for 24 h, and the medium was then removed and fresh medium without drug was added for 24 h. Cells were then treated as above.

Cell viability was estimated by the Trypan Blue exclusion method (Gorer & O'Gorman, 1956).

Labelling with [¹⁴C]putrescine

Chick embryo fibroblasts (1.5×10^6 cells) were seeded on to 10 cm-diameter Petri dishes and incubated at 37°C for 72 h. The labelling with putrescine was carried out by adding 20 μl of sterile [¹⁴C]putrescine to 10 ml of medium. After incubation at 37°C for 15 min, 1.0 ml of concentrated SIBA stock solution in Eagle's medium was added to each plate to final concentrations of 0.5 mM and

1.0 mM respectively. In the control experiment 1 ml of Eagle's medium was added instead. After different exposure times the cells were treated as above. After Lin *et al.* (1978) we define the uptake as the amount of radioactivity derived from the labelled putrescine within the cell, without discriminating between simple diffusion, cross-membrane transport, metabolic conversion or covalent incorporation.

Polyamine analysis

To each sample of freeze-dried cells, 0.5 ml of 3% HClO₄ was added. After shaking, insoluble material was sedimented by centrifugation at 6000 rev./min for 10 min. The supernatant fluid, which contained the extracted polyamines, was dansylated by the method of Seiler (1970). T.l.c. plates (silica gel G) were run in cyclohexane/ethyl acetate (3:2, v/v) as solvent. Material corresponding to standards of dansyl-polyamines was scraped off the plates from the respective spots, extracted with 3.0 ml quantities of ethyl acetate and assayed fluorimetrically as described by Bachrach (1980).

Radioactive material was similarly treated, except that plates were exposed to X-ray films (for 14 days), which were then developed. Material was also scraped off the plates as described above, suspended in scintillation fluid (Bray, 1960) and counted for radioactivity.

The total radioactivity of each sample was measured by suspending 10 μl portions of the original cellular suspension in scintillation fluid (Bray, 1960) and counting its radioactivity.

Proteins were determined by dissolving the HClO₄ precipitate in 1.0 M-NaOH, followed by a colorimetric assay (Lowry *et al.*, 1951).

Results

Effect of SIBA on the uptake of [¹⁴C]putrescine and its conversion into labelled polyamines

Fig. 2 shows that untreated chick embryo fibroblasts incorporated [¹⁴C]putrescine and that the amount taken up increased with incubation time. It is also evident that SIBA inhibited the uptake of the radioactive diamine and that the inhibition was dose-dependent (Fig. 2).

After showing that [¹⁴C]putrescine is taken up by chick embryo fibroblasts and demonstrating the inhibition of this reaction by SIBA, it was of interest to find out whether [¹⁴C]putrescine is further metabolized by the cells and whether this reaction is also affected by SIBA. [¹⁴C]Putrescine and SIBA were added to the cells as described above and polyamines were then extracted from the cells with HClO₄. Dansyl-derivatives were prepared as described under 'Methods'. They were then separated

Fig. 2. Effect of SIBA on the uptake of [^{14}C]putrescine by cultured chick embryo fibroblasts

Chick embryo fibroblasts (1.5×10^6 cells) were seeded on to 10cm Petri dishes and incubated at 37°C for 72h. [^{14}C]Putrescine ($1\mu\text{Ci}$; sp. radioactivity 60Ci/mol) was then added to the cultures. After 15 min, SIBA was added to some cultures. ●, Control experiment; ■, cells exposed to 0.5mM -SIBA; □, 1.0mM -SIBA.

by t.l.c. on silica-gel plates, which were finally subjected to autoradiography.

Fig. 3 is an autoradiogram that shows that [^{14}C]putrescine was readily converted into [^{14}C]spermidine and [^{14}C]spermine in untreated cells. This reaction was time-dependent and more significant at 48h than after 24h of incubation (samples 2 and 5). It may be seen (Fig. 3) that SIBA inhibited the conversion of [^{14}C]putrescine into [^{14}C]spermidine or [^{14}C]spermine. As expected, the degree of inhibition was higher when 1.0mM -SIBA (samples 4 and 7) was used, compared with a concentration of 0.5mM (samples 3 and 6).

Fig. 3 also shows that the synthesis of some other radioactive compounds was very efficiently inhibited by SIBA. The dansyl-derivatives of those unidentified compounds migrated on the chromatograms below dansyl-spermine.

To quantify the results, radioactive materials, corresponding to dansyl-polyamines, were scraped off the respective spots and counted for radioactivity. The ratio of the radioactivities of the various spots is given in Table 1. It may be seen that the formation of [^{14}C]spermidine and [^{14}C]spermine from [^{14}C]putrescine was markedly inhibited by SIBA. This is well reflected by a significant decrease

Fig. 3. Effect of SIBA on the conversion of [^{14}C]putrescine into [^{14}C]spermidine and [^{14}C]spermine

Chick embryo fibroblasts were incubated with [^{14}C]putrescine in the absence (lanes 2, 5 and 8) or in the presence of 0.5mM -SIBA (lanes 3 and 6) or 1.0mM -SIBA (lanes 4 and 7). Incubation was carried out for 24h (lanes 2–4) or 48h (lanes 5–7). In the reversion experiment (lanes 8–10) cells were treated with either 0.5mM -SIBA (lane 9) or 1.0mM -SIBA (lane 10) for 24h. The drug was then removed and cultures (lanes 8–10) were incubated without the inhibitor for another 24h. Abbreviations: PUT, putrescine; SPD, spermidine; SP, spermine; STD, standards. The Figure shows an autoradiogram. O = origin.

Table 1. *Effect of SIBA on the biosynthesis of ^{14}C -labelled polyamines from ^{14}C putrescine by cultured chick embryo fibroblasts*

Cells were seeded at a density of 1.5×10^6 cells per 10 cm-diameter dish and grown for 72 h. Labelling was carried out as described under 'Methods'. For reversal, cultures were treated with the inhibitor at final concentrations of 0.5 mM and 1.0 mM for 24 h. The culture medium, with the inhibitor, was then removed and cultures were incubated with fresh medium (without inhibitor) for another 24 h.

Treatment	Ratio of radioactive polyamines					
	Spermidine/putrescine		Spermine/putrescine		Spermine/spermidine	
	(c.p.m.)	(% of control)	(c.p.m.)	(% of control)	(c.p.m.)	(% of control)
Control (24 h)	3.60	—	0.83	—	0.23	—
SIBA (0.5 mM, 24 h)	2.9	80	0.52	62	0.18	78
SIBA (1.0 mM, 24 h)	2.6	72	0.53	64	0.20	87
Control (48 h)	4.8	—	2.34	—	0.49	—
SIBA (0.5 mM, 48 h)	2.8	58	1.16	49	0.42	86
SIBA (1.0 mM, 48 h)	2.0	41	0.66	28	0.33	67
Reversal (0.5 mM-SIBA)	8.7	187	5.37	229	0.61	124
Reversal (1.0 mM-SIBA)	9.2	191	6.05	258	0.65	132

in the spermidine/putrescine and spermine/putrescine ratios. It is noteworthy that a similar inhibition was observed after exposing the cells to either 1.0 mM- or 0.5 mM-SIBA for 24 h. The dose-dependent effect was mainly manifested after drug treatment for 48 h. It may also be seen (Table 1) that the effect of SIBA on chick embryo fibroblasts was reversible and that removal of the drug after 24 h resulted in an increased polyamine biosynthesis.

The above results indicate that SIBA inhibits the conversion of radioactive putrescine into radioactive polyamines. This is further reinforced by the following. Since a lowered uptake of labelled putrescine would lead to a decreased specific radioactivity of the intracellular putrescine pool and consequently to a decreased incorporation of radioactivity into spermidine and spermine, we calculated the specific radioactivities of these polyamines. After 48 h of incubation, specific radioactivities in the control experiment were 2360, 6160, 3109 c.p.m./nmol of putrescine, spermidine and spermine respectively. After exposure to 1.0 mM-SIBA, the values were 571, 945, 523 for putrescine, spermidine and spermine. If the synthesis of polyamine is not inhibited, the ratio of specific radioactivities should be similar in the SIBA-treated cells and the control. The ratios of specific radioactivities of spermidine/putrescine were 2.6 and 1.6 and of spermine/putrescine were 1.3 and 0.91 for the control and drug-treated cells respectively. Thus the decreased incorporation of radioactivity into spermidine and spermine is not just a reflection of inhibited uptake of the putrescine in the presence of SIBA.

Effect of SIBA on cellular polyamine content

The above described experiments indicated that SIBA inhibited the conversion of radioactive put-

rescine into radioactive polyamines. To confirm the assumption that SIBA-treated cells contained less polyamines than the untreated controls, the following experiment was carried out. Chick embryo fibroblasts were cultured and treated with SIBA as described above. Polyamines were then extracted, dansylated and estimated as described under 'Methods'. The cellular content of polyamines was expressed as nmol/mg of protein to avoid complications stemming from possible cell growth inhibition by the drug.

It is possible that inhibition of cell growth could influence the rate of polyamine synthesis, but at present we do not know which event is first (variation of cell growth or variation of polyamine synthesis).

Results of a typical experiment are given in Table 2, which shows that SIBA inhibited the growth of chick embryo fibroblasts mainly after exposure for 48 h.

This inhibition was reversed by removing the drug. SIBA caused the accumulation of putrescine and a decrease in cellular spermine levels. Removal of the drug caused a reduction in putrescine content (Table 2).

When chick embryo fibroblasts were transformed by Rous sarcoma virus and then treated with SIBA the following picture emerged (Table 2). As expected (Bachrach *et al.*, 1973; Don & Bachrach, 1975; Bachrach & Seiler, 1981) transformed chick embryo fibroblasts were richer in putrescine. As in the normal chick embryo fibroblasts, SIBA caused a significant decrease in cellular spermine levels.

Changes in cellular polyamines might also be expressed in terms of their molar ratios. When this was done, a consistent decrease in spermidine/putrescine or spermine/putrescine ratios was observed, when normal chick embryo fibroblasts were

Table 2. *Effect of SIBA on polyamine content in cultured chick embryo fibroblasts*

Normal or Rous-sarcoma-virus-transformed cultures (1.3×10^6 cells, per 35 mm-diameter Petri dish) were treated with the inhibitor for 24 or 48 h. For reversal, the culture medium with inhibitor was removed after 24 h and cultures were incubated with fresh medium (without inhibitor) for another 24 h. Values in parentheses indicate cell viability (%).

Treatment	Polyamines per sample (nmol)			Protein per sample (mg)	Polyamines (nmol/mg of protein)		
	Putrescine	Spermidine	Spermine		Putrescine	Spermidine	Spermine
Normal cells							
Control (24 h)	2.0	3.4	2.3	0.60 (96)*	3.3	5.6	3.8
SIBA (0.5 mM, 24 h)	2.6	3.4	1.8	0.55 —	4.7	6.2	3.3
SIBA (1.0 mM, 24 h)	3.3	3.8	2.5	0.70 (95)*	4.7	5.4	3.6
Control (48 h)	1.8	3.3	3.2	0.85 (98)*	2.1	3.9	3.8
SIBA (0.5 mM, 48 h)	1.4	2.6	1.3	0.50 —	2.8	5.2	2.6
SIBA (1.0 mM, 48 h)	1.8	2.2	1.3	0.50 (97)*	3.6	4.4	2.6
Reversal (0.5 mM-SIBA)	1.3	3.8	2.7	0.75	1.7	5.1	3.6
Reversal (1.0 mM-SIBA)	1.7	4.0	2.5	0.80	2.1	5.0	3.1
Transformed cells							
Control (24 h)	2.9	2.0	1.4	0.30 (96)*	9.6	6.8	4.7
SIBA (0.5 mM)	2.0	1.8	1.0	0.28 —	7.2	6.3	3.6
SIBA (1.0 mM)	2.5	1.9	1.1	0.28 (99)*	8.4	6.2	3.7

* Cell viability was measured by the Trypan Blue method.

Table 3. *Effect of SIBA on polyamine ratio in cultured chick embryo fibroblasts*

Cells were grown as described in the legend to Table 1. Expt. A: 0.8×10^6 cells per 35 mm Petri dish. Expt. B: 1.3×10^6 cells per 35 mm Petri dish. In the reversal experiments culture medium was removed after 24 h and all the cultures were subsequently incubated with fresh medium (without inhibitor) for another 24 h.

Treatment	Polyamine ratio					
	Spermidine/putrescine		Spermine/putrescine		Spermine/spermidine	
	(nmol)	(% of control)	(nmol)	(% of control)	(nmol)	(% of control)
Expt. A						
Control (24 h)	2.8	—	0.87	—	0.31	—
SIBA (0.5 mM, 24 h)	3.1	111	0.76	87	0.25	81
SIBA (1.0 mM, 24 h)	1.8	64	0.50	75	0.27	95
Control (48 h)	3.0	—	1.33	—	0.44	—
SIBA (0.5 mM, 48 h)	1.6	53	0.69	52	0.42	95
SIBA (1.0 mM, 48 h)	0.96	32	0.42	32	0.44	100
Reversal (control)	2.27	—	0.86	—	0.38	—
Reversal (0.5 mM-SIBA)	2.90	127	0.86	100	0.30	79
Reversal (1.0 mM-SIBA)	0.74	33	0.16	19	0.17	45
Expt. B						
Control (24 h)	3.74	—	0.78	—	0.21	—
SIBA (0.5 mM, 24 h)	3.32	89	0.68	87	0.20	95
SIBA (1.0 mM, 24 h)	1.54	41	0.39	50	0.25	119
Control (48 h)	3.18	—	1.18	—	0.37	—
SIBA (0.5 mM, 48 h)	1.17	37	0.41	35	0.35	95
SIBA (1.0 mM, 48 h)	1.40	44	0.50	42	0.36	98
Reversal (control)	3.47	—	1.37	—	0.39	—
Reversal (0.5 mM-SIBA)	3.35	96	1.29	94	0.38	97
Reversal (1.0 mM-SIBA)	4.40	127	0.91	66	0.21	54

analysed (Table 3). Again, the inhibition was most significant after treating the cells with SIBA for 48 h. The effect was reversed (mainly in sub-confluent cultures) by removing the drug. Sub-confluent cultures of chick embryo fibroblasts behaved like

non-confluent cultures; the polyamine content was similarly affected by SIBA (Table 3).

SIBA also affected polyamine biosynthesis in chick embryo fibroblasts transformed by Rous sarcoma virus (Table 4). In general, transformed

Table 4. *Effect of SIBA on polyamine ratio in chick embryo fibroblasts transformed by Rous sarcoma virus*
 Infected chick embryo fibroblasts (2.5×10^6 cells) were seeded on to 10 cm-diameter Petri dishes and grown for 72 h; then cells were treated with SIBA as described under 'Methods'. In the reversal experiments culture medium was removed after 24 h and all the cultures were subsequently incubated with fresh medium (without inhibitor) for another 24 h.

Treatment	Polyamine ratio					
	Spermidine/putrescine		Spermine/putrescine		Spermine/spermidine	
	(nmol)	(% of control)	(nmol)	(% of control)	(nmol)	(% of control)
Control (24 h)	1.48	—	0.48	—	0.33	—
SIBA (0.5 mM, 24 h)	1.44	97	0.44	92	0.31	94
SIBA (1.0 mM, 24 h)	1.24	84	0.27	56	0.22	66
Control (48 h)	1.28	—	0.53	—	0.41	—
SIBA (0.5 mM, 48 h)	0.81	63	0.21	42	0.26	63
SIBA (1.0 mM, 48 h)	0.56	44	0.16	30	0.28	68
Reversal (control)	3.17	—	1.29	—	0.41	—
Reversal (0.5 mM-SIBA)	5.50	173	1.08	84	0.20	49
Reversal (1.0 mM-SIBA)	3.00	95	0.61	47	0.20	49

cells contain more putrescine than their normal controls (Bachrach *et al.*, 1973; Don & Bachrach, 1975). Therefore, the spermidine/putrescine ratio values of the transformed cells should be lower than those of the normal controls. This is indeed the case (cf. Tables 3 and 4). Nevertheless, SIBA caused a further reduction in spermidine/putrescine and spermine/putrescine ratios, mainly after treating the cells with the drug for 48 h (Table 4). A decrease in spermine synthesis was observed by incubating the transformed cells with 1.0 mM-SIBA for 24 h (Table 4).

Discussion

Results of experiments described in the present paper suggest that SIBA is not a specific inhibitor of transmethylation. It appears that other reactions, related to the metabolism of SAM, are also affected by the drug. The mechanism of action of SIBA, which is known to inhibit oncogenic virus-induced cell transformation, virus replication etc. (see review by Robert-Géro *et al.*, 1980), cannot be explained exclusively by the inhibition of transmethylation. Our studies strongly suggest that SIBA inhibits the conversion of putrescine into spermidine and/or spermine (Table 1) and that this inhibition is a reversible one. Because of this inhibition, putrescine tends to accumulate in the treated cells (Table 2). The cellular content of putrescine is rigorously regulated and further uptake of [^{14}C]putrescine by putrescine-rich cells is not readily facilitated. The inhibition of [^{14}C]putrescine uptake by SIBA-treated cells (Fig. 2) may be explained by the elevated cellular putrescine levels in the presence of the drug. These relatively high concentrations of putrescine may prevent further uptake.

Another possible explanation for the inhibition of putrescine uptake by SIBA is the action of the drug on membrane function. This nucleoside has been shown to inhibit the uptake of chemically unrelated molecules such as 2-deoxyglucose and uridine into chick embryo fibroblasts (Pierré & Robert-Géro, 1979).

The accumulation of putrescine in SIBA-treated cells may be due either to a decrease in the rate of its conversion into spermidine and spermine, or, alternatively, by enhanced biosynthesis. The activation of ornithine decarboxylase, which catalyses the synthesis of putrescine, by various drugs and among them some purine analogues, has been documented (Canellakis & Theoharides, 1976; Costa, 1978). Furthermore it has recently been reported (Schorderet-Slatkine *et al.*, 1981) that in *Xenopus* oocytes, SIBA causes an increase in the intracellular level of cyclic AMP. This phenomenon is explained by an activation of membrane-bound adenylate cyclase. As cyclic AMP regulates ornithine decarboxylase activity (Bachrach, 1975; Byus & Russell, 1975), one could postulate that SIBA increases the activity of ornithine decarboxylase by changing the cellular levels of cyclic AMP.

Chick embryo cells treated with SIBA are rich in putrescine (Table 2) but this diamine is not effectively metabolized to spermidine or spermine (Table 1 and Fig. 3). These findings may suggest that the drug or one of its metabolites inhibits polyamine biosynthesis by blocking the aminopropyltransferase reactions.

To measure the activity of these enzymes the protein has to be purified (at least partially) to avoid the degradation of decarboxylated SAM and to prevent the metabolism of the products of the reaction. Because of the limited number of cells,

cultured chick embryo fibroblasts cannot be used as the source of enzyme. However, the effect of SIBA on purified spermine synthase and spermidine synthase from rat ventral prostate has been documented by Pegg *et al.* (1981). Under their conditions of assay 0.5 mM-SIBA inhibits spermidine synthase activity at 45% and spermine synthase activity at 88%.

It is not yet clear whether SIBA can serve alone or in combination with other compounds as a useful chemotherapeutic agent. A better understanding of its mode of action might permit such an approach, which will take into account the processes blocked by each drug.

These investigations were supported in part by the Ligue Nationale Française contre le Cancer, and by the Foundation de la Recherche Médicale Française. The valuable assistance of Mrs. H. Wiener is gratefully acknowledged.

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