INHIBITION OF NUCLEOSIDE AND SUGAR TRANSPORT INTO CELLS BY AN ONCOSTATIC METHYLASE INHIBITOR, 5′-DEOXY-5′-*S*-ISOBUTYL-THIOADENOSINE (SIBA)

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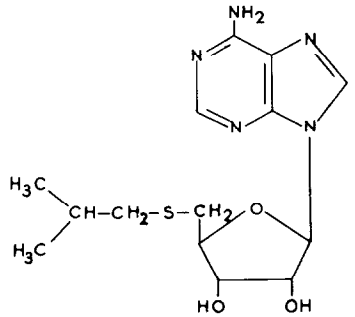
1. Introduction

Viral transformation of normal cells in vitro is accompanied by a wide variety of chemical and biological alterations of cell surface, including cross membrane transport [1]. For instance, Rous sarcoma virus (RSV)-transformed chick embryo fibroblasts (CEF) take up 2-deoxy-D-glucose (2-DG) or 3-*O*-methyl-D-glucose much more rapidly than normal cells [2]. This is due to a structural change in the plasma membrane [3] and assumed to be an early event following infection [4].

In view of studying the relationship between transmethylation and oncogenic cell transformation, many structural analogues of *S*-adenosylhomocysteine (SAH) have been synthesized in our laboratory [5]. One of these, 5′-deoxy-5′-*S*-isobutylthioadenosine (SIBA)* was shown to inhibit cell transformation and replication of several oncogenic RNA and DNA viruses [6,7] as well as methylation of tRNA and proteins [8,9]. When the effect of SIBA on intracellular protein and nucleic acid synthesis was studied, a great decrease of the uptake of some radioactive precursors was observed. We thus examined the effect of SIBA on the uptake of nucleosides and of 2-DG in normal and RSV-transformed CEF.

Our results show that the transport process, rather than the subsequent phosphorylation of these molecules, is inhibited by the drug. The inhibition is reversible and identical in normal and transformed cells. However, the increase of 2-DG uptake by transformed cells is irreversibly inhibited by SIBA, when the drug remains in contact for 24 h with infected cells. As this compound inhibits the transport of structurally unrelated molecules, its effect may be due to an interaction with a membrane constituent, involving alterations in the transport systems of the cell.



SIBA

* SIBA has been patented by ANVAR (Agence Nationale pour la Valorisation de la Recherche, patent no. 75 177 972 (1975))

2. Materials and methods

2.1. *Cells and viruses*

Growth of secondary CEF, in 35 mm Petri dishes, the infection by RSV and the media used were as in [6]. Uptake experiments were performed with 6×10^5 cells/dish corresponding to 150 μg protein.

2.2. *Inhibitors*

SIBA and SAH are products of Sefochem Fine Chemicals, Israel. 5′-deoxy-5′-*S*-isobutylthioinosine (SIBI), 5′-deoxy-5′-*S*-methyl-allylthioadenosine (SMAA) and 5′-deoxy-5′-*S*-isobutylthiouridine (SIBU) were synthesized in our laboratory by Dr P. Blanchard and Dr M. Vuilhorgne. 3-deaza-SIBA (3d-SIBA) [10] was a gift of Dr P. K. Chiang, NIH, Bethesda, MD.

5-[^{3}H]uridine (U) 28 Ci/mM, [*methyl*-^{3}H]thymidine (T) 49 Ci/mM were purchased from the Commissariat à l'Energie Atomique, Saclay, France.

2.3. *Nucleoside uptake*

This was measured by adding the adequate amount of radioactive precursor in 1 ml culture medium or phosphate-buffered saline (PBS) to each dish. After incubation at 37°C or at 20°C the liquid was removed, cells rinsed with cold PBS, and 1 ml cold 5% trichloroacetic acid (TCA) was added per dish for 15 min. Radioactivity was counted in an aliquot of this (TCA)-soluble pool. Incorporation was then estimated after hydrolysing the cells in 1 ml 0.5 N NaOH at 80°C for 15 min.

2.4. *Transport of nucleosides*

This was measured after incubating the cells in 50 mM 2-DG at 37°C for 30 min, followed by 2 washes with PBS. This treatment reduces the intracellular level of ATP by ~70%. Nucleosides cannot be phosphorylated under these conditions, so the intracellular radioactivity corresponds to transport [11]. The device used for the rapid labelling experiments ($\leqslant$ 5 s) developed in our laboratory will be described elsewhere.

2.5. *The phosphorylated intermediates of nucleosides*

These were identified as in [12].

2.6. *Uridine and thymidine kinase*

Activity was measured in cell-free extracts by the method in [13].

2.7. *Hexose uptake*

Cultures were supplemented with 1 or 2 μCi 2-D[^{3}H]G (20 Ci/mM, Amersham) in 1 ml PBS and incubated for 10 min at 20°C. Tracer was then removed, cells were washed with cold PBS and total sugar uptake was determined by measuring the radioactivity in 100 μl cell hydrolysate as described for nucleosides.

2.8. *Phosphorylation of 2-DG*

This was measured in cells incubated at 37°C for 10 min with 2 mM 2-DG containing 2 μCi/ml of labelled sugar. The total amount of 2-DG + 2-DG-6-phosphate (2-DG-6-P) was solubilized in 1 ml boiling water for 1 min [14]. Radioactivity in 50 μl of this fraction was counted. The amount of free sugar was estimated after selective precipitation of phosphorylated sugar with $BaSO_4$ [3].

2.9. *Hexokinase*

Activity was measured in cell-free extracts as in [3]. Proteins were determined by the Lowry procedure [15].

3. Results and discussion

Experiments on the effect of SIBA on nucleic acid synthesis showed that the molecule interferes strongly with the uptake of radioactive precursors into the cells. As shown in fig.1a the uptake of [^{3}H]U into TCA-soluble and -insoluble cell fraction is affected to the same extent by different concentrations of SIBA;

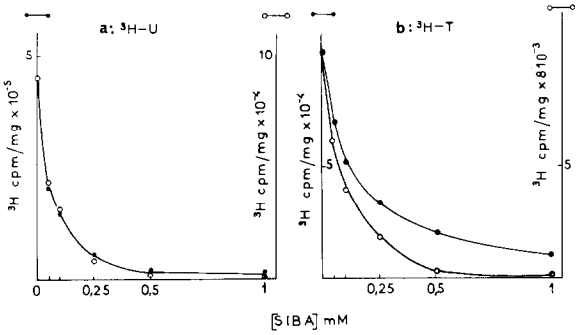


Fig.1. Effect of increasing concentrations of SIBA on (a) uridine (U) and (b) thymidine (T) uptake by normal CEF. Tritiated precursor (2 μCi) in 1 ml of Eagle's medium with the indicated amount of SIBA were added to each plate and incubated at 37°C for 30 min. The cells were rinsed, and radioactivity in TCA-soluble (●———●) and TCA-precipitable (○———○) fraction counted. The same results were obtained with transformed cells. Results are expressed in cpm/mg protein.

half-maximal inhibition was reached with 50 μM. The uptake of [^{3}H]T into TCA-soluble material is inhibited to a lesser degree than incorporation into TCA-insoluble fraction. The half maximal inhibition was reached, respectively, in the two fractions with 100 μM and 75 μM SIBA. Thus in this case an effect on DNA synthesis cannot be ruled out (fig.1b). The phosphorylation of both nucleosides was then studied in vitro and in whole cultures (table 1A).

Table 1A
Effect of SIBA on phosphorylation of uridine and thymidine in whole cells

Metabolites	Untreated cpm (% total)	Treated cpm (% total)
U	2572 (53)	1002 (75)
UMP	264 (5)	46 (3)
UDP	1407 (29)	214 (16)
UTP	613 (13)	64 (5)
Total counts	4856 (100)	1326 (100)
T	95 (1)	115 (14)
TMP	3210 (53)	502 (59)
TDP	856 (14)	72 (8)
TTP	1854 (31)	155 (18)
Total counts	6015 (100)	844 (100)

Cultures of normal and transformed cells were exposed to Eagle's medium containing 0.5 μM [^{3}H]T or 5 μM [^{3}H]U (4 μCi/ml) with 1 mM and 0.5 mM, respectively, or without SIBA. Incubation time at 37°C was 15 min (normal cells) and 30 min (transformed cells). Monolayers were rinsed with PBS, extracted with perchloric acid 0.5 N and the extract chromatographed with unlabelled carriers. Spots were localized, extracted with H_2O and counted

Table 1B
Effect of SIBA on phosphorylation of uridine and thymidine on the two kinases in vitro

SIBA (mM)	Uridine kinase (pmol/mg protein/30 min)	Thymidine kinase (pmol/mg protein/30 min)
0	3.0	6.2
0.10	3.2	6.3
0.25	3.5	6.1
0.50	3.3	6.8
1.25	2.9	6.8

Cell free extracts of normal and transformed cells were assayed for uridine and thymidine kinase activity by measurement of the amount of tritiated phosphorylated metabolites retained on positively charged filters, in the presence and absence of SIBA

The reduced level of phosphorylated intermediates was at first thought to be due to the inhibition of the kinases by SIBA. However, as shown in table 1B the drug has no effect on these enzymes in vitro even at 1.25 mM. Considering this fact we conclude that there is no direct action of SIBA on phosphorylating enzymes. We therefore measured the effect of the drug on nucleoside transport under conditions where no phosphorylation takes place, i.e., when there is rapid labelling and ATP depletion. Rapid labelling experiments (fig.2) demonstrated that 0.5 mM SIBA inhibits completely the transport of both nucleosides as early as 15 s. In table 2 the effects of different concentrations of SIBA are compared on nucleoside transport process in phosphorylating and non-phosphorylating (2-DG-treated) cells. With ⩽ 500 μM (0.5 mM) SIBA the inhibitions are identical and very strong (reaching 84–95% at 500 μM) indicating that this compound affects mostly the transport process.

Thus, SIBA has doubtlessly a very strong and quick effect on nucleoside transport. We can not explain at this stage of our work the reason for the reduced level of phosphorylated nucleosides in SIBA-treated cells. As the kinases are not affected this may be due to an indirect, unknown effect of the drug.

One of the characteristic features of RSV-trans-

Table 2
Inhibition by SIBA of uridine and thymidine uptake in untreated (a) and ATP-depleted cells (b)

SIBA (μM)	U		I	
	a	b	a	b
–	20 960 (–)	3215 (–)	100 950 (–)	11 560 (–)
25	11 110 (47)	2515 (22)	56 745 (44)	7685 (34)
50	9010 (57)	1350 (58)	38 410 (58)	5200 (55)
100	6915 (67)	1120 (65)	30 520 (70)	3755 (68)
200	4610 (78)	875 (73)	18 171 (82)	2312 (80)
500	1675 (92)	175 (95)	13 123 (87)	1849 (84)

Cells were incubated with 50 mM 2DG at 37°C for 30 min and labelled with 10 μCi/ml of U or T at 20°C for 30 s, (b) in order to measure the transport process alone. Phosphorylating cells were labelled for 10 min with 2 μCi/ml of U or T at 20°C (a) in order to obtain maximum inhibition of all the events: transport, uptake and phosphorylation. SIBA at different concentrations was added with the precursor. Radioactivity was measured in TCA-soluble pool (> 95% of total radioactivity). Results are expressed in cpm/mg protein; % of inhibition are given in brackets

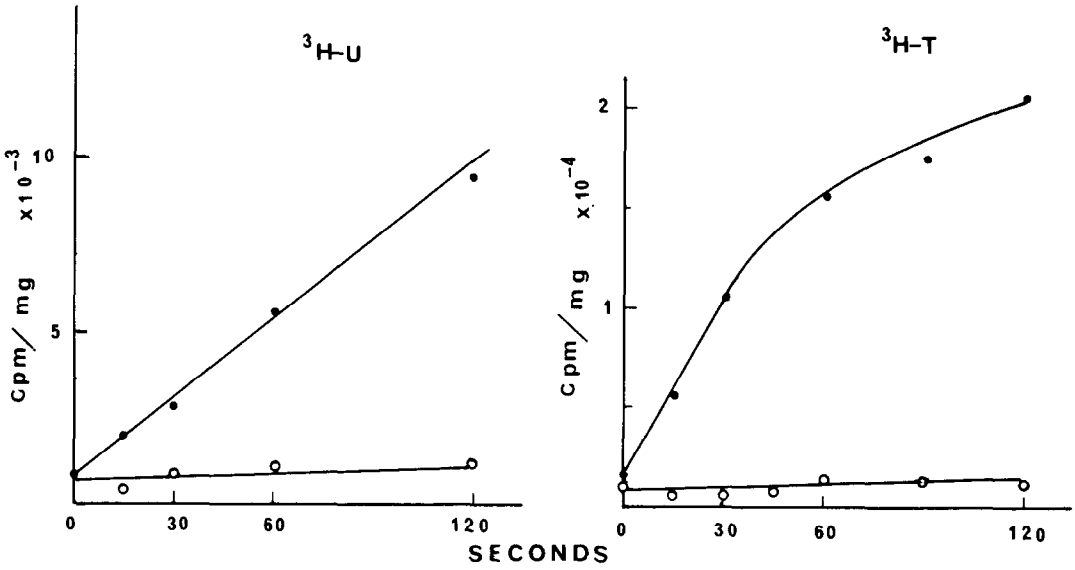


Fig.2. Transport of uridine and thymidine in ATP-depleted cells. Cells were incubated with 50 mM 2-DG in 1 ml PBS at 37°C for 30 min, rinsed 2 times with PBS at room temperature, and [^{3}H]U or [^{3}H]T (10 μCi/ml PBS) were added. Radioactivity in soluble pool, containing > 95% of total cellular radioactivity, counted. (•---•) Without SIBA; (○---○) 0.5 mM SIBA. Results are expressed in cpm/mg protein.

formed cells is the increased uptake of 2-DG [2]. In the presence of 1 mM SIBA sugar uptake in normal or transformed cells was lowered by ~80%. Phosphorylation in vitro was not affected. When the concentrations of free and phosphorylated 2-DG were determined in SIBA-treated cells, the proportion of 2 DG-6-P with respect to 2-DG did not change (table 3).

Table 3
Uptake and phosphorylation of 2-deoxy-D-glucose in whole cells and phosphorylation by a cell-free extract in the presence and in the absence of SIBA

		SIBA (mM)			
		0	0.25	0.5	1
Uptake	(nmol/mg protein)	122	97	73	40
Free 2-DG	(nmol/mg protein)	49	36	29	16
2DG-6-P	(nmol/mg protein)	73	61	44	24
$\frac{\text{2DG}}{\text{2DG-6P}}$		0.67	0.59	0.66	0.67
% inhibition of hexokinase in vitro		–	2	4	5

Normal cells were loaded with 2 mM 2-D[^{3}H]G (2 μCi/ml) in 1 ml PBS and incubated at 37°C for 10 min. Then the total sugar was solubilized with 1 ml boiling water, and the amount of free 2-DG was determined by selective precipitation of phosphorylated sugar with $BaSO_4$. Hexokinase activity in crude extracts was measured after 90 min incubation at 37°C. The reaction was stopped by $Ba(OH)_2$ and the free 2-DG in the supernatant determined as above

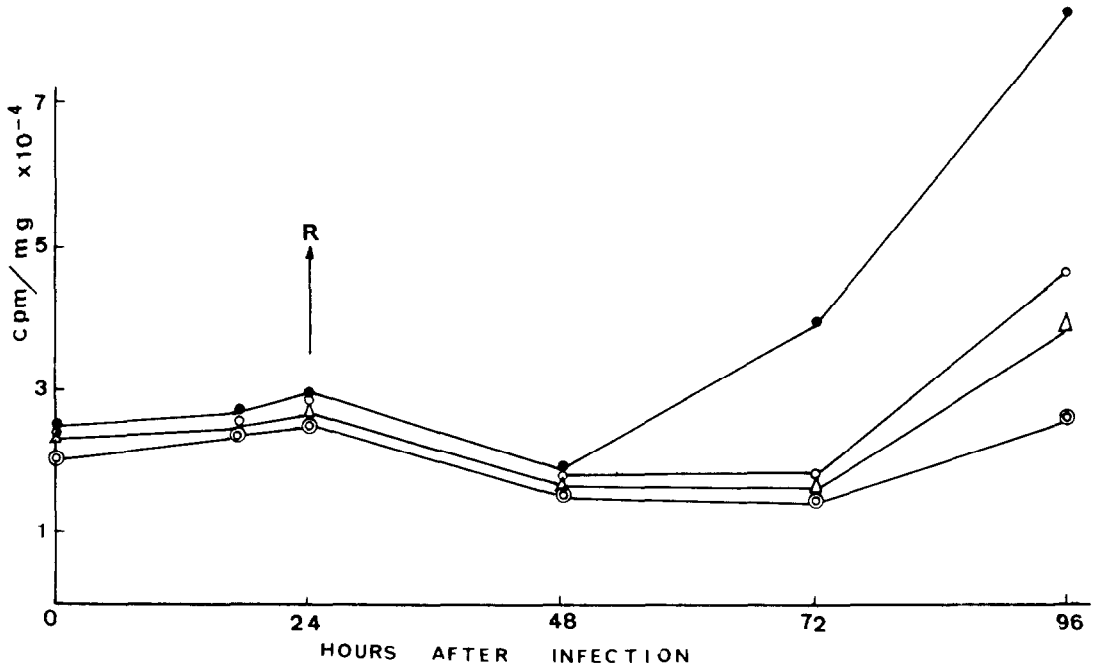


Fig.3. The irreversible inhibition by SIBA of the increase of 2-DG uptake following infection. Normal cells were infected with RSV (0 time). After the adsorption of the virus (1 h), the medium was removed and 1 ml Eagle's medium without (●———●) or with SIBA added: (○———○) 0.25 mM; (△———△) 0.5 mM; (⊙———⊙) 1 mM. After 24 h incubation the medium was removed from each dish (indicated by the arrow and R) and 1 ml medium without SIBA was added. Cells were further incubated at 37°C, and 2-DG uptake was measured at indicated times as follows: medium was removed, and 1 μCi 2-D[^{3}H]G in ml PBS was added. After 10 min incubation at 37°C, sugar uptake was measured as before. Results are expressed in cpm/mg protein.

The increase of sugar uptake by RSV-transformed CEF is thought to be due to structural changes in the cell membrane [3]. To know whether the effect of SIBA on transformation parallels its effect on increase of sugar transport, cells were infected with RSV in order to obtain fully transformed cells in 5 days. As shown in fig.3 the rate of 2-DG uptake begins to increase after 48 h. On day 3 several Rous cells appeared and 96 h after infection 60% of the cells were transformed. The rate of 2-DG uptake at this time was 3-fold higher than in normal cells. It was shown [6] that 1 mM SIBA after a 24 h contact with infected cells inhibits further transformation at least for 8 days. Thus, different amounts of SIBA were added for 24 h to infected cells and sugar uptake was measured in the absence of the drug. As shown in fig.3, with 1 mM SIBA no significant increase in 2-DG uptake occurred indicating that inhibition of cell transformation parallels the inhibition of increased

Table 4
Inhibition by adenosine (A), *S*-adenosylhomocysteine (SAH) and its synthetic analogues of the uptake of uridine and 2-deoxy-D-glucose and of cell transformation

Compounds	Uridine I_{50} (μM)	2-Deoxy-D-glucose I_{50} (mM)	Cell transformation I_{50} (mM)
A	120	> 10	n.i.
SAH	> 1000	> 10	> 1
SIBA	30	0.6	0.2
3d-SIBA	80	n.d.	0.1
SIBI	140	> 10	> 1
SIBU	300	1.5	n.i.
SMAA	10	0.7	0.1

Analogues are added with the radioactive precursor in PBS and incubated at 20°C for 10 min. Results are given in concentrations producing 50% inhibition (I_{50}). Concentrations were 0.01–1 mM for U uptake, 0.2–5 mM for 2-DG uptake and 0.05–1 mM for cell transformation. n.d., not done; n.i., no inhibition. The analogues are enumerated in section 2

uptake. The effect of SIBA was concentration dependent. In order to know whether the effect on membrane permeability is related to the oncostatic action, the I_{50} of several synthetic analogues on U and 2-DG uptake as well as their effect on cell transformation was determined. As shown in table 4 the best inhibitors of cell transformation have the lowest I_{50} concentration for both precursors. The fact that SIBA (and the other active analogues) inhibit the uptake of structurally unrelated molecules suggests an interaction between the drug and the cell surface. This can produce conformational changes in the membrane affecting to various extents transport processes and the virus receptors. The purification of a 'SIBA-binding membrane factor' is now under way in our laboratory.

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