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SHORT COMMUNICATIONS

Effect of S-Adenosylhomocysteine and S-Tubercidinylhomocysteine on Catecholamine Methylation in Neuroblastoma Cells

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SUMMARY

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Methylation of dopamine in neuroblastoma cells was studied by measuring the formation of [^{3}H]3-methoxytyramine from [^{3}H]dopamine. Analyses of the cellular extracts by high-pressure liquid chromatography afforded a means of monitoring cellular dopamine metabolism. *S*-Adenosylhomocysteine and the 7-deaza analogue, *S*-tubercidinylhomocysteine, both known to inhibit catechol *O*-methyltransferase *in vitro*, were used to block the methylation of dopamine in these cells. Both drugs inhibited the formation of 3-methoxytyramine, with an accompanying increase in the synthesis of a material tentatively identified as dopamine 3-sulfate. In this work and in previous work on tRNA methylation in phytohemagglutinin-stimulated rat lymphocytes [(1975) *Mol. Pharmacol.*, 11, 701-707], the 7-deaza analogue was consistently more effective than the natural product inhibitor, *S*-adenosylhomocysteine. These results are discussed in terms of possible differences in transport and/or metabolism of the drugs.

Circulating catecholamines are metabolized via oxidative deamination catalyzed by monoamine oxidase (EC 1.4.3.4) and/or methylation catalyzed by catechol *O*-methyltransferase (EC 2.1.1.6). The use of monoamine oxidase inhibitors to maintain elevated catecholamine levels *in vivo* is well documented (1). However, the consequences of inhibiting catecholamine methylation *in vivo* are not known. We have been developing a new type of catechol *O*-methyltransferase inhibitor based on the potent inhibition of this enzyme by one of its reaction products, *S*-adenosyl-L-homocysteine (2, 3). The most potent SAH[3]-like inhibitor of catechol *O*-methyltransferase developed in our laboratory (4) or others' (5) is the 7-deaza analogue, *S*-tubercidinylhomocysteine. We have shown that the 7-deaza analogue inhibits catechol *O*-methyltransferase with K_i = 30 μM, compared with K_i = 20 μM for SAH inhibition (4). In the present work we have used murine neuroblastoma cells as a model system, since they have many properties and contain many of the enzymes found in adrenergic and dopaminergic neurons (6), and the metabolism of catecholamines in these cells is well studied (7). The effect of the catechol *O*-methyltransferase inhibi-

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[3] The abbreviations used are: SAH, *S*-adenosylhomocysteine; STH, *S*-tubercidinylhomocysteine (7-deaza analogue of SAH).

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tors, SAH and its 7-deaza, analogue on the metabolism of [^{3}H]dopamine in neuroblastoma cells is the subject of this communication.

Dulbecco's modified Eagle's medium, phosphate-buffered NaCl, penicillin and streptomycin solution, and 2.5% trypsin solution were purchased from Grand Island Biological Company; fetal calf serum, from Flow Laboratories; trypan blue (direct blue 14), from Matheson, Coleman, and Bell; and plastic flasks and Petri dishes, from Falcon Plastics Company [^{3}H]Dopamine (2–10 Ci/mmole) was purchased from New England Nuclear Corporation. *S*-Adenosyl-L-homocysteine was obtained from Boehringer/Mannheim, and *S*-tubercidinyl-DL-homocysteine was synthesized in this laboratory (4). Adenosine was purchased from Nutritional Biochemicals Corporation, tubercidin was a gift of Dr. John Whitfield of the Upjohn Company, and DL-homocysteine was synthesized by Dr. Wolfgang Gunther. Nialamide, ascorbic acid, 3-methoxytyramine, vanillylmandelic acid (DL-4-hydroxy-3-methoxymandelic acid), and homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) were purchased from Sigma. Dopamine was obtained from Mann Research Laboratories, and L-norepinephrine, from Aldrich Chemical Company.

Stock cultures of N-18 and N1E-115 neuroblastoma cells were maintained in 75-cm^2 plastic flasks in growth medium supplemented with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2.2 g of sodium bicarbonate per liter in an atmosphere of 5% CO_2 and 95% air saturated with H_2O at 37°. Cells were subcultured by mechanically dislodging them from the plastic surface without the aid of trypsin. For each experiment, the cells were plated on 100-mm plastic Petri dishes at a concentration of 10^4–10^5 cells/ml, in 10 ml of medium supplemented with 10% fetal calf serum. After 4 days in this medium, the cells were fed with medium supplemented with 5% fetal calf serum and fed at 1–2-day intervals for 1–2 weeks prior to experimentation, at which time monolayers were 80% confluent (10^6–10^7 cells) and the majority of cells had formed extensive neurites. For viability studies, the medium was decanted off, and suspensions of cells were obtained by incubation of the monolayer cultures for 5 min at 37° in a solution of 0.25% trypsin in phosphate-buffered NaCl. The growth of the cell population was determined by counting the cells in a suspension with a hemocytometer or Coulter counter. The viability of the cells in suspension was determined after staining the cells with a solution of 0.4% trypan blue (8).

Immediately upon removal from the incubator, monolayers were rinsed gently three times with 1 ml of an isotonic modified Dulbecco's phosphate-buffered NaCl medium (9) containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM glucose, 46 mM sucrose, 0.1 mM ascorbic acid, and 0.02 mM nialamide, pH 7.2. Cells were incubated in this buffer (3 ml/dish) in an atmosphere of 5% CO_2–95% air for 90 min at 37°. In some experiments, cells were incubated for 5 min with drug (see Table 1) before incubation with [^{3}H]dopamine (10–20 μCi; 0.64–1.28 μM). Cell viability in the presence of 56 μM SAH and the 7-deaza analogue was shown to be at least 90% of drug-free controls grown under the conditions described above.

After incubation, the dishes were frozen (at approximately −4°) for at least 1 hr. The dishes were thawed at 37° on a water bath (or gently with an air dryer), and the solutions were pipetted into test tubes, then frozen and thawed three times with an acetone–Dry Ice bath. Microscopic observation of the mixtures showed that all cells were broken after this treatment. When measuring the catechol *O*-methyltransferase activity of the homogenates, we estimated the concentration of protein by determining the optical density at 260 and 280 nm (10). To each homogenate were added 100 μl of 10% trichloracetic acid, to give a mixture of about pH 3. These mixtures were kept for 10 min in a water bath at 37° to flocculate the protein, and the acidified homogenates were centrifuged at 27,000 × *g* for 90 min. After this centrifugation, the radioactivity remaining in the

supernatant was 77–99% of the initial radioactivity in the incubation medium. An aliquot of each supernatant was kept frozen for chromatography (solution A). The supernatants (2.0 ml) were then neutralized with 0.5 ml of Tris buffer (4 M, pH 8.0) and poured into test tubes, each containing 1.0 g of alumina (3). The solutions were stirred vigorously (Vortex mixer) for 1 min and centrifuged. The supernatants, which were kept frozen for chromatography (solution B), contained 20–36% of the radioactivity originally in the incubation solution.

Thin-layer chromatography of catecholamines is a well-documented technique (11–14). We worked primarily with coated Eastman cellulose plates eluted with 1-butanol–acetic acid–water (100:20:60) according to Vahidi and Sankar (12). Authentic samples of catecholamine metabolites were spotted with aliquots of solutions A and B (10–20 μl; 10,000–20,000 cpm), and the plates were then eluted and cut into small strips (2 × 0.5 cm). Each strip was extracted for 10 min in a scintillation vial with 200 μl of 0.1 N HCl; 10 ml of Aquasol were added, and the solutions were counted for radioactivity determination. The radioactivity of each strip was plotted against its location on the chromatogram. Under these conditions the typical graphs for solutions A and B showed essentially three different metabolite peaks of varied intensities: (a) noneluted compounds at the origin, (b) 3-methoxytyramine plus metanephrine, and (c) homovanillic acid and vanillylmandelic acid. In our preliminary work we focused on the 3-methoxytyramine peak, since other metabolites, present in larger amounts, are poorly separated by the thin-layer chromatographic technique. Because of this poor separation of many catecholamine metabolites (see below) we developed an analytical method utilizing high-pressure liquid chromatography (15). Qualitative, preliminary results reported in this paper were obtained using thin-layer chromatography, whereas quantitative analyses of the drug effects (Table 1) were obtained by high-pressure liquid chromatography with a microparticle reverse-phase column operated isocratically in 1.0 M sodium acetate, pH 4.6, at a flow rate of 0.46 ml/min.

Although catechol *O*-methyltransferase activity has been demonstrated previously in neurobastoma cells (16, 17), we felt it necessary to establish that it was similar to the rat liver enzyme in terms of product inhibition by SAH (3) and its 7-deaza analogue (4). A crude cell homogenate was obtained as previously described (16), and was shown to have catechol *O*-methyl-

TABLE 1

Analysis of [^{3}H]dopamine metabolites isolated from neuroblastoma cells

Results are expressed as percentage of total isotope recovered from the column (15). The final concentration of SAH and the 7-deaza analogue was 56 μM. The final concentrations of adenosine, tubercidin, and homocysteine were 48, 45, and 57 μM, respectively.

Drug	N-18				N1E-115 TG6			
	3-Methoxytyramine	Homovanillic acid	Dopamine 3-sulfate	Inhibition of 3-methoxytyramine formation	3-Methoxytyramine	Homovanillic acid	Dopamine 3-sulfate	Inhibition of 3-methoxytyramine formation
	%	%	%	%	%	%	%	%
None	41.3[a]	9.7[a]	48.0[a]		30.2	0	66.8	
SAH	26.0	12.0	60.4	37	13.3	0	83.9	56
STH	10.6	13.6	74.2	74	6.0	0	90.8	80
Adenosine	27.1	13.1	55.2	34	21.1	0	75.0	30
Tubercidin	27.2	13.6	56.4	34	23.0	0	74.5	24
Homocysteine	33.4	13.6	50.4	19	29.1	0	67.4	4

[a] May be compared with data of ref. 15 for reverse-phase chromatography of a non-drug-treated extract of N-18 cells: 42.4% 3-methoxytyramine, 8.8% homovanillic acid, and 47.8% dopamine 3-sulfate.

transferase activity when assayed by the method of Nikodejevic *et al.* (18). The rate of methylation was shown to be linearly dependent on time of incubation (up to 2 hr) and protein concentration (up to 0.4 mg/ml), as previously reported (16), and was markedly diminished in the presence of either SAH or the 7-deaza analogue. However, the apparent inhibition of neuroblastoma catechol *O*-methyltransferase by these drugs was not as potent as previously observed with the rat liver enzyme (2–4). Analysis of the kinetic data obtained at saturating levels of both substrates (1.1 mM *S*-adenosylmethionine and 2.0 mM 3,4-dihydroxybenzoic acid) revealed that "partial" inhibition (19) was occurring. At high concentrations (above 3 mM) of either SAH or the 7-deaza analogue, only 75% inhibition of the reaction was observed. Unfortunately, a more detailed investigation of the kinetic properties of the neuroblastoma enzyme was precluded by the low catechol *O*-methyltransferase activity of the cell homogenates (16). However, given the observed value of $I_{50} \cong 0.5$ mM for both SAH and STH, and the finding that, as the concentration of inhibitor approached infinity, $v = 0.25\ V_{max}$, one can calculate an approximate value of $K_i = 0.015$ mM. This value is comparable to that previously obtained for inhibition of rat liver catechol *O*-methyltransferase by SAH and the 7-deaza analogue, based on a more detailed kinetic study (3, 4).

In order to study the effects of these drugs on the catechol *O*-methyltransferase-catalyzed methylation of catecholamines in neuroblastoma cells, we chose to use [^{3}H]dopamine as a radioactive marker to monitor this metabolism. Initially we investigated several thin-layer chromatographic solvent systems (12, 20) in order to obtain maximal separation of the metabolites of primary interest in this study; i.e., the *O*-methylated derivatives. Solvent system I of Vahidi and Sankar (12) was found to be the most satisfactory in this regard when synthetic standards were applied. However, we were unable to use either thin-layer chromatography (11–14, 20, 21) or thin-layer electrophoresis (22) to analyze cellular extracts routinely. Nonreproducibility and low recovery of the applied radiolabeled metabolites rendered these techniques of little value for quantitative analysis. In an earlier study of catecholamine metabolism in neuroblastoma cells (23), the authors used a combination of ion-exchange and paper chromatography to identify the metabolites of [^{3}H]-tyrosine and [^{3}H]dopamine. This combination of techniques appeared somewhat cumbersome for routine analysis of drug-treated cells, and we decided to attempt a direct analysis of the combined medium–cell lysate by high-pressure liquid chromatography. We have recently described the details of this analytical technique (15), and in the experimental data presented below, high-pressure liquid chromatographic analyses were used exclusively to analyze the effects of catechol *O*-methyltransferase inhibitors, SAH and the 7-deaza analogue, on dopamine metabolism.

Table 1 shows the results of a typical analysis of the combined medium–cell lysate, following alumina treatment (solution B), in the presence of various drugs, together with a non-drug-treated control. It is apparent that the inhibition of dopamine methylation was accompanied by an increase in the amount of dopamine 3-sulfate formed. Although an increase in the amount of homovanillic acid formed was also observed with SAH and the 7-deaza analogue, a similar increase in homovanillic acid synthesis was observed in the presence of the SAH and STH fragments, adenosine, tubercidin, and homocysteine, and therefore may not be related to methyltransferase inhibition, since these fragments are known to be poor inhibitors of methyl transfer in cell-free systems (4). It is possible that the observed inhibition by these fragments may be due to their action on the enzyme adenosylhomocysteinase (EC 3.3.1.1), giving rise to increased amounts of intracellular SAH, either by inhibition of SAH hydrolysis or by driving the equilibrium in the direction of SAH synthesis. It is not yet known whether neuroblastoma cells contain adenosylhomocysteinase. A mutant line of neuroblastoma, N1E-115 TG6, which is very low in monoamine oxidase activity (24), was studied in order to avoid the complication of the additional metabolite, homovanillic

acid. In these cells, as in the N-18 cells, decreased synthesis of the methylation product, 3-methoxytyramine, in the presence of SAH and the 7-deaza analogue was accompanied by increased synthesis of the conjugate (Table 1). These data strongly argue for the existence of parallel pathways of dopamine methylation and conjugation in neuroblastoma cells. By inhibiting the methylation pathway, conjugation becomes the predominant metabolic reaction, and an increase in the amount of the conjugate is observed. We have previously shown that N-18 cell extracts not treated with alumina (solution A) contain small amounts of dihydroxyphenylacetic acid (15). The presence of large amounts of unmetabolized dopamine in solution A limited the accuracy of a quantitative analysis of dihydroxyphenylacetic acid by this method. Therefore we did not routinely monitor solution A for dihydroxyphenylacetic acid. It is possible that inhibition of methylation by SAH and the 7-deaza analogue might lead to increased dihydroxyphenylacetic acid synthesis. The observation that the percentage of radioactivity bound to alumina (i.e., dopamine, dihydroxyphenylacetic acid, etc.) did not increase substantially during a given experiment, between control and drug-treated cells, suggests that dihydroxyphenylacetic acid synthesis is not markedly enhanced in drug-treated N-18 cells. Since no dihydroxyphenylacetic acid is formed in N1E-115 TG6 cells (24), the conclusions drawn in regard to 3-methoxytyramine as opposed to dopamine 3-sulfate synthesis in that line are not clouded by any ambiguities regarding dihydroxyphenylacetic acid synthesis.

Table 1 also shows data on 3-methoxytyramine formation, expressed as percentage inhibition of methylation compared with a drug-free control. SAH inhibition of dopamine methylation in N-18 cells was not appreciably greater than the inhibition elicited by the control drug fragments, adenosine, tubercidin, and homocysteine. In contrast, inhibition of dopamine methylation by the 7-deaza analogue in N-18 cells was much greater than that observed with any of the fragments. In the N1E-115 TG6 line, inhibition of dopamine methylation by SAH was somewhat higher than observed with the fragments, and inhibition by the 7-deaza analogue was again much more pronounced than inhibition by SAH or any of the controls. These data on the relative efficacy of SAH and the 7-deaza analogue as methylation inhibitors in neuroblastoma cells are similar to those obtained previously with the same two drugs in phytohemagglutinin-stimulated rat lymphocytes (25). Since SAH and the 7-deaza analogue have very similar K_i values in several cell-free enzyme systems (4), it would appear that the enhanced efficacy of the 7-deaza analogue in whole cells is due to differences in transport and/or metabolism. Careful studies on the transport of SAH in mammalian cells have yet to be done, although it is known that SAH is present in tissues of various organs (26). Thus it is not possible to discuss with any certainty the differences in transport between SAH and the 7-deaza analogue. However, differences in the biochemistry of adenosine and tubercidin derivatives are well documented (27, 28). For the purposes of this discussion, it is sufficient to note that the tubercidin derivatives do not act as substrates for enzymes which catalyze deamination of the 6-amino group (27) or phosphorolytic cleavage of the purine-ribose bond (28) of adenosine. In previous work with stimulated lymphocytes, we suggested that differences observed between SAH and the 7-deaza analogue when monitoring DNA synthesis in long-term (up to 64-hr) cultures might be due to enzyme-catalyzed cleavage of the 5′-thioether bond. However, recent studies[4] indicate that STH is not a substrate for purified adenosylhomocysteinase isolated from rat liver. Thus accumulated data suggest that the 7-deaza analogue cannot be metabolized via deamination of the 6-amino group or hydrolysis of the purine-ribose or 5′-thioether bonds. It is possible that the enhanced efficacy of the 7-deaza analogue compared with SAH as a methyltransferase inhibitor in whole cells is due to its stability toward metabolic degrada-

[4] G. L. Cantoni, personal communication.

tion, as indicated above. Further studies using radiolabeled drug to study its transport and metabolism are required to explore this possibility.

Note added in proof: Since this manuscript was submitted for publication, we have become aware of a recent paper in which it is suggested that conjugation and methylation at the 3-hydroxyl group of dopamine compete as major routes of metabolism (29). This conclusion, based on analyses of urine samples from patients with Parkinson's disease, is in accord with one of the conclusions of the present work.

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