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PERTURBATION OF BIOCHEMICAL TRANSMETHYLATIONS BY 3-DEAZAADENOSINE *IN VIVO*

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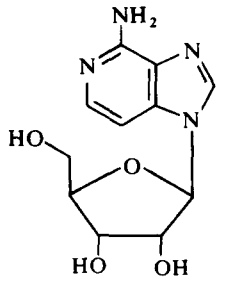
Abstract—Elevated levels of adenosylmethionine (AdoMet) and adenosylhomocysteine (AdoHcy) were observed in livers of rats injected with 3-deazaadenosine, an inhibitor of adenosylhomocysteine hydrolase. A marked appearance of 3-deazaadenosylhomocysteine was observed. The ratio of AdoMet/AdoHcy in the liver was reduced from a normal value of 4.5 to 1.6 after two injections, and to 1.3 after three injections. Perturbation of biochemical transmethylations was achieved in rats injected with 3-deazaadenosine, as evidenced by: (a) a reduction in the level of creatine in the liver; (b) a decrease in the urinary excretion of 3-methoxy-4-hydroxymandelic acid; and (c) a drastic reduction in the methylation of lipids, as measured by methyl-^{3}H-incorporation from [methyl-^{3}H]AdoMet. These observations support the hypothesis that the ratio AdoMet/AdoHcy may be of critical importance in the regulation of biological methylations. The enzyme, adenosylhomocysteine hydrolase, therefore, may be considered a prime biological target in terms of *in vivo* regulation of transmethylation reactions.

Adenosylhomocysteine (AdoHcy) was shown by Mann and Mudd [1] in 1963 to be a strong competitive inhibitor of tyramine *N*-methyltransferase, with a K_i of 5 μM compared to a K_m of 20μM for adenosylmethionine (AdoMet). Since then, a variety of methyltransferases have been shown to be inhibited by AdoHcy [2–11], including N^5-methyltetrahydrofolate-homocysteine methyltransferase which is activated by AdoMet [12].

The effects of synthetic analogs of AdoHcy on cellular systems have been investigated by several laboratories. Of special interest were the observations of the inhibition of growth and replication of viruses, and the inhibition of oncogenic transformation induced by Rous sarcoma virus [13–16]. These effects have been attributed to inhibition of viral mRNA methylation, and evidence supporting this hypothesis has been reported by Jacquemont and Huppert [17] for herpes simplex virus. Synthetic analogs of AdoHcy, however, are not as effective as the natural congener, AdoHcy itself, as inhibitors of methyltransferases *in vitro*, with the possible exception of tubercidinylhomocysteine in the case of mRNA (Gua-7)-methyltransferase [18]. This led us to search for inhibitors of AdoHcy hydrolase [19], with the expectation that inhibition of AdoHcy hydrolase *in vivo* would result in intracellular accumulation of AdoHcy, and subsequent inhibition of one or more methyltransferases.

3-Deazaadenosine was found by us to be the most potent inhibitor of AdoHcy hydrolase, as well as being resistant to adenosine deaminase, among a large number of compounds tested [19]. The reaction catalyzed by AdoHcy hydrolase is reversible, and the equilibrium favors the direction of synthesis [20]. When isolated rat hepatocytes were incubated with 3-deazaadenosine, increased cellular levels of AdoMet and AdoHcy were observed, with the simultaneous appearance of a new product, 3-deazaadenosylhomocysteine (3-deaza-AdoHcy) [19]. These results are assumed to be due to the inhibition of AdoHcy hydrolase by 3-deazaadenosine; the increased levels of AdoHcy together with the appearance of 3-deaza-AdoHcy could then inhibit transmethylation processes in the hepatocytes and result in AdoMet accumulation. The ability of AdoHcy hydrolase to catalyze the formation of novel congeners of AdoHcy from purine nucleoside analogs in the presence of homocysteine, such as we have demonstrated for 3-deazaadenosine [19, 21], has also been observed by Guranowski and Pawelkiewicz [22] using AdoHcy hydrolase from yellow lupin seeds, and by Hoffman [23], who recently demonstrated the formation of N^6-methyl-AdoHcy from N^6-methyladenosine and homocysteine by mouse liver homogenates.



3-Deazaadenosine.

Enzymatic studies with AdoMet synthetase revealed that it is a regulatory enzyme under complex control mechanisms *in vitro* [24, 25]. Little is known, however, about the control of the utilization of AdoMet and/or factors that influence *in vivo* the activity of different methyltransferases. In eukaryotes, as far as is known, AdoHcy is metabolized exclusively by AdoHcy hydrolase to adenosine and homocysteine. Although the equilibrium of the reaction lies far in the the direction of synthesis [20], under physiological conditions

AdoHcy is hydrolyzed because adenosine, a potent inhibitor of AdoHcy hydrolase, is removed by adenosine deaminase to become inosine, and homocysteine is removed either by condensation with serine to yield cystathionine or by remethylation to methionine. Because 3-deazaadenosine is a potent inhibitor of AdoHcy hydrolase [19], it was of interest to determine whether and how the administration of 3-deazaadenosine *in vivo* would lead to changes in the intracellular concentrations of AdoMet and AdoHcy and any attendant alterations of transmethylation processes.

MATERIALS AND METHODS

Male Wistar rats (about 200 g) and female golden Syrian hamsters (about 90 g) from Charles River (Wilmington, MA) were used. All chemicals were dissolved in 0·9% NaCl for intraperitoneal injection. Control animals were injected with the 0.9% NaCl vehicle. Rats were killed by decapitation, and each liver or organ was homogenized with 5% sulfosalicylic acid, 4 ml/g wet weight. Levels of AdoMet, AdoHcy and 3-deaza-AdoHcy were then determined by an adaptation of a high pressure liquid chromatography [16, 19] using VYDAC cation exchanger [26]. The sulfosalicylic acid extract was applied onto a column of 0.6 × 30 cm of VYDAC. For the control animals, 4 ml of the acid extract was used, but for the animals injected with 3-deazaadenosine, 2 ml was used. After washing with 95 ml of 0.01 M ammonium formate, pH 4.0, elution was started by a 100-ml linear gradient of 0.01 to 0.8 M ammonium formate, pH 4.0. Column effluents were monitored at 254 nm by a Glenco 5480 u.v. monitor equipped with a 1.6 mm × 10 mm flow cell. The VYDAC cation column was calibrated with radioactive standards.

Urine samples were collected from rats placed in metabolic cages; each collection beaker contained 0.5 ml of 6 N HCl. Creatinine was determined by a Beckman creatinine analyzer 2, based on a Jaffe alkaline picrate reaction. Vanilmandelic acid (VMA; 3-methoxy-4-hydroxymandelic acid) was determined according to the method of Pisano *et al.* [27]. When assaying for the amount of creatine in liver samples, livers were homogenized with 1 N HCl, 4 ml/g wet weight. After centrifugation, the supernatant fraction was autoclaved for 30 min at 15 lb pressure for conversion of the creatine present to creatinine [28], which was then determined by Beckman creatinine analyzer 2. The procedure of Folch *et al.* [29] was used for the extraction of total lipids from livers. The total lipids extracted in the chloroform layer were then measured quantitatively by a colorimetric method [30], using a test kit from Boehringer Mannheim (Cat. No. 124303).

RESULTS

Administration of 3-deazaadenosine to rats fed *ad lib.* resulted in dramatic increases in the concentrations of AdoMet and AdoHcy in the liver, and the appearance of a novel product, 3-deaza-AdoHcy (Figs. 1 and 2). The normal liver concentration of AdoHcy was about 20 nmoles/g, and about 90 nmoles/g for AdoMet, in close agreement with previously reported measurements [26, 31]. After two injections of 3-deazaadenosine, AdoHcy increased about 10-fold

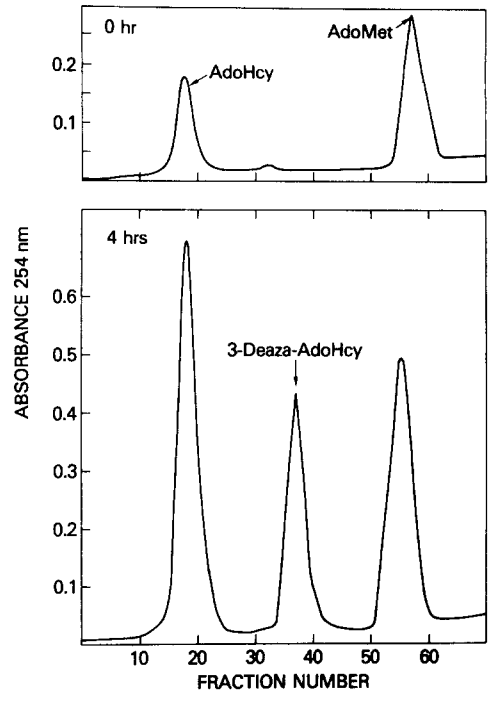


Fig. 1. High pressure liquid chromatography of AdoMet, AdoHcy and 3-deaza-AdoHcy from liver extracts on VYDAC cation exchanger. (Top) 0 hr; (bottom) 4 hr. 3-Deazaadenosine (20 mg/rat) was injected at 0 and 3 hr; liver was extracted 1 hr after the second injection. Each fraction contained 1.2 ml. In this chromatographic run, 4 ml of the sulfosalicylic extract was used.

while AdoMet increased about 2-fold; after three injections, AdoHcy increased 12-fold while AdoMet increased 3-fold. The 3-deaza-AdoHcy accumulating in the liver was 151 ± 9 nmoles/g after two injections, and 326 ± 65 nmoles/g after three injections. The ratio of AdoMet/AdoHcy was reduced from a normal value

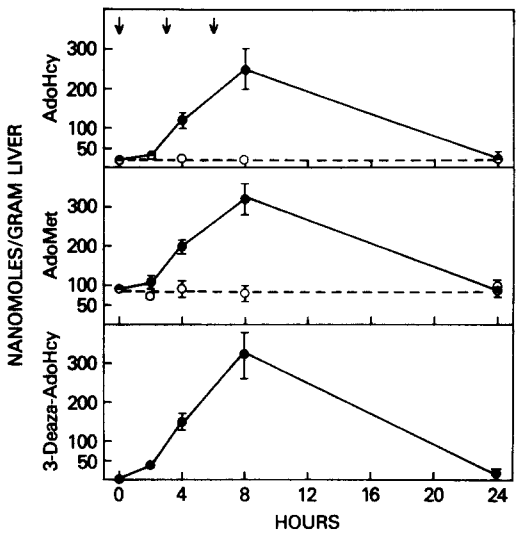


Fig. 2. Time course of the levels of AdoMet, AdoHcy and 3-deaza-AdoHcy in livers of rats injected with 3-deazaadenosine. Each arrow indicates time of injection (20 mg each). Each data point represents the mean ± S.E.M. for four animals; open circles represent control rats.

Table 1. Effects of 3-deaza-Ado on the levels of AdoHcy, AdoMet and 3-deaza-AdoHcy in rats tissues*

Tissues	AdoHcy (nmoles/g)	AdoMet (nmoles/g)	3-Deaza-AdoHcy (nmoles/g)
Liver			
Control	23 ± 2	90 ± 12	0
Treated	125 ± 14	200 ± 4	151 ± 9
Spleen			
Control	13 ± 1	45 ± 2	0
Treated	138 ± 40	62 ± 7	39 ± 5
Heart			
Control	17 ± 5	37 ± 2	0
Treated	24 ± 5	51 ± 5	Trace
Lung			
Control	16 ± 6	17 ± 1	0
Treated	12 ± 5	21 ± 3	Trace
Kidney			
Control	34 ± 4	32 ± 5	0
Treated	20 ± 7	40 ± 6	36 ± 4

* Rats received injections of 3-deaza-Ado (20 mg) at 0 and 3 hr. Organs were removed at 4 hr. Results are expressed as means ± S.E.M. for four animals.

of 4.5 to about 1.6 after two injections, and to 1.3 after three injections. If 3-deaza-AdoHcy was taken into consideration, i.e. AdoMet/(AdoHcy + 3-deaza-AdoHcy), the ratio was 0.7 after two injections and 0.6 after three injections. It is notable that, in the isolated rat hepatocytes, the ratio of AdoMet/AdoHcy approximated that of the liver *in vivo* [19]. The effect of 3-deazaadenosine was of relatively short duration since the concentrations of AdoMet and AdoHcy returned practically to normal by 24 hr, and by then 3-deaza-AdoHcy disappeared almost completely (Fig. 2).

It is interesting that different rat tissues responded dissimilarly to the administration of 3-deazaadenosine (3-deaza-Ado) (Table 1). There was a 10-fold increase in AdoHcy in the rat spleen without any attendant alteration in the level of AdoMet. No changes in AdoMet or AdoHcy levels could be observed in the heart, lung and kidney. In each of these tissues, only a trace amount of 3-deaza-AdoHcy was observed. In contrast to the results obtained from the rat liver, the liver of hamsters showed a different response to the administration of 3-deazaadenosine (Table 2). The increase in the concentration of AdoMet was not correlated with an increase in AdoHcy, but rather with the appearance of 3-deaz-AdoHcy. However, the normal levels of AdoMet and AdoHcy in the hamster liver correspond well with those of rat liver. Whether the different responses to 3-deazaadenosine can be explained on the basis of isozyme patterns differing in affinities for 3-deazaadenosine, or other cellular differences, will have to be determined.

The last step in the biosynthesis of creatine as shown by Cantoni and Vignos [28] is the methylation of guanidoacetic acid by AdoMet. The content of creatine in the liver of rats fed *ad lib.* or starved for 18 hr was reduced by about 50 per cent after 3-deazaadenosine administration (Table 3). When the urinary excretion of creatinine was measured, the difference between the controls and the 3-deazaadenosine-treated rats was not statistically significant at a 95 per cent confidence level. Because creatinine excreted in the urine is formed nonenzymatically in proportion to the amount of phosphocreatine stored in muscle [32], it is then not surprising that creatinine excretion cannot serve as a sensitive

Table 2. Effects of 3-deaza-Ado on levels of AdoHcy, AdoMet and 3-deaza-Adohcy in livers of hamsters*

Injected with	AdoHcy (nmoles/g)	AdoMet (nmoles/g)	3-Deaza-AdoHcy (nmoles/g)
Saline, 2 hr	14 ± 4	89 ± 8	0
3-Deaza-Ado, 2 hr	18 ± 4	157 ± 14	60 ± 6
Saline, 4 hr	19 ± 3	103 ± 6	0
3-Deaza-Ado, 4 hr	21 ± 4	141 ± 9	108 ± 20

* Female hamsters (90–100 g) were injected with 6 mg each of 3-deaza-Ado; in 2-hr experiments, hamsters received one injection at 0 hr; in 4-hr experiments, hamsters received injections at 0 and 2 hr. Results are expressed as the means ± S.E.M. for four animals.

Table 3. Effects of 3-deaza-Ado on levels of creatine in livers of rats fed *ad lib.* or starved

Experiments	Creatine (μg/g liver ± S.E.M.)
Fed *ad lib.*	
Saline control, 4 hr*	51 ± 2†
3-Deaza-Ado, 4 hr	21 ± 3†
Saline control, 8 hr‡	48 ± 2§
3-Deaza-Ado, 8 hr	33 ± 4§
Starved‖	
Saline control, 4 hr*	48 ± 5¶
3-Deaza-Ado, 4 hr	22 ± 3¶

* Rats received injections of either saline or 3-deaza-Ado (20 mg) at 0 and 2 hr; number of rats used was four.
† $P < 0.01$.
‡ Rats received injections of either saline or 3-deaza-Ado (20 mg) at 0, 2 and 3 hr; number of rats used was four.
§ $P < 0.05$.
‖ Rats were starved for 18 hr before receiving injections; number of rats used was four.
¶ $P < 0.01$.

index of perturbation in creatine biosynthesis. In recent studies by us,* it was found that guanidoacetate methyltransferase is quite sensitive to inhibition by AdoHcy or 3-deaza-AdoHcy.

The urinary excretion of VMA was decreased significantly, by about 40 per cent (Table 4). Since quantitatively the most important norepinephrine metabolite in the periphery is VMA [33], a decreased excretion of VMA in the rats is probably the result of perturbation in the methylation of catecholamines and/or catechols.

Mudd and Poole [32] have expounded on the enzymatic conservation of the homocysteine moiety back to methionine after the labile methyl group of methionine has been transferred to an acceptor molecule. Experiments were conducted by injecting [^{3}H]methionine and [^{35}S]methionine simultaneously to label the AdoMet pool in the rats after administration of 3-deazaadenosine. A ratio, [^{35}S]AdoMet specific radioactivity/[methyl-^{3}H]AdoMet specific radioactivity, is used as an indicator of the rate of the utilization of the methyl-^{3}H-moiety in the AdoMet pool by methyltransferases. A drop in this ratio is, therefore, suggestive of a decrease in the rate of the utilization of the methyl-^{3}H-moiety of the AdoMet pool, relative to the recycling of the ^{35}S-labeled homocysteinyl moiety into the AdoMet molecule. As shown in Table 5, the ratio of [^{35}S]/[methyl-^{3}H] in the AdoMet pool was almost halved in the liver of rats treated with 3-deazadenosine. There

* Y. S. Im, P. K. Chiang and G. L. Cantoni, unpublished observations.

Table 4. Effects of 3-deaza-Ado on levels of creatinine and 3-methoxy-4-hydroxymandelic acid (VMA) in urine of rats*

Injected with	Creatinine (mg/24 hr)	VMA (μg/24 hr)
Saline	6.0 ± 1.3	28 ± 2
3-Deaza-Ado	4.4 ± 1.0	18 ± 2†

* Rats received injections of 3-deaza-Ado (20 mg) at 0, 3 and 6 hr. Results are expressed as means ± S.E.M. for eight animals.
† $P < 0.001$.

was an increase in the treated livers in the specific radioactivity of AdoMet in both the ^{35}S and methyl-^{3}H labels of about 2-fold. The observation that both the specific activity and the pool size of AdoMet were increased after the administration of 3-deazaadenosine seems, at first glance, to be difficult to interpret. However, if as a consequence of 3-deazaadenosine administration, the utilization of the methyl group of AdoMet for transmethylation reactions were inhibited and hence the turnover time of AdoMet were increased, it would be expected that under pulse-labeling conditions, the specific activity and the pool size of AdoMet would be increased simultaneously. In fact, the occurrence of the latter event further supports our hypothesis that 3-deazaadenosine interrupted the normal pathway for the disposition of the [methyl-^{3}H]-moiety of the AdoMet pool.

Since the drop in the ratio of [^{35}S]/[methyl-^{3}H] in the AdoMet pool was an indication of impairment in the utilization of the methyl group of AdoMet by methyltransferases, extracts of rat livers were made to determine the rate of methyl-^{3}H-incorporation into total lipids. Measurements of the specific radioactivity of lipids labeled by methyl-^{3}H (Table 5) provide other striking evidence of impaired methylation after administration of 3-deazaadenosine. It can be seen that the methylation of lipids in the rat liver treated with 3-deazaadenosine was reduced by almost 20-fold, after adjusting for the increase in the specific radioactivity of [methyl-^{3}H]AdoMet. The lipid content in both groups remained about the same, approximately 30 mg/g wet weight.

DISCUSSION

Although AdoHcy, as pointed out in the introduction, is the most potent known inhibitor of AdoMet-dependent methyltransferases *in vitro*, its effectiveness in inhibiting methyltransferases or as an anti-viral agent when applied extracellularly is less than that of several

Table 5. Specific radioactivities of AdoMet and lipids from rat livers*

Treatment	[Methyl-^{3}H]AdoMet (cpm/nmole)	[^{35}S]AdoMet (cpm/nmole)	[^{35}S]AdoMet / [Methyl-^{3}H]AdoMet	Methyl-^{3}H-labeled lipids (cpm/mg)
Control	41 ± 4	204 ± 16	5.1 ± 0.3	9557 ± 1504
3-Deazaadenosine	109 ± 12	377 ± 28	3.4 ± 0.2	1030 ± 262

* Rats received injections of 3-deaza-Ado (20 mg) at 0 and 3 hr. Radioactive L-methionine solution containing 40 μCi L-[methyl-^{3}H]methionine (200 mCi/m-mole), 22 μCi L-[^{35}S]methionine (0.95 mCi/m-mole) and 16 mg L-serine was injected at 3.5 hr; at 4.5 hr, the rats were killed. Results are expressed as means ± S.E.M. for four animals.

less potent inhibitors [13, 34, 35]. It is not yet clear whether this is due to a problem of cellular transport, or to the removal of AdoHcy once it enters the cell. In the present investigation, we showed that by the use of an inhibitor of AdoHcy hydrolase, i.e. 3-deazadenosine, perturbations in cellular transmethylations can be achieved.

The physiological consequences of the administration of 3-deazaadenosine are: (1) a reduction in the concentration of creatine in the liver; (2) a decreased urinary excretion of VMA; and (3) a large decrease in the rate of methylation of lipids. These overall effects are consistent with our hypothesis that, upon inhibition of AdoHcy hydrolase *in vivo*, the ratio AdoMet/AdoHcy or, more correctly, AdoHcy/PuoHcy,* is altered. When the ratio AdoMet/PuoHcy drops, transmethylation processes are inhibited. The large decrease in the rate of the methylation of lipids is surprising to us, and indicates that this reaction is very sensitive to control by PuoHcy. Inhibition of fatty acid synthetases by AdoHcy has been reported by Law *et al.* [2, 6]. Biosynthesis of choline or phosphatidylchline (lecithin) was shown to involve AdoMet as the methyl donor [36, 37], and liver is the main choline-synthesizing system [36]. Hirata *et al.* [38, 39] recently reported the involvement of two methyltransferases in the methylation of phosphatidylethanolamine to form phosphatidylcholine. These two methyltransferases possibly participate in membrane functions [39], and the phosphatidylethanolamine methyltransferase is inhibited by AdoHcy, with a K_i of 1.6 μM compared to a K_m of 1.4 μM for AdoMet [38]. The role of AdoHcy in regulating the biosynthesis of lipids is being studied further.

AdoMet participates in a multitude of biological reactions [40, 41]. A change in the ratio AdoMet/AdoHcy may be reflected by perturbations in these reactions, depending upon their sensitivities. Further experimentation with inhibitors of AdoHcy hydrolase, *in vitro* and *in vivo*, may help establish a hierarchy of different thresholds for perturbation of reactions involving AdoMet. By employing metabolic inhibitors of AdoHcy hydrolase, molecular events involving the interplay between AdoMet and AdoHcy can be elucidated.

We have suggested that inhibition of AdoHcy hydrolase may be useful for purposes of chemotherapy [21]. This possibility is supported by the findings that 3-deazaadenosine will prevent replication and growth of the Rous sarcoma virus in chick embryo cells, and under certain conditions will reverse malignant transformation by this oncogenic virus [16]. 3-Deazaadenosine is also effective against other RNA or DNA viruses: Sindbis virus, Newcastle disease virus and vesicular stomatitis virus [16]; influenza virus and herpes virus.† 3-Deazaadenosine can also prevent transformation induced by Simian virus 40 in rat embryo cells and Simian sarcoma virus in normal rat kidney cells.† Antimalarial activity is also exhibited by 3-deazaadenosine against the human malarial parasite, *Plasmodium falciparum*, in culture.† Another inhibitor of AdoHcy hydrolase, 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine, is also an effective anti-viral agent against the Rous sarcoma virus and the Gross murine leukemia virus [15].

The drastic reduction in the level of adenosine deaminase in cells transformed by Rous sarcoma virus [42] poses intriguing questions as to how these cells metabolize AdoHcy, or how these cells adapt to this new biochemical environment in comparison to normal cells. In this connection, the ability of 3-deazaadenosine or an inhibitor of adenosine deaminase, erythro-9-(2-hydroxy3-nonyl)adenine, to inhibit chemotaxis in neutrophils [43], and phagocytosis in mouse macrophages [44], points to possible pathological involvement of AdoHcy in combined immunodeficiency disease that is accompanied by either an absence of, or much reduced levels of, adenosine deaminase. In fact, a possible connection between AdoHcy and the molecular pathogenesis of immune defect in adenosine deaminase deficiency has been considered by Kredich and Martin [45].

Because 3-deazaadenosine can neither be incorporated into nucleic acids, nor phosphorylated [16], the physiological consequences in rats caused by the administration of 3-deazaadenosine can be attributed primarily to the inhibition of AdoHcy hydrolase and the formation of a large amount of 3-deaza-AdoHcy. When tested *in vitro*, 3-deazaadenosine was found by us to be a relatively weak inhibitor of adenylate cyclase from human fibroblast or neuroblastoma cells, with an I_{50} of about 10 μM for both.† The perturbation in the biochemical methylations reported here by us is consistent with the observation that the ratio of AdoMet/PuoHcy was altered substantially by 3-deazaadenosine.

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