Mechanism of the Cytostatic Activity of 3-Deazaaristeromycin, an Inhibitor of Adenosylhomocysteine Hydrolase*

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3-Deazaaristeromycin (100 μM) is cytostatic for the RAW264 mouse macrophage cell line. In cells incubated with 3-deazaaristeromycin, cell replication is effectively stimulated by 0.5 μM homocysteine thiolactone and no further stimulation occurs at concentrations of homocysteine thiolactone greater than 50 μM. Cell replication occurs when homocysteine thiolactone is administered either simultaneously with, or 4 days after, the administration of 3-deazaaristeromycin. In the presence of 3-deazaaristeromycin, cell replication also occurs when either folate (10 to 100 μM) or hypoxanthine and thymidine are included in the medium. Incorporation of [3H]formate into trichloroacetic acid-precipitable material is inhibited when cells are incubated with 3-deazaaristeromycin, and this inhibition is partially reversed by inclusion of either homocysteine thiolactone or folate in the medium. Experiments showed that adenosylhomocysteine hydrolase is inhibited to the same extent after the cells have been incubated for 4 days with either 3-deazaaristeromycin or 3-deazaaristeromycin and homocysteine thiolactone. The findings suggest that in cells incubated with 3-deazaaristeromycin, the formation of homocysteine from 3-adenosylhomocysteine is insufficient for the regeneration of tetrahydrofolate for the synthesis of purines and pyrimidines.

In recent years, S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) has been the object of several studies in this laboratory (1, 2). As first established by de la Haba and Cantoni (3), AdoHcy1 hydrolyase catalyzes the reversible hydrolysis of AdoHcy. Although the equilibrium of this reaction is strongly in the direction of synthesis, AdoHcy is efficiently hydrolyzed in vivo because the products of hydrolysis, adenosine and thymidine, are removed very efficiently by a number of different enzymatic reactions. Our renewed interest in AdoHcy hydrolyse stemmed from two considerations. First, it has been proposed that AdoHcy hydrolyase might play a key role in the regulation of biological methylation reactions by modulating the AdoMet/AdoHcy ratio (4). Second, AdoHcy is the only source of homocysteine in vertebrates. A supply of homocysteine is an absolute requirement for the biosynthesis of cystathionine and cysteine through the transsulfuration pathway and for regeneration of tetrahydrofolate from methylenetetrahydrofolate through the Bi$_4$-dependent synthesis of methionine catalyzed by 5-methyltetrahydropteroyl-L-glutamate-L-homocysteine S-methyltransferase (EC 2.1.1.13).

We have examined a relatively large number of adenosine analogs for the capacity to interact with AdoHcy hydrolase as inhibitors, alternate substrates, or both (1, 5–8). 3-Deazaadenosine and 3-deazaaristeromycin are particularly interesting because they exhibit some common features, yet differ significantly both from a biochemical and biological standpoint (1, 6, 7). 3-Deazaadenosine and 3-deazaaristeromycin appear to interact with a single enzymatic target, AdoHcy hydrolase, since neither analog is a substrate for adenosine deaminase or adenosine kinase. 3-Deazaaristeromycin is a potent reversible inhibitor of the enzyme, and its administration results in a large accumulation of AdoHcy that reflects the rate of biological utilization of AdoMet for transmethylation reactions (7). By contrast, 3-deazaadenosine can function both as a strong inhibitor and as a good substrate for the enzyme (1, 5). Thus, its administration results both in the accumulation of AdoHcy and in the intracellular formation of an AdoHcy cogen, 3-deaza-AdoHcy. For reasons that are not yet entirely clear, in some tissues, the administration of 3-deazaadenosine results predominantly in the accumulation of AdoHcy, which indicates that 3-deazaadenosine functions as an inhibitor (8). In others, the intracellular level of AdoHcy is not altered, and 3-deaza-AdoHcy accumulates, indicating that in this case, 3-deazaadenosine is utilized as a substrate (8). Accumulation of AdoHcy, whether brought about by 3-deazaadenosine or 3-deazaaristeromycin, is generally accompanied by an increase in the AdoMet level, indicating that a number of transmethylation reactions, such as phospholipid methylation, are inhibited (1, 8, 9). Biologically, both compounds exhibit potent antiviral activity against retroviruses such as Rous sarcoma virus (6, 10, 11). However, only 3-deazaadenosine has been found to inhibit chemotaxis by a mouse macrophage cell line, a finding that has been interpreted by Aksamit et al. (7) as indicating that 3-deaza-AdoHcy is a more potent inhibitor of one or more transmethylation reactions that are essential for the chemotactic response. Another fundamental difference between these two adenosine analogs can be predicted from what is known of their mode of action. The accumulation of 3-deaza-AdoHcy upon administration of 3-deazaadenosine indicates that the inhibition of AdoHcy hydrolase has not been complete and that some homocysteine has been generated by cleavage of AdoHcy. On the other hand, at sufficiently high concentrations of 3-deazaaristeromycin, it might be expected that formation of homocysteine might be completely inhibited. This would secondarily result in inhibition of cystathionine synthesis and in accumulation of methyltetrahydrofolate. We show here that the cytostatic effect of 3-deaaza-

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1 The abbreviations used are: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; 3-deaza-AdoHcy, 3-deazaadenosylhomocysteine.

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isteromycin is reversed by minute quantities of homocysteine that regenerate tetrahydrofolate or by administration of purines and thymidine whose synthesis requires the recycling of tetrahydrofolate. Thus, it is concluded that inhibitors of AdoHcy hydrolase in vitro result in the accumulation of methyltetrahydrofolate.

**MATERIALS AND METHODS**

**Cell Culture and Chemicals**—The RAW264 macrophage cell line was cultured in modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and subcultured twice weekly as previously described (12). For growth curves, 2 × 10^4 cells in 1 ml of medium were plated in 16-mm wells and cultured at 37 °C in a humid atmosphere of 5% CO2 and 95% air. The cells were suspended with a pipette and counted on a Coulter counter. 3-Deaza-AdoHcy was obtained from the Southern Research Institute, Birmingham, AL.

**Determination of 35S-labeled AdoMet and AdoHcy**—Cells (2 × 10^5) in 60-mm dishes. [35S]Methionine (16.5 μCi) was added to each dish and the dishes were incubated at 37 °C for 90 min. The dishes were placed on ice and the cells were removed by scraping, followed by two 1-ml washes with phosphate-buffered saline. The cell suspension and washes were combined and centrifuged, and the pellets were suspended in 1 ml of 5% sulfosalicylic acid. The radioactivity in AdoMet and AdoHcy was determined after chromatography of the sulfosalicylic acid extract on a Vydac (Applied Sciences Inc.) cation exchange column (13).

**Incorporation of [14C]Formate into Trichloroacetic Acid precipitable Material**—Cells were incubated for 1 h with 15 μCi of [14C]formate, and the labeled cells were collected and washed as above for the determination of radioactivity in AdoMet. The cell pellet was suspended in 1 ml of phosphate-buffered saline at 0 °C, and macromolecules were precipitated by addition of 1 ml of 20% trichloroacetic acid at 0 °C. The suspension was vigorously mixed, incubated in an ice bath for 15 min, and centrifuged. The pellet was washed three times with ice-cold 10% trichloroacetic acid and dissolved in 9.3 M NaOH, and the radioactivity was counted.

**RESULTS**

Replication of RAW264 cells was progressively inhibited by increasing concentrations of 3-deaza-AdoHcy until cytostasis was obtained at approximately 100 μm 3-deaza-AdoHcy (Fig. 1A). The inhibition was reversed by micromolar concentrations of homocysteine thiolactone (Fig. 1B). Homocysteine thiolactone itself did not affect cell replication (data not shown), and, in the presence of 100 μM 3-deaza-AdoHcy and 100 μM homocysteine thiolactone, the number of RAW264 cells increased, although at a slower rate than uninhibited cells, to a density similar to that in the absence of 3-deaza-AdoHcy. Homocysteine thiolactone reversed 3-deaza-AdoHcy inhibition of cell replication when added at the same time as 3-deaza-AdoHcy or as late as 4 days after addition of 3-deaza-AdoHcy (longer times were not tested) (Fig. 2). Inhibition was reversible in that 3-deaza-AdoHcy could be removed after 3 days of treatment and the cells began to replicate (data not shown). Homocysteine, homocysteine, and, to a lesser extent, cystathionine also reversed the inhibition of replication by 3-deaza-AdoHcy (Fig. 1C). The effects of cystathionine are probably due to the fact that the reaction catalyzed by cystathionine synthase is reversible, even though the equilibrium is in favor of synthesis (14). Increasing the concentration of methionine or cysteine in the medium from 0.1 to 0.3 mm or addition of 0.2 mm cysteine to the medium did not reverse the inhibition of cell replication by 3-deaza-AdoHcy. The failure of cysteine to reverse the inhibition shows that homocysteine does not exert its effect simply by virtue of being a sulphydryl compound.

Homocysteine is metabolized via 1) the transsulfuration pathway that first involves condensation with serine to form cystathionine and then cleavage of cystathionine to yield cysteine, 2) the sulfur conservation pathway that involves remethylation of homocysteine by a cobalamin-dependent methyltransferase that catalyzes the transfer of the methyl group of methy1tetrahydrofolate to form methionine, and 3) condensation with adenosine to form AdoHcy. Conversion of homocysteine to cystathionine and cysteine did not appear to account for the capacity of homocysteine to reverse the inhibition of replication by 3-deaza-AdoHcy since cysteine did not reverse the inhibition and cystathionine had only a small effect. In a previous study (7), it has been shown that 3-deza-AdoHcy plas 0 (O), 0.5 (■), 1.0 (▲), 5.0 (□), 10 (▲), 50 (□), or 100 μM (○) 3-deaza-AdoHcy. B, cells incubated with 100 μM 3-deaza-AdoHcy plus 0 (■), 0.5 (■), 1.0 (▲), 5.0 (□), 10 (▲), 50 (□), or 100 μM (○) 3-deaza-AdoHcy and either 100 μM cystathionine (△), 200 μM D,L-homocysteine (●), 200 μM D,L-homocysteine (■), or 100 μM homocysteine thiolactone (▲). C, Cells in medium without any additions.
Dezaaisteromycin inhibits AdoHcy hydrolase, and, as a result, AdoHcy accumulates to levels that inhibit some methylation reactions. To determine whether incubation with homocysteine reversed the inhibition of AdoHcy hydrolase, cells treated for 4 days with 3-deazaisteromycin or 3-dezaaisteromycin and homocysteine were incubated with [35S]methionine for 90 min in the absence or presence of 3-deazaisteromycin and homocysteine. Table I shows that there was an accumulation of AdoHcy in cells treated with 100 μM 3-deazaisteromycin and that the level of AdoHcy was not decreased by incubation with homocysteine thiolactone. These results show that AdoHcy hydrolase remained inhibited and the inhibition was not decreased by a concentration of homocysteine thiolactone that reversed the cytostatic effect of 3-deazaisteromycin.

The remaining possibility was that treatment of cells with 3-deazaisteromycin reduced the in vivo formation of homocysteine to a point where insufficient homocysteine was available for remethylation to methionine. As a result, methylytetrahydrofolate would accumulate and tetrahydrofolate, a product of remethylation of homocysteine, would not be available to the cells. To test this hypothesis, folic acid was added to the 3-deazaisteromycin-treated cells (Fig. 3A). Folate reversed the inhibition of replication by 3-deazaisteromycin and an optimal concentration of folate was nearly as good at reversal as an optimal concentration of homocysteine thiolactone. Since folate is utilized in the *de novo* synthesis of pyrimidines and purines, addition of thymidine and hypoxanthine to 3-deazaisteromycin-treated cells was also tested (Fig. 3B). There was a small reversal of the inhibition of replication by thymidine but no reversal by hypoxanthine; however, when hypoxanthine and thymidine were both added, significant reversal of inhibition of replication occurred. A further indication that homocysteine facilitated folate-methionine to 3-deazaaristeromycin-treated cells was also tested in the presence of 3-deazaaristeromycin and homocysteine. To determine whether incubation with homocysteine thiolactone reversed the inhibition of AdoHcy hydrolase, cells treated for 100 μM 3-deazaaristeromycin and 100 μM homocysteine thiolactone were added to each dish. After incubation with [35S]methionine for 90 min, the radioactivity in AdoMet and AdoHcy was determined. Cell number after 4 days was determined in replicate dishes.

**Table I**

| Additions to growth mediuma | Cells | AdoMet | AdoHcy |
|-----------------------------|-------|--------|--------|
| None                        | 1.26 × 10^7 | 4140   | 190    |
| Homocysteine thiolactone    | 1.20 × 10^7 | 5190   | 160    |
| 3-Dezaaristeromycin         | 4.80 × 10^6 | 5560   | 4400   |
| 3-Dezaaristeromycin plus ho-| 1.00 × 10^6 | 6800   | 4500   |
| mocysteine thiolactone      |        |        |        |

a The concentration for either homocysteine thiolactone or 3-deazaaristeromycin was 100 μM.

![Fig. 2. Reversal of 3-deazaaristeromycin inhibition of cell replication by homocysteine thiolactate at various times after the addition of 3-deazaaristeromycin. RAW264 cells were plated in the presence of 100 μM 3-dezaaristeromycin, and 100 μM homocysteine thiolactate was added at the time the cells were plated (A) or 1 day (C), 2 days (C), or 3 days (A) after plating. O, homocysteine thiolactone was not added; C, cells in medium without any additions.](image)

**Fig. 3. Reversal of 3-deazaaristeromycin inhibition of cell replication by folate or by thymidine and hypoxanthine. RAW264 cells were plated in the presence of 100 μM 3-deazaaristeromycin (O). A, when the cells were plated, 10 μM, 20 μM, 100 μM, or 200 μM folate was added. B, when the cells were plated, 100 μM hypoxanthine (B), 20 μM thymidine (Δ), 100 μM hypoxanthine plus 20 μM thymidine (D), or 100 μM homocysteine thiolactone (A) was added. Cells in medium without any additions.**

**Table II**

| Additions to growth medium | [14C]Formate incorporation (dpm/10^6 cells) |
|---------------------------|---------------------------------------------|
| None                      | 93,100 ± 2,500                              |
| 3-Deazaaristeromycin      | 23,100 ± 800                                |
| 3-Deazaaristeromycin plus homocysteine thiolactone | 47,800 ± 700 |
| 3-Deazaaristeromycin plus folate | 34,600 ± 1,900 |

a The concentration for either 3-deazaaristeromycin, homocysteine thiolactone, or folate was 100 μM.

The results presented above indicate that administration of 3-deazaaristeromycin in vivo results in a very profound, pos-
sibly complete, inhibition of AdoHcy hydrolase. The cyto-
static effects of 3-dezaaasteromycin are a consequence of
this inhibition because when deprived of an adequate supply
of homocysteine, the cells cannot recycle methyltetrahydro-
folate and regenerate tetrahydrofolate for use in de novo
synthesis of purines and pyrimidines needed for nucleic acid
biosynthesis (15). Aside from the intrinsic biochemical interest
of these observations, our findings could have important clin-
cal significance. It has been shown that in a variety of clinical
conditions, AdoHcy hydrolase activity becomes severely re-
duced. Thus, in patients suffering from adenosine deaminase
deficiency disease, the AdoHcy hydrolase of erythrocytes is
completely and irreversibly inhibited by the accumulation of
2'-deoxyadenosine, an irreversible suicidal inhibitor of
AdoHcy hydrolase (16).

Adenine arabinoside together with an inhibitor of adenosine
deaminase, such as erythro-9-(2-hydroxy-3-nonyl)-adenine or
deoxycoformycin, is currently being tested in a series of clinical
trials in cancer chemotherapy. It has been suggested that the
inhibition of AdoHcy hydrolase that is observed upon
administration of AdoHcy would inhibit a variety of biologically
important transmethylation reactions (17). While this possibility
undoubtedly must be considered, we would suggest that perhaps an equally important consequence of the
inhibition of AdoHcy hydrolase in vivo might be its effects on
the sulfur conservation pathway. Mudd and Poole (18) have
shown that in normal healthy adults, the homocysteine moiety
of methionine cycles between homocysteine and methionine
two to four times (depending on the amount of methionine in
the diet) before being utilized by the enzymes of the transsul-
furation pathway for the synthesis of cystathionine and cyste-
ine. It would be of great interest to determine if the blood
level of methionine is abnormally low in patients suffering
with untreated adenosine deaminase deficiency or in patients
undergoing chemotherapy with adenine arabinoside plus an
adenosine deaminase inhibitor. Dietary supplementation
might be indicated in these clinical situations where the
patients might be close to, or actually in, negative nitrogen
balance. On the other hand, the trapping of tetrahydrofolate
as methyltetrahydrofolate might contribute to the oncostatic
activity of adenine arabinoside by limiting the amount of
tetrahydrofolate available for purine and pyrimidine biosyn-
thesis. It should also be pointed out that the combination of
adenine arabinoside, erythro-9-(2-hydroxy-3-nonyl)-adenine,
and methotrexate should be avoided since the methyltetra-
hydrofolate rescue therapy would fail due to insufficient ho-
mcysteine.

A deficiency of homocysteine would also result in a reduc-
tion of cystathionine, an amino acid that is present in brain in
high levels (19). Its regional distribution in the brain has been
studied (20–22), and a particularly high level is found in the
pineal gland (22). No specific functions have been assigned to
brain cystathionine, and it is noteworthy that patients with
cystathionine synthetase deficiency do not show any symp-
toms that can be attributed to a deficiency of brain cystathio-
nine. However, the possibility remains that cystathionine
might have a hitherto unrecognized role as a neurotransmitter
in the brain or that its absence might cause neurological
deficits with a long time delay.

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