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Differential Effects on Growth, Homocysteine, and Related Compounds of Two Inhibitors of *S*-Adenosylhomocysteine Catabolism, 3-Deazaadenosine, and 3-Deazaaristeromycin, in C3H/10T1/2 Cells[1]

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ABSTRACT

The growth of nontransformed (Cl 8) and malignant (Cl 16) C3H/10T1/2 mouse embryo fibroblasts was inhibited by 3-deazaadenosine (c^3Ado) (LD_{50} = 195 μM for Cl 8 and 30 μM for Cl 16 cells) and 3-deazaaristeromycin (c^3Ari) (LD_{50} about 36 μM for Cl 8 and 9 μM for Cl 16 cells). Both compounds inhibited in a dose-dependent manner *S*-adenosylhomocysteine (AdoHcy) catabolism and homocysteine production, measured as homocysteine egress, and c^3Ari was most potent in this respect. c^3Ado gave rise to its congener, 3-deazaadenosylhomocysteine (c^3AdoHcy). Addition of homocysteine thiolactone (Hcy-tl) to the medium enhanced AdoHcy (and c^3AdoHcy) accumulation but did not affect the cell growth at concentrations of inhibitor less than 10 μM. At high concentrations (30–300 μM) both compounds were cytotoxic and decreased cell count when added during midexponential growth. When Hcy-tl was supplemented under these conditions it partly rescued the malignant cells exposed to c^3Ari, did not affect the cytotoxicity of this agent towards the nontransformed cells, but greatly potentiated the cytotoxicity of c^3Ado against both cell types. Differential metabolic effects were also observed in that high concentrations of c^3Ado, but not c^3Ari, induced build-up of c^3AdoHcy and modulated cellular glutathione level. Growing cells contained the highest amount of glutathione, and in such cells c^3Ado induced a significant increase in glutathione whereas the cytotoxic combination of c^3Ado plus Hcy-tl decreased the amount of the reduced form. Quiescent confluent cells, which were less sensitive to the toxic effect of c^3Ado, contained low glutathione, and under these conditions neither c^3Ado alone nor in combination with Hcy-tl affected cellular glutathione. Remarkably, Hcy-tl alone induced an increase in glutathione in nondividing cells. These data suggest that homocysteine or some agents affecting homocysteine metabolism may modulate glutathione metabolism, but differently in dividing and nondividing cells.

INTRODUCTION

The cytotoxicity of several adenosine analogues is dependent on their phosphorylation to the corresponding nucleotide, which in turn are inhibitors of DNA synthesis (1). A nucleotide-independent mechanism of action of such compounds was first suggested by Hershfield, showing that 9-β-D-arabinofuranosyladenine and related compounds also may block *S*-adenosylhomocysteine hydrolase, the enzyme responsible for the catabolism of the endogenous transmethylase inhibitor, AdoHcy[4] (2). In the years from 1979 to 1985 numerous adenosine analogues were shown to interfere with the function of AdoHcy hydrolase, and this enzyme was used as a target for design of biological active compounds (3, 4). c^3Ari is a result of such rational design (5).

Adenosine analogues may function as competitive inhibitor, substrate, or inactivator (irreversible inhibitor) of AdoHcy hydrolase (2). In principle, the metabolic derangements induced by agents interacting with this enzyme can be divided into two types:

(*a*) Inhibition of AdoHcy catabolism results in accumulation of large amount of this metabolite, and nucleosides serving as substrate of this enzyme may be metabolized to substantial amounts of the corresponding nucleosidylhomocysteine. AdoHcy and some of its analogues are potent inhibitors of numerous AdoMet-dependent transmethylation reactions, and some metabolic effects of nucleoside analogues have been related to inhibition of methyltransferase reactions (2, 3).

(*b*) AdoHcy catabolism is the only known source of homocysteine in vertebrates (6), and both inhibitors and substrates of AdoHcy hydrolase may therefore lead to depletion of cellular supply of this amino acid (7, 8). Since homocysteine serves as methyl acceptor in the 5-methyltetrahydrofolate homocysteine methyltransferase reaction, lack of homocysteine may trap reduced folates as 5-methyltetrahydrofolate and thereby interfere with several metabolic pathways dependent on reduced folates (6). Lack of homocysteine has been suggested as a factor responsible for inhibition of cell growth in the presence of some adenosine analogues, including c^3Ari (9, 10).

Among numerous nucleosides interacting with the enzymatic breakdown of AdoHcy (3), c^3Ado and c^3Ari have proven to be effective both *in vitro* and *in vivo* (2, 3). c^3Ari is a competitive inhibitor of AdoHcy hydrolase, while c^3Ado serves as a substrate for the enzyme in direction of nucleosidylhomocysteine synthesis. Both compounds cause cellular build up of AdoHcy. In addition c^3Ado induces accumulation of the nucleosidyl analogue c^3AdoHcy. Neither compound are readily phosphorylated to their corresponding nucleotides (5), indicating that their effects on cellular metabolism are mediated by the nucleoside.

AdoHcy hydrolase was long considered as the prime cellular target of c^3Ado and c^3Ari (3), but recent data suggest that c^3Ado may interfere with other metabolic functions as well (11–13). Such diverse modes of action has not been demonstrated for c^3Ari. These properties of c^3Ado should be related to the finding that several biological effects seem to be confined to this agent. These effects include immunosuppression (14), antichemotaxis (15), induction of myoblast differentiation (16), and disruption of microfilaments (17).

The present paper compares the effects of c^3Ado and c^3Ari on nontransformed and malignant C3H/10T1/2 mouse embryo fibroblasts with respect to growth and metabolic responses, including homocysteine depletion and accumulation of nucleosidyl amino acids. The effect on total (GSH + GSSG + GSSR) and reduced glutathione (GSH) was also determined since

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[4] The abbreviations used are: Hcy-tl, homocysteine thiolactone; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; c^3Ari, 3-deazaaristeromycin; c^3Ado, 3-deazaadenosine; c^3AdoHcy, 3-deazaadenosylhomocysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; GSSR, soluble glutathione mixed disulfides; PBS, phosphate buffered saline; PE, plating efficiency; TCN, total cell number; LD_{50}, dose inhibiting survival by 50%; IC_{50}, dose inhibiting cell growth by 50%.

glutathione plays an important role in cellular response to numerous toxic agents (18), and recent data suggest that AdoHcy may affect glutathione content (19). The growth and metabolic responses were tested in the absence and presence of added homocysteine to evaluate whether they are related to homocysteine depletion, and in the case of c^3Ado, accumulation of c^3AdoHcy (20).

MATERIALS AND METHODS

Chemicals. Hcy-tl, L-methionine, AdoHcy, dithioerythritol, GSH, and GSSG were obtained from Sigma Chemical Co., St. Louis, MO, and AdoMet was from Koch-Light Laboratories, Colnbrook, UK. c^3Ado and c^3Ari were kindly supplied by Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL. Sodium borohydride was from Fluka Chemie AG, Switzerland, and monobromobimane was from Calbiochem, Behring Diagnostics, La Jolla, CA.

Cell Lines and Culture Conditions. Nontransformed C3H/10T1/2 Cl 8 (21) and chemically transformed C3H/10T1/2 Cl 16 (22) mouse embryo fibroblasts were obtained from the laboratory of Dr. J. R. Lillehaug, Department of Biochemistry, University of Bergen. Stock cultures were grown on tissue culture flasks (Nunc, Denmark) in Basal Medium Eagle (GIBCO, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (Sera-Lab, Ltd., Sussex, UK) at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 95%.

Toxicity of Nucleoside Analogues. Toxicity of c^3Ari and c^3Ado was estimated by PE or by determination of TCN.

For PE, 200 cells were seeded per dish (6 cm, Nunc, Denmark). One day later, the medium was replaced by fresh medium containing increasing concentrations of nucleoside analogue with or without coaddition of Hcy-tl (100 μM). The thiolactone form of homocysteine was chosen because when added to the medium it is nontoxic and is metabolized in these cells (23).

After 24 h the medium was removed and replaced with fresh medium without any drugs. 10 days after seeding, the medium was removed, the cells washed with 0.9% NaCl, fixed with methanol and stained with Giemsa. Colonies were scored and PE expressed as number of colonies in test group as percentage of control. Each group consisted of at least five dishes.

For TCN, cells were seeded in multiwell dishes (24-well cluster plate, Costar, USA) at a density of 1000 cells per well in medium supplemented with increasing concentrations of nucleoside analogues as above. On Day 5 (Cl 8) or Day 8 (Cl 16) after seeding the cells were trypsinized and counted using a Coulter Counter Model ZM (Coulter Electronics Ltd., Luton, UK). TCN was expressed as net increase in cell number in test group as percentage of net increase in control. Each group consisted of three wells.

Dose-Response Effects on Metabolites. Cells were seeded at a density of 5,000–20,000 per dish (10 cm, Costar). Late in logarithmic growth phase the medium was replaced by fresh medium containing increasing concentrations of nucleoside analogue with or without coaddition of 100 μM Hcy-tl. 24 h later the cells were harvested by removal of the culture medium, gently washed twice with ice-cold PBS and immediately frozen at −85°C. Samples of the medium were frozen at −20°C for determination of extracellular metabolites. Parallel dishes from each group were used to determine the cell number at harvesting.

Determination of Extracellular Homocysteine. The medium was mixed with perchloric acid to remove proteins, neutralized, and treated twice with dextran-coated charcoal to remove nucleosides and AdoHcy which would interfere with the homocysteine assay (7, 8). Homocysteine was then determined as described previously (24).

Determination of AdoHcy, c^3AdoHcy, and AdoMet. The frozen cells were extracted with perchloric acid and the adenosylamino acids assayed according to procedures described previously (7, 8).

Analysis of Cell Growth and Metabolites at High Drug Level. Cells were seeded at a density of 1500–5000 per dish (6 cm; Nunc, Denmark). In mid-log phase the medium were replaced by fresh medium containing c^3Ado (200 μM) or c^3Ari (100 μM) with or without coaddition of Hcy-tl (100 μM). At the times indicated two parallel dishes from each group were trypsinized and counted. For determination of metabolites parallel dishes were harvested as described above at the times indicated.

Determination of GSH and Total Glutathione. Cells were seeded at a density of 5000 cells per dish (10 cm, Costar). During mid-log phase (13% confluence), late-log phase (40% confluence) or 3 days after reaching confluence, the cells were treated for 24 h with c^3Ari or c^3Ado at the concentrations indicated and with or without coaddition of Hcy-tl (100 μM). The treatment was started by replacing the medium with fresh medium containing these compounds. At the beginning and at the end of each treatment period, two parallel dishes were harvested. Parallel dishes from each group were used to determine the cell number at these time points.

The frozen cells (−85°C) were extracted within 3 days after harvesting with ice-cold 5% sulfosalicylic acid, scraped off the dish with a rubber policeman and the precipitated proteins removed by centrifugation.

GSH was determined in the acid extract by a slight modification of a published method (25). Briefly, free sulfhydryl groups were derivatized with monobromobimane (Kosower's reagent), and the GSH-bimane derivative was quantitated by chromatography on a 3-μm ODS Hypersil column, which was equilibrated and eluted with 14.2% methanol and 43.5 mM acetic acid adjusted to pH 3.9 with NaOH. The column was washed by increasing the methanol concentration to 90%. The retention time of the GSH-bimane derivative was 3.2 min.

Total soluble glutathione (GSH + GSSG + GSSR) was determined by a procedure which has recently been developed in our laboratory. The procedure involves reduction of GSSG and GSSR to GSH by borohydride and subsequent derivatization of the free sulfhydryl groups with monobromobimane. The incubation mixture contained in a final volume of 280 μl: 30 μl acid extract, 80 mM HBr, 180 mM ethylmorpholine, 3.6 mM monobromobimane, and 150 mM sodium borohydride. After standing in the dark at room temperature for 20 min, 20 μl of 5.82 M perchloric acid was added. Total GSH-bimane concentration was determined by chromatography as described above for reduced GSH.

Determination of Protein. Protein was determined according to Bradford (26) using the Bio-Rad Protein Assay Kit. Bovine γ-globulin was used as protein standard.

RESULTS

Determination of the Cytotoxicity of c^3Ari and c^3Ado. We determined the cytotoxic effect of c^3Ari and c^3Ado on Cl 8 and Cl 16 cells in the absence and presence of Hcy-tl. The plating efficiency following drug exposure is shown in Fig. 1.

c^3Ari was slightly more toxic towards both Cl 8 (LD_{50} about 36 μM) and Cl 16 cells (LD_{50} = 9 μM) than was c^3Ado (LD_{50} = 195 μM, for Cl 8 cells and 30 μM for Cl 16 cells). Notably, under these conditions (24 h of drug exposure followed by cell growth in drug free medium for 10 days) Hcy-tl did not affect the cytotoxicity of c^3Ari (data not shown), while it enhanced the cytotoxicity of high concentrations of c^3Ado against both cell types (Fig. 1).

When the cytotoxicity was determined as total cell number, the relative cytotoxicity of c^3Ari and c^3Ado against both Cl 8 and Cl 16 cells was similar as above, but the assay was more sensitive, due to different experimental design. The IC_{50} values are listed in Table 1.

Dose-Response Effects on Homocysteine Egress and Cellular Adenosylamino Acids. The dose-response effect of c^3Ado and c^3Ari on metabolites primarily affected during inhibition of AdoHcy catabolism was investigated to evaluate their possible role in cytotoxicity. These metabolites include homocysteine, AdoHcy, c^3AdoHcy, and AdoMet (Figs. 2 and 3).

Both c^3Ari and c^3Ado induced a dose-dependent decrease in homocysteine egress, measured as extracellular homocysteine. There was a parallel increase in intracellular and extracellular

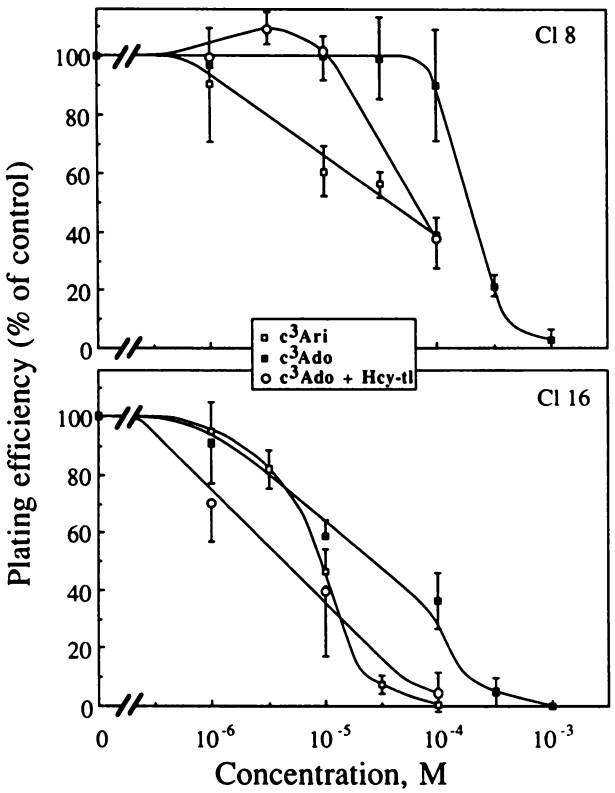

Fig. 1. Dose-response relationship for toxicity of c^3Ari and c^3Ado on C3H/10T1/2 cells with or without coaddition of 100 μM Hcy-tl. The plating efficiency is expressed as number of colonies as percentage of control 10 days after seeding of 200 cells per dish. The results are from a representative experiment, and each point represents the average of at least five determinations ± SD. *Top*, nontransformed Cl 8 cells; *bottom*, malignant Cl 16 cells.

Table 1 *Toxicity of c^3Ari and c^3Ado towards C3H/10T1/2 cells*

The net increase in TCN was determined 5 days (Cl 8) or 8 days (Cl 16) after seeding of 1000 cells per well (multiwell dishes) in medium containing the appropriate concentrations of the compounds to be tested. Control groups (100%) contained 39,000 cells (Cl 8) or 178,000 cells (Cl 16). The IC_{50} values listed are the concentration inhibiting cell growth (determined as TCN) by 50% compared to control.

Compound	IC_{50} (μM)	
	Cl 8	Cl 16
c^3Ari	5	0.6
c^3Ado	24	11

AdoHcy. c^3Ari was somewhat more potent in this respect than c^3Ado, but at high concentrations the latter agent gave rise to large amounts of c^3AdoHcy, which were almost quantitatively exported into the extracellular medium. AdoMet was unaffected by concentrations of c^3Ari or c^3Ado up to 100 μM (Figs. 2 and 3). However, at 300 μM c^3Ado a 5-fold increase in AdoMet was observed (data not shown).

Since Hcy-tl seemed to enhance the cytotoxicity of high concentration of c^3Ado (Figs. 1 and 5) the dose response effect of this agent was investigated also after addition of Hcy-tl (100 μM) to the culture medium. This resulted in an almost selective increase in c^3AdoHcy production, whereas the amount of AdoHcy was only moderately affected.

Cytotoxic and Metabolic Effects of High Concentrations of c^3Ari or c^3Ado. The growth curves were determined for Cl 8 and Cl 16 cells during c^3Ari or c^3Ado exposure for up to 11 days. The experiments were performed in the absence and presence of Hcy-tl, and concentrations of nucleoside analogue were ≧100 μM.

When c^3Ari (100 μM) was added to Cl 8 or Cl 16 cells during midexponential growth, an inhibition of growth was observed. Coaddition of Hcy-tl (100 μM) did not affect the growth reduction of the Cl 8 cells but decreased the growth inhibitory effect of c^3Ari against the transformed Cl 16 cells (Fig. 4).

A similar experiment was done with 200 μM c^3Ado. This analogue caused a marked inhibition of growth of both Cl 8 and Cl 16 cells. Addition of 10 μM Hcy-tl to the culture medium did not affect growth inhibition (data not shown). By increasing the Hcy-tl concentration to 100 μM there was a drastic decrease in cell count (Fig. 5), suggesting a pronounced toxic effect leading to cell lysis. When the drug was removed by medium replacement, both Cl 8 and Cl 16 cells resumed an almost normal growth rate within 72 h (data not shown).

Under experimental conditions as described above, the amounts of intra- and extracellular *S*-adenosylamino acids were determined after 48-h exposure to c^3Ado. As shown in Table 2, c^3Ado induced a marked increase in AdoHcy, but did not affect AdoMet. Coaddition of 100 μM Hcy-tl and 200 μM c^3Ado induced decreased intracellular level of AdoHcy and a 4–5-fold increase in c^3AdoHcy. Again c^3AdoHcy was almost quantitatively exported into the medium. Notably, AdoMet levels increased 4–5-fold in the nontransformed Cl 8 cells and twofold in the malignant Cl 16 cells under these conditions.

Cellular Glutathione. We determined reduced (GSH) and total glutathione (GSH + GSSG + GSSR) in growing cells exposed to c^3Ado or c^3Ari. c^3Ari was essentially without effect in the concentration range 3–100 μM, whereas c^3Ado above 10 μM altered cellular glutathione content (data not shown). The effect of 200 μM c^3Ado was studied in some detail in Cl 8 cells during various phases of growth. This cell type was used because Cl 8 cells (but not the transformed Cl 16 cells) show complete growth arrest at confluence.

Glutathione content was highest during mid-exponential growth and decreased markedly in non-dividing, confluent cells (Fig. 6). Similar results have been obtained by others (27, 28).

In proliferating cells c^3Ado induced a marked increase in glutathione whereas the combination of c^3Ado plus Hcy-tl decreased the amount of reduced GSH and to a lesser degree total glutathione when compared to c^3Ado alone. Thus the combination, which is highly cytotoxic under these conditions (Fig. 5), increased the fraction of glutathione in its oxidized form (GSSG + GSSR). Hcy-tl alone was without effect (Fig. 6, *A* and *B*).

In nonproliferating, confluent cells the response to c^3Ado was quite different. Both c^3Ado alone or in combination with Hcy-tl only marginally increased cellular glutathione. Notably, Hcy-tl alone increased the content to amounts approaching those seen in cells in late log phase (Fig. 6*C*).

DISCUSSION

The nucleoside analogue c^3Ado has several biological effects not shared by c^3Ari (14–17). This suggests that the former compound has some properties which may not be related to inhibition of AdoHcy catabolism. Formation of the metabolite c^3AdoHcy is a well established consequence of cellular exposure to c^3Ado (2), but other unique metabolic effects of c^3Ado have been described. These include elevation of cellular cyclic AMP content (12) and formation of trace amount of the corresponding monophosphate (11).

The present paper compares c^3Ado and c^3Ari with respect to cytostasis and metabolic effects in nontransformed mouse fibroblasts and a malignant cell line obtained by chemical transformation of the former. Particular attention is paid to the

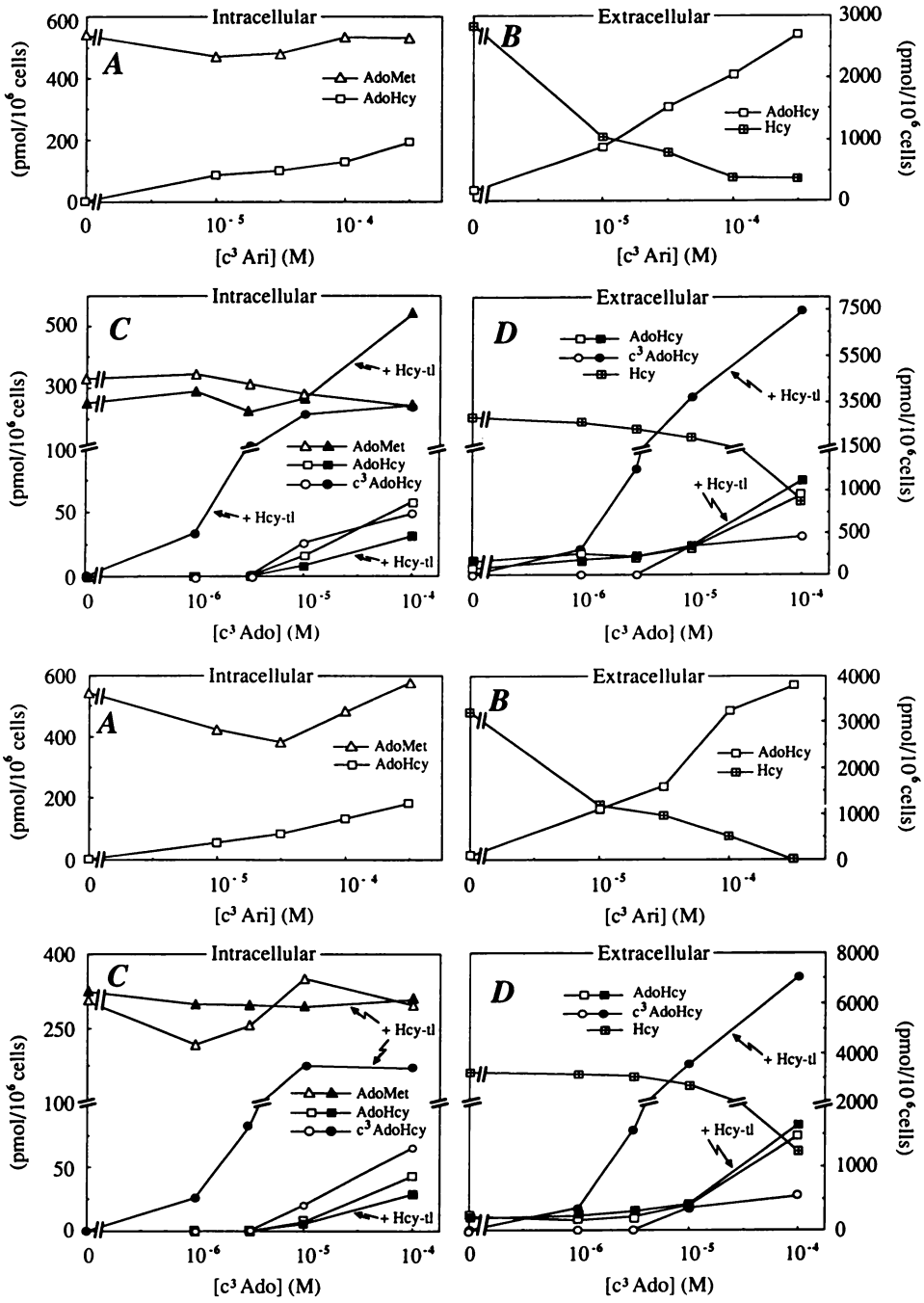

Fig. 2. Dose-response relationship for the effect of c³Ari and c³Ado on intra- and extracellular content of metabolites in nontransformed C3H/10T1/2 Cl 8 cells. 8000 cells were seeded per dish (10 cm) and treated for 24 h in late-log phase by replacing the medium by fresh medium containing either no additions, c³Ari or c³Ado at the concentrations indicated. The results are from a representative experiment. *A*, intracellular metabolites after 24 h exposure to c³Ari; *B*, extracellular metabolites (content of medium) after 24-h exposure to c³Ari; *C*, intracellular metabolites after 24-h exposure to c³Ado with or without coaddition of 100 μM Hcy-tl; *D*, extracellular metabolites (content of medium) after 24 h exposure to c³Ado with or without coaddition of 100 μM Hcy-tl.

Fig. 3. Dose-response relationship for the effect of c³Ari and c³Ado on intra- and extracellular content of metabolites in malignant C3H/10T1/2 Cl 16 cells. 20,000 cells were seeded per dish (10 cm) and treated for 24 h in late-log phase by replacing the medium by fresh medium containing either no additions, c³Ari or c³Ado at the concentrations indicated. The results are from a representative experiment. *A*, intracellular metabolites after 24-h exposure to c³Ari; *B*, extracellular metabolites (content of medium) after 24 h exposure to c³Ari; *C*, intracellular metabolites after 24-h exposure to c³Ado with or without coaddition of 100 μM Hcy-tl; *D*, extracellular metabolites (content of medium) after 24 h exposure to c³Ado with or without coaddition of 100 μM Hcy-tl.

possible role of homocysteine depletion following inhibition of AdoHcy catabolism, since this metabolic derangement has been assigned a role in the cytostatic effect of nucleoside analogues towards several cell lines (9, 10). In addition, lack of homocysteine may be responsible for inhibition of purine nucleotide biosynthesis by adenosine dialdehyde in T- and B-human lymphoblasts, and this effect is partly reversed by homocysteine (29).

Both c³Ado and c³Ari showed cytostatic effects towards nontransformed and malignant fibroblasts, and c³Ari was slightly more potent in this respect. The growth inhibitory effects were associated with a pronounced, dose-dependent decline in homocysteine production, measured as homocysteine egress, and there was a parallel increase in AdoHcy (Figs. 2 and 3). Cytostatic effect (Fig. 1) and homocysteine depletion (Figs. 2 and 3) occurred within the same concentration range, suggesting that these phenomena may be related. However, addition of Hcy-tl during exponential growth to the non-transformed cells did not rescue cells exposed to c³Ari, and only partly counteracted the effect of c³Ari towards the malignant Cl 16 cells, while Hcy-tl addition potentiated the cytostatic effects of c³Ado towards both cell types (Fig. 5). This suggests that lack of homocysteine was not responsible for the cytostatic effect of the 3-deazanucleosides towards the mouse embryo fibroblasts. However, some reservations should be made, because compartmentalization and transportation of homocysteine may result in different metabolic effects of extracellular and endogenously formed homocysteine. Availability of added Hcy-tl to the intracellular compartment is demonstrated by enhancement of the formation of c³AdoHcy (Figs. 2 and 3; Table 2).

The observation that the partial protection of cells towards the cytostatic effect of c³Ari was confined to the malignant Cl

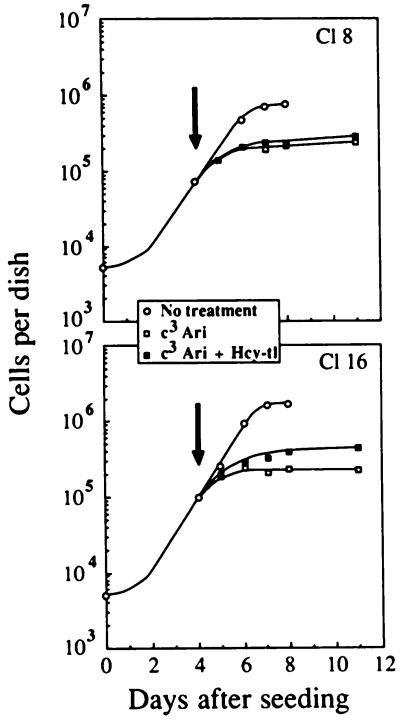

Fig. 4. Growth of C3H/10T1/2 cells exposed to c³Ari in mid-log phase. 5000 cells were seeded per dish (6 cm), and at Day 4 (*arrows*) the medium was replaced with fresh medium containing either no additions, 100 μM c³Ari or 100 μM c³Ari + 100 μM Hcy-tl. The results are from a representative experiment. *Top*, nontransformed Cl 8 cells; *bottom*, malignant Cl 16 cells.

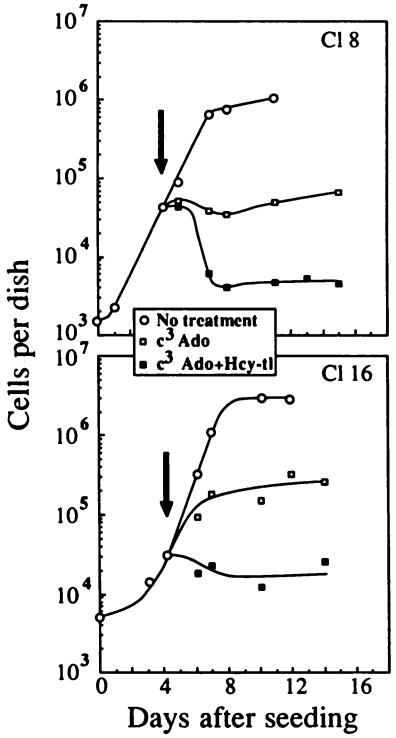

Fig. 5. Growth of C3H/10T1/2 cells exposed to c³Ado in mid-log phase. 1500 cells (Cl 8) or 5000 cells (Cl 16) were seeded per dish (6 cm), and at Day 4 (*arrows*) the medium was replaced with fresh medium containing either no additions, 200 μM c³Ado or 200 μM c³Ado + 100 μM Hcy-tl. The results are from a representative experiment. *Top*, nontransformed Cl 8 cells; *bottom*, malignant Cl 16 cells.

16 cells is in accordance with the previous reports (9, 10) on the protection of cells by homocysteine against the cytotoxic effect of purine nucleosides. Obviously, the role of homocysteine depletion may vary between cell types, and even between closely related cells as nontransformed and malignant mouse fibroblasts.

Table 2 *Metabolic effects of high concentration of c³Ado in absence and presence of Hcy-tl*

The experiment was performed under conditions similar to those described in Fig. 5. 10,000 cells (Cl 8) or 30,000 cells (Cl 16) were seeded per dish (10 cm) and treated for 48 h in mid-log phase by replacing the medium with fresh medium containing either no additions, 200 μM c³Ado or 200 μM c³Ado + 100 μM Hcy-tl. Treatment was initiated 6 days (Cl 8) or 5 days (Cl 16) after seeding, and 48 h later intra- and extracellular content of *S*-adenosylamino acids was determined. Results represent the average from two separate dishes.

		pmol/10⁶ cells				
		Intracellular			Extracellular	
Cells	Treatment	Ado Met	Ado Hcy	c³Ado Hcy	Ado Hcy	c³Ado Hcy
Cl 8	No addition, 6 days	271	ND[a]		0	
	No addition, 8 days	272	ND		190	
	c³Ado, 8 days	311	114	37.3	3,294	950
	c³Ado + Hcy-tl, 8 days	1,455	20.3	159	4,064	71,659
Cl 16	No addition, 5 days	315	ND		0	
	No addition, 7 days	370	ND		113	
	c³Ado, 7 days	333	82	35.2	3,213	1,049
	c³Ado + Hcy-tl, 7 days	615	47	191	2,797	26,260

[a] ND, not detectable.

In addition to partial rescue of the Cl 16 cells exposed to c³Ari described above, the most significant differences between c³Ado and c³Ari were that the cytotoxic effect of prolonged exposure (>24 h) to high concentration of c³Ado, but not c³Ari, was greatly potentiated by Hcy-tl (Figs. 4 and 5), the formation of c³AdoHcy by cells exposed to c³Ado, and finally, high concentration of c³Ado increased the amount of glutathione in proliferating cells (Fig. 6).

There are consistent reports that cellular glutathione content fluctuates during the cell cycle. Intracellular glutathione is elevated during cell proliferation, while quiescent cells contained low glutathione (27, 28). Thus, cellular glutathione metabolism may be differently regulated during the cell cycle. We therefore investigated the effect of c³Ado in both proliferating and non-proliferating confluent cells (Fig. 6).

Our data confirmed that cellular glutathione was higher in dividing than quiescent cells and most intracellular glutathione exists in its reduced form (GSH) (Fig. 6). Also, the glutathione response to c³Ado and Hcy-tl, alone or in combination, was different in growing and confluent cells. This observation adds support to the idea (27) that regulation of glutathione metabolism may be linked to cell division.

In growing cells c³Ado induced an increase in glutathione. We investigated the possibility that this was related to homocysteine depletion by testing the combination c³Ado plus Hcy-tl. This treatment decreased the content of GSH even below that observed in control cells (Fig. 6*A*). However, this combination was highly cytotoxic (Fig. 5), and decreased glutathione was probably an unspecific event related to cellular injury. This possibility was supported by the finding that a larger fraction (30–40%) of total glutathione is oxidized in these cells compared with controls.

Some speculations could be made on the mechanism behind the effect of c³Ado on cellular glutathione. Well-known metabolic consequences of c³Ado exposure are accumulation of AdoHcy and c³AdoHcy (30) and homocysteine depletion (30, Figs. 2 and 3). Since the effect on glutathione was observed at high c³Ado concentration and not in the presence of c³Ari, AdoHcy is probably not a mediator of this effect. c³AdoHcy on the other hand is a possible effector, and potentiation of this effect by supplementing Hcy-tl may be obscured by pronounced

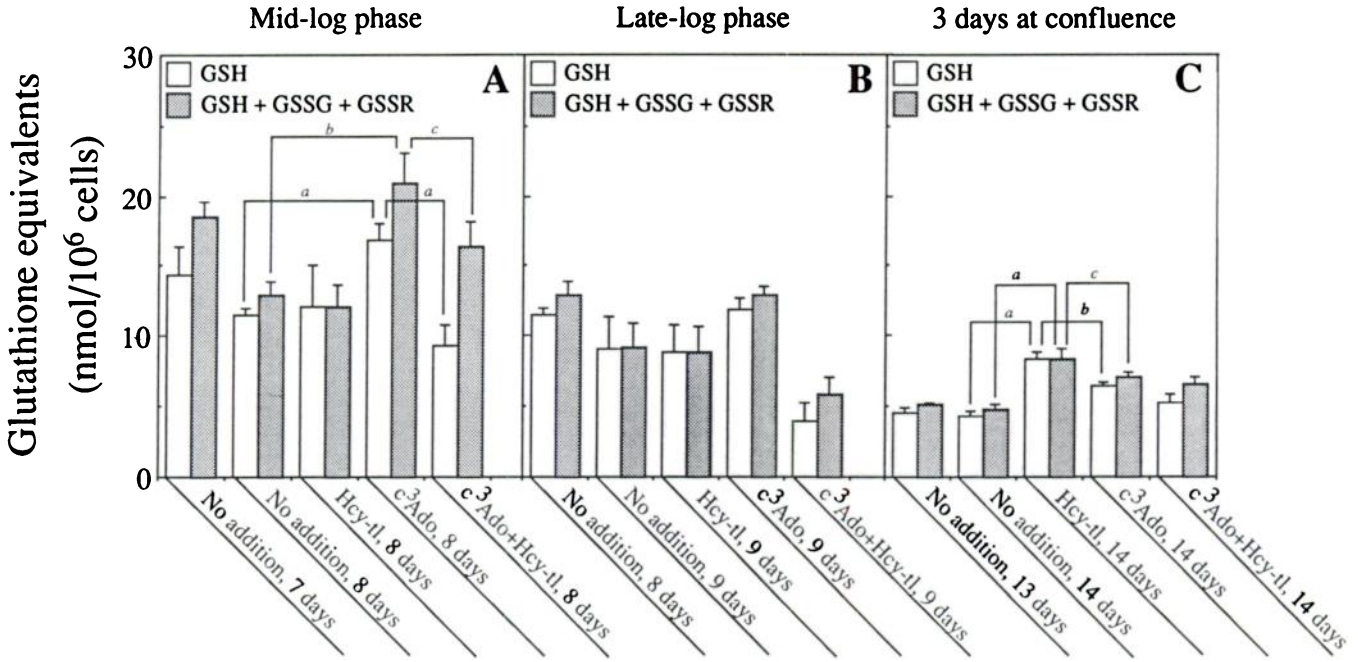

Fig. 6. Contents of reduced and total glutathione in nontransformed C3H/10T1/2 Cl 8 cells after 24-h exposure to c^3Ado and Hcy-tl during various phases of growth. 5000 cells were seeded per dish (10 cm), and at the times indicated the medium was replaced by fresh medium containing either no additions, 100 μM Hcy-tl, 200 μM c^3Ado or 200 μM c^3Ado + 100 μM Hcy-tl. *A*, treatment initiated 7 days after seeding (mid-log phase, 13% confluence); *B*, treatment initiated 8 days after seeding (late-log phase, 40% of confluence); *C*, treatment initiated 13 days after seeding (3 days at confluence). Results are expressed as equivalents of reduced glutathione and represent the average of four determinations ± SD. Significance levels were calculated using Student's *t* test. $^aP < 0.001$; $^bP < 0.002$; $^cP < 0.05$.

toxic effect (Fig. 5). High concentration of c^3Ado also efficiently traps homocysteine as c^3AdoHcy and blocks its formation (30) and the involvement of homocysteine in the regulation of glutathione metabolism during cell cycle may form the basis for the c^3Ado effect. This intriguing possibility should be considered in the light of fact that homocysteine production showed great variations during the cell cycle with maximal rate during S phase (31). Secondly, Hcy-tl alone did not affect glutathione in proliferating cells but increased the content in confluent cells (Fig. 6). Finally, methionine serves as a precursor for glutathione synthesis via homocysteine in some cells (32, 33). The possible influence of homocysteine on glutathione metabolism may be an important area for further research.

Modulation of glutathione metabolism may be an important consequence of cellular exposure to c^3Ado. GSH is a cytoprotective agent toward numerous toxic xenobiotics, including several drugs used in cancer chemotherapy (18), the cellular effects of which may therefore be altered in the presence of c^3Ado. Furthermore, the biological activity of several proteins, including enzymes, are dependent on free sulfhydryl groups, and it has been suggested that enzymatic and metabolic activity may be regulated according to the relation between reduced and oxidized glutathione (18). Notably, the cytoskeletal organization seems to be affected by glutathione (34), and the question of involvement of glutathione in the microfilament disrupting property of c^3Ado (17) should be considered. In general, it is possible that some biological effects of c^3Ado are a result of altered thiol status.

In conclusion, both c^3Ari and c^3Ado are potent inhibitors of AdoHcy catabolism and some effects of both compounds may therefore be related to inhibition of AdoMet-dependent transmethylation reactions (2) or depletion of intracellular homocysteine (6, 30). The present report adds to data (11) suggesting that c^3Ado has additional effects on vital cellular function. The potentiation of cytotoxicity by Hcy-tl associated with massive build up of c^3AdoHcy are properties unique to c^3Ado. In addition, the modulation of glutathione status by c^3Ado is a new aspect of this drug which should be further investigated.

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