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ELEVATION OF CYCLIC AMP LEVELS IN HL-60 CELLS ACCUMULATED IN G_1 OR G_2 BY TRANSMETHYLATION INHIBITORS

PER S. PRYTZ,* BERIT E. BANG, PETTER C. ENDRESEN, CONNY MØLLER and JARLE AARBAKKE

Department of Pharmacology, Institute of Medical Biology, University of Tromsø, P.O. Box 977, N-9001 Tromsø, Norway

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Abstract—Effects of the transmethylation inhibitors 3-deazaadenosine (c^3Ado) and 3-deaza-(±)-aristeromycin (c^3Ari) on cell cycle and cyclic AMP (cAMP) concentrations in human promyelocytic leukemia cells (HL-60) were studied by flow cytometry and radioimmunoassay techniques. Previously described cell cycle accumulations, after incubation with drugs (25 μM) for two cell doublings (36 hr), were localized to G_1 and G_2 after incubation with c^3Ado and c^3Ari, respectively. cAMP levels were elevated in cells treated with c^3Ado (35%) and c^3Ari (92%) for 36 hr. Addition of the phosphodiesterase (PDE) inhibitor theophylline, increased cAMP levels further, while cAMP responsiveness to the β-adrenergic stimulator isoproterenol was attenuated after c^3Ado and c^3Ari incubation. Homocysteine thiolactone (Hcy) alone reduced cell growth slightly (5%) and increased cAMP levels (17%). Hcy increased the growth inhibitory effects of c^3Ado, while no modulating effect was seen in combination with c^3Ari, nor did Hcy counteract the effects on the cell cycle perturbations. The results suggest that c^3Ado- and c^3Ari-induced cell cycle accumulation is, at least in part, mediated through cAMP elevation, possibly due to PDE inhibition secondary to *S*-adenosyl-homocysteine hydrolase inhibition and *S*-adenosyl-homocysteine build-up.

The adenosine analogues 3-deazaadenosine (c^3Ado†) and 3-deaza-(±)-aristeromycin are inhibitors of AdoHcyase (EC 3.3.1.1), which catabolizes the endogenous transmethylase inhibitor AdoHcy [1]. c^3Ari is a competitive inhibitor of AdoHcyase, while c^3Ado also serves as a substrate for the enzyme in the nucleosidyl-homocysteine direction. The prime cellular target for c^3Ari is considered to be AdoHcyase, while c^3Ado has additional modes of action [2].

Previously we have shown that c^3Ado and c^3Ari induce differentiation [3, 4] and cause G_0/G_1 and G_2 + M accumulation, respectively, in HL-60 cells [5]. The first objective of the present study was to locate whether the previously reported [5] HL-60 cell accumulation after 36 hr exposure to 25 μM c^3Ado was confined to either G_0 or G_1, and after 36 hr exposure to 25 μM c^3Ari to either G_2 or M. Secondly, it was to investigate levels of the second messenger cAMP after the same exposure regimes. The rationale for the latter experiments is the c^3Ado-induced increase in cAMP levels of mouse neutrophils [6] and PGE1 stimulated mouse lymphocytes [7] after short-term exposure, and the observation of increased levels of cAMP associated with differentiation induction [8, 9] and perturbation of cell cycle [10, 11]. Thirdly, we wanted to test the possibility that cell cycle perturbation could be due to cellular homocysteine depletion as a consequence of AdoHcyase inhibition [12].

We herein report that c^3Ado-treated cells accumulated in G_1 and c^3Ari-treated cells accumulated in G_2. These perturbations of cell cycle, maximal after 36 hr exposure to drug, were associated with increased cAMP concentrations, a hitherto unrecognized effect of c^3Ari. Addition of Hcy did not impair cell cycle perturbations or growth inhibition induced by c^3Ari, but enhanced c^3Ado toxicity.

MATERIALS AND METHODS

Drugs. c^3Ado was kindly supplied by Dr Peter K. Chiang, Walter Reed Army Institute of Research, Washington, DC, U.S.A. and c^3Ari was obtained from Dr John Montgomery, Southern Research Institute, Birmingham, AL, U.S.A. Theophylline was from Norsk Medisinaldepot, Harstad, Norway and (−)-isoproterenol (+)-bitartrate salt from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cells. HL-60 cells were grown in RPMI 1640 (Gibco Ltd, Paisley, U.K.) supplemented with 10% heat inactivated horse serum in a fully humidified atmosphere of 5% CO_2 at 37°. When harvested cell counts were determined in duplicate by a hemocytometer, and cell viability was assessed by trypan blue exclusion.

Experiments. Suspensions of cells were adjusted to concentrations of early logarithmic growth. The following day, cell suspensions were transferred to 50 mL culture flasks, and drug (10-fold of final

* To whom correspondence should be addressed.

† Abbreviations: c^3Ado, 3-deazaadenosine; c^3Ari, 3-deaza-(±)-aristeromycin; Ado, adenosine; Hcy, homocysteine thiolactone; AdoHcy, *S*-adenosyl-homocysteine; c^3AdoHcy, *S*-3-adenosylhomocysteine; AdoHcyase, *S*-adenosyl-homocysteine hydrolase; cAMP, adenosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; PGE1, prostaglandin E1.

concentration) or medium added to a final volume of 10 mL. Cultures were then incubated for 36 hr before harvest. Cells used as positive controls of mitotic accumulation were added vinblastine sulfate (Velbe, Eli Lilly & Co., Indianapolis, IN, U.S.A.) to a final concentration of 11 μM 4 hr before harvest.

Measurements of RNA content. Measurements were performed by the method of Darzynkiewicz *et al.* [13]. Briefly, at harvest cell density was adjusted to 1×10^6 cells/mL. Cells were permeabilized by Triton X-100 (Sigma) and stained with chromatographically purified acridine orange (Polysciences Inc., Warrington, PA, U.S.A.) at a final dye concentration of 13 μM in the presence of 1 mM Na-EDTA. The red fluorescence arising from RNA was measured immediately in single cells by an Argus Flowcytometer (Skatron, Lierbyen, Norway) equipped with a Skatron datasystem. The results were presented as fluorescence histograms.

Measurements of mitotic accumulation. Measurements were performed by the method of Darzynkiewicz *et al.* [14]. In short, at harvest cells were fixed in 70% (v/v) ethanol and stored refrigerated. Fixative was removed by centrifugation and cells were resuspended in phosphate buffered saline (Dulbecco) to a density of 5×10^6 cells/mL. After addition of 2×10^3 units/mL of RNase A (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) cells were incubated at 37° for 1 hr before being washed and resuspended. Cell DNA was partly denaturated by addition of 0.1 M HCl before being stained with acridine orange (Polysciences Inc.) at a final dye concentration of 11 μg/mL. The red and green fluorescence arising from single- and double-stranded DNA were measured simultaneously in single cells by an Argus Flowcytometer. The results were presented as bivariate contour plots of red versus green fluorescence.

Measurements of cAMP content. At harvest aliquots of cell suspensions were added to ice-cold ethanol to a final concentration of 65% (v/v) ethanol. The samples were analysed further by the protocol of a RPA.509 radioimmunoassay kit (Amersham International plc, Amersham, U.K.).

Calculations. Calculations for statistical significance by Student's *t*-test were performed on a microcomputer with the Microstat (Ecosoft Inc., Indianapolis, IN, U.S.A.) program.

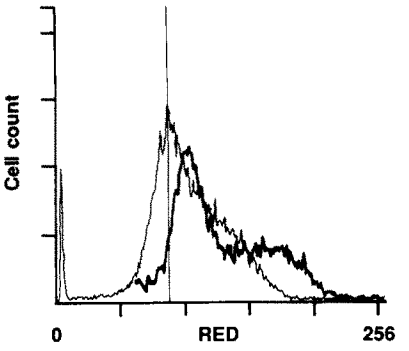

Fig. 1. Effect of 25 μM 3-deazaadenosine on RNA distribution in HL-60 cells incubated in the absence (———) or presence (———) of drug for 36 hr. Cells were stained with acridine orange as described in Materials and Methods, and the red fluorescence from RNA was measured in single cells by flow cytometry.

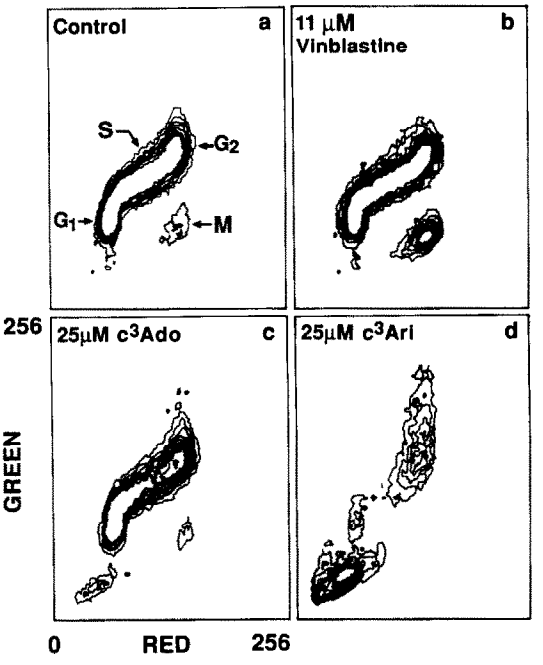

Fig. 2. Bivariate flow cytometric contour plots of red and green fluorescence of HL-60 cells after RNase treatment and acridine orange staining as described in Materials and Methods. Cells were incubated for 36 hr. Vinblastine was added 4 hr before harvest, other drugs were added at incubation start.

RESULTS

Effects of c³Ado and c³Ari on HL-60 cell cycle accumulation

HL-60 cells incubated with 25 μM c³Ado for 36 hr showed an increase in RNA content compared to controls (Fig. 1). The red fluorescence peak shifted to 119.0 ± 3.1% (N = 3) of the control peak channel, indicating that cells are accumulated in mid or late G_1 rather than in quiescent G_0 or early G_1 state [13].

The contour plots of red versus green fluorescence (Fig. 2) show the mitotic population separated from other cells. Control cells (panel a) and c³Ado-treated cells (panel c) had the same proportion of mitotic cells, 2.3 and 2.1%, respectively. The c³Ado treated cultures contained more possibly dead cells than control cultures. Vinblastine-treated cultures (panel b), acting as positive mitotic accumulation controls, had an increased proportion of mitotic cells (10.1%). HL-60 cells exposed to 25 μM c³Ari for 36 hr (panel d) showed no mitotic accumulation, which left G_2 as the compartment where cells accumulated. There was, however, a large proportion of possibly dead cells (approximately 45%) emerging in the plot.

Table 1. cAMP concentration of HL-60 cells incubated in absence or presence of 25 μM 3-deazaadenosine (c^3Ado) for 36 hr

Compound added	cAMP concentration (% ± SD)	N
None	100.0 ± 15.8	15
c^3Ado	135.1 ± 28.3*	15
Theo	192.4 ± 26.5	15
c^3Ado + Theo	250.4 ± 99.7*	15
IPR	472.2 ± 128.3	12
c^3Ado + IPR	384.8 ± 109.0*	11
Theo + IPR	5221.8 ± 1555.1	12
c^3Ado + Theo + IPR	4717.3 ± 1128.0	12

Theophylline (4 mM) (Theo) and isoproterenol (10 μM) (IPR) were given 30 and 5 min before harvest, respectively.

Results are presented as percentage of mean control cAMP levels (1.54 ± 0.26 pmol/10^6 cells).

* Value significantly different from its respective control ($P < 0.05$).

Table 2. cAMP concentration of HL-60 cells incubated in absence or presence of 25 μM 3-deaza-(±)-aristeromycin (c^3Ari) for 36 hr

Compound added	cAMP concentration (% ± SD)	N
None	100.0 ± 11.6	18
c^3Ari	192.1 ± 55.8*	18
Theo	197.8 ± 41.6	18
c^3Ari + Theo	270.4 ± 47.7*	18
IPR	684.6 ± 167.4	13
c^3Ari + IPR	489.6 ± 123.9*	15
Theo + IPR	5138.4 ± 1206.5	14
c^3Ari + Theo + IPR	3289.8 ± 720.5*	15

Theophylline (4 mM) (Theo) and isoproterenol (10 μM) (IPR) were given 30 and 5 min before harvest, respectively.

Results are presented as percentage of mean control cAMP levels (1.59 ± 0.43 pmol/10^6 cells).

* Value significantly different from its respective control ($P < 0.05$).

Effects of c^3Ado and c^3Ari on baseline and stimulated HL-60 cAMP concentrations

HL-60 cells incubated for 36 hr with 25 μM c^3Ado showed a significant increase ($P < 0.05$) in cAMP content to 135% of control values (Table 1). Cells exposed to 4 mM of the cAMP phosphodiesterase-inhibitor theophylline for the last 30 min of the 36 hr incubation, nearly doubled their cAMP content (Tables 1 and 2). Theophylline further increased cAMP levels compared to controls when added to c^3Ado treated cells. Exposure to 10 μM of the β-adrenergic stimulator isoproterenol, for 5 min before harvest, increased cellular cAMP content to nearly five times the baseline level of control cells. Pretreatment with c^3Ado reduced the isoproterenol-induced cAMP elevation to about four times the baseline level. Control and c^3Ado-incubated cells, receiving both theophylline and isoproterenol treatment before harvest, increased their cAMP content to approximately 50 times the baseline level, and c^3Ado treatment tended to attenuate the response.

Cells incubated with 25 μM c^3Ari for 36 hr also significantly increased their cAMP content to 192% of control values (Table 2). The cAMP content in c^3Ari-exposed cells was significantly higher than in cells incubated with c^3Ado. Thus, both c^3Ari and theophylline treatment nearly doubled cAMP concentration in HL-60 cells. When given in combination the cAMP concentration reached almost three times the baseline level. Isoproterenol treatment increased cAMP concentration in control and c^3Ari-exposed cells to approximately seven and five times the baseline level, respectively. Control cells receiving both theophylline and isoproterenol treatment increased, as the controls in the c^3Ado-incubated series, cAMP concentrations to about 50 times the baseline level. c^3Ari reduced the cAMP concentration elevation to approximately 33 times the baseline level.

Effects of homocysteine alone and in combination with c^3Ado and c^3Ari on HL-60 cell growth and cAMP concentration

Incubation of HL-60 cells with 1 mM Hcy for 36 hr raised cAMP concentrations slightly (Table 3). Hcy in combination with 25 and 5 μM c^3Ado caused massive cell death which prevented any useful determination of cAMP or cell cycle distribution. Co-exposure of cells to Hcy with 1 and 0.1 μM c^3Ado was not cytotoxic. For 0.1 μM c^3Ado this combination treatment increased cAMP levels above the levels seen for Hcy alone.

Incubation of HL-60 cells for 36 hr with 25 μM c^3Ari in combination with 1 mM Hcy did not produce the cytotoxic effect seen with c^3Ado. The cAMP concentration increased, but not to the levels seen when cells were incubated with 25 μM c^3Ari alone. Cell cycle distributions, when measurable, were never modified by addition of Hcy to cells incubated with or without transmethylation inhibitors (data not shown).

DISCUSSION

We present here data specifying the cell cycle accumulation effects of transmethylation inhibitors reported previously [5]. The shift of the HL-60 cell population to a higher level of RNA content after c^3Ado treatment indicates, according to the G_1 subcompartment model of Darzynkiewicz *et al.* [13], that the majority of c^3Ado-treated G_1 cells were located in a later stage of G_1 than control cells. Furthermore, a shift of cells from medium RNA content to a population of high RNA content was seen. HL-60 cells exposed to 25 μM c^3Ari for 36 hr showed literally no sign of mitotic cells (Fig. 2d), rendering that the observed accumulation in G_2 + M are in G_2 alone. We therefore conclude that HL-60 cells accumulate in late G_1 after c^3Ado and in G_2 after c^3Ari treatment for 36 hr.

Data from a variety of cell systems, among them

Table 3. cAMP concentration of HL-60 cells incubated in absence or presence of homocysteine thiolactone (Hcy) and 3-deazaadenosine (c^3Ado) or 3-deaza-(±)-aristeromycin (c^3Ari) for 36 hr

Compound added	cAMP concentration (% ± SD)	N
None (Control)	100.0 ± 6.4	9
Hcy (1 mM)	116.1 ± 23.1*	9
c^3Ado (25 μM)	126.3 ± 13.7*	9
c^3Ado (25 μM) + Hcy (1 mM)	ND	9
c^3Ado (5 μM) + Hcy (1 mM)	ND	9
c^3Ado (1 μM) + Hcy (1 mM)	128.2 ± 35.1*	9
c^3Ado (0.1 μM) + Hcy (1 mM)	141.7 ± 29.6*†	9
None (Control)	100.0 ± 11.6‡	18
Hcy (1 mM)	117.5 ± 23.7‡	17
c^3Ari (25 μM)	192.1 ± 55.8‡	18
c^3Ari (25 μM) + Hcy (1 mM)	144.7 ± 54.1‡	18

Results are presented as percentage of mean control cAMP levels.
* Values significantly different from control ($P < 0.05$).
† Value significantly different from incubation with Hcy ($P < 0.05$).
‡ All values significantly different ($P < 0.05$).
ND, not detectable due to cytotoxicity.

cell cycle mutants derived from mammalian cell lines, indicate the existence of several arrest points in the course of cell cycle [15]. Points of arrest seem to be confined mainly to the G_1 and G_2 phases. Evidence of a "restriction point" in G_1 and a presumably weaker point in G_2 have been reported [16]. A universal control mechanism of eukaryotic cell cycle, involving control points in G_1 and G_2, is believed to be based on cell division cycle (CDC) genes [17]. It is tempting to speculate that c^3Ado-induced accumulation in G_1 is by interference at a "restriction point" in late G_1, and that c^3Ari-induced accumulation of HL-60 cells in G_2 is due to an interaction with a G_2 "restriction point".

Various mammalian cell types are reported to be blocked in G_1 or G_2 as a result of treatment with cAMP elevating agents [10]. These blocks are not necessarily mutally exclusive. The adenylate cyclase activator forskolin provoked both a G_1 arrest and temporary delay through G_2 + M in the B-lymphoid precursor cell line Reh [11]. A similar prolongation of G_2 have previously been reported after cAMP elevation in synchronized HeLa cells [18]. Though cAMP generally is described as a growth inhibitor, stimulatory effects are also reported [19]. A link between CDC genes and cAMP signalling have been established [20].

Our finding that increased cAMP levels are correlated with differential cell cycle accumulation induced by transmethylation inhibitors, could indicate that cAMP elevation is a common denominator. The temporary accumulation of cells in G_2 after c^3Ari treatment reported previously [5], and the reports of prolongation of G_2 after treatment with cAMP elevating compounds [11, 18], strengthen this explanation. Down regulation of cAMP-dependent protein kinase, like that reported for hepatocytes [21], could be responsible for the temporary effect. As c^3Ari raised cAMP to a higher level than c^3Ado, the possibility of a cAMP concentration-dependent effect on cell cycle perturbation should not be ruled out. However, since c^3Ado interactions have been associated with several cellular targets while the only known target of c^3Ari is AdoHcyase [2], the differential effects on cell cycle could also be explained by interactions with additional different cellular targets.

Increased cAMP levels caused by c^3Ari are reported here for the first time, while c^3Ado has been shown to affect cAMP levels in other *in vitro* systems [6, 7, 22]. Mouse lymphocytes incubated with c^3Ado or isoproterenol alone show no sign of cAMP elevation [7]. Combined pretreatment with Ado and Hcy increased lymphocyte AdoHcy concentration and augmented cAMP responsiveness to isoproterenol and PGE1. Human platelets incubated with c^3Ado for 30 min showed a slight decrease in cAMP concentration and cAMP responsiveness to PGE1, while c^3Ari had no effect on cAMP levels [22].

We found increased levels of cAMP after treatment with c^3Ado, c^3Ari, theophylline and isoproterenol alone. Treatment with theophylline augmented cAMP concentrations of c^3Ado and c^3Ari exposed cells. HL-60 cells exposed to c^3Ado and c^3Ari showed reduced cAMP responsiveness after adenylate cyclase stimulation with isoproterenol alone, and in combination with theophylline. These data show that c^3Ado- and c^3Ari-treated cells are responsive to further stimulation of baseline cAMP level, and they indicate that the compounds attenuate responsiveness in the β-adrenergic receptor–G-protein–adenylate cyclase complex.

Taken together, these data and the finding of increased cAMP levels in HL-60 cells pretreated with c^3Ado or c^3Ari followed by incubation with theophylline, indicate that at least part of the transmethylation inhibitor-induced elevation of cAMP is through inhibition of PDE. Furthermore, such a mechanism is supported by results with

homogenates of mouse lymphocytes pretreated with c^3Ado and Hcy, showing that both AdoHcy and c^3AdoHcy were competitive inhibitors of the high-affinity cAMP PDE [7].

HL-60 cells exposed to 1 mM Hcy grew slightly (5%) less than controls (data not shown), and had increased cAMP concentrations after 36 hr (Table 3), compatible with increased cAMP levels as a result of elevated AdoHcy concentrations [7].

The growth inhibitory effect of c^3Ado was increased by Hcy, probably due to formation of c^3AdoHcy, a potent inhibitor of AdoHcyase [23]. Furthermore, the elevated cAMP levels seen after coexposure to 0.1 μM c^3Ado and 1 mM Hcy, indicated that the cAMP increase was mediated through the build-up of AdoHcy and c^3AdoHcy, and not by c^3Ado itself.

We found no modifying influence of Hcy on the growth inhibitory and cycle perturbation effect of c^3Ari on HL-60 cells. This observation, and the fact that c^3Ari is a strong inhibitor and a poor substrate for AdoHcyase [23], indicate that the effects of 25 μM c^3Ari is due to the build-up of AdoHcy observed after 36 hr in HL-60 cells (Loennechen T, personal communication).

There are conflicting reports in the literature regarding the course of cell growth upon co-administration of c^3Ari and Hcy. In mouse macrophages the cytostatic effect of c^3Ari was diminished by Hcy, while Hcy alone had no effect [12]. The authors concluded that the cytostatic effect of c^3Ari was due to depletion of cellular homocysteine, hence it could be restored by addition of Hcy. On the other hand, in several tumor cell lines Hcy increased the growth inhibitory effect of c^3Ari [24]. Hcy alone reduced cell growth far less, suggesting that Hcy exerts its potentiating effect through elevation of intracellular AdoHcy levels. In mouse embryo fibroblasts, Hcy had no effect on cytotoxicity of c^3Ari on non-transformed cells, while a malignant clone was partly rescued [25].

Our studies do not support a role for Hcy depletion in the cell cycle perturbation after c^3Ado or c^3Ari treatment. Rather they point to cAMP elevation secondary to perturbation of transmethylation metabolites. Further investigations will have to clarify if cAMP elevation is an early and primary event. Finally, our previous data on c^3Ado- and c^3Ari-induced differentiation in HL-60 cells [3, 4] may also be linked to cAMP elevation. cAMP elevating agents have been shown to induce differentiation in numerous cell types [10], including HL-60 cells [8, 9]. In our laboratory, elevation of cAMP by epinephrine and theophylline to levels reported herein after c^3Ado or c^3Ari incubation, caused differentiation of HL-60 cells.*†

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