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DIFFERENTIAL CELL CYCLE PERTURBATION BY TRANSMETHYLATION INHIBITORS

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Abstract—Cell cycle distribution of HL-60 cells was studied by flow cytometry after incubation with the transmethylation inhibitors 3-deaza-(±)-aristeromycin (c^3Ari) and 3-deazaadenosine (c^3Ado). Cells were incubated with the drugs (25 μM) for two cell doublings in control cells (36 hr). The presence of c^3Ari caused a dose-dependent, reversible G_2+M arrest, whereas c^3Ado-treated cells accumulated in G_0/G_1. The G_2+M arrest was also found in NIH/3T3 cells incubated for 36 hr with 25 μM c^3Ari, but not in U937 and K562 cells. Possible mechanisms for the described effects of c^3Ari are discussed from the perspective that inhibition of *S*-adenosyl homocysteine hydrolase, and subsequent inhibition of transmethylation reactions, at present is the only known site of action of c^3Ari.

The adenosine analogues c^3Ado† and c^3Ari have been extensively studied as inhibitors of AdoHcyase (E.C.33.22.1) and transmethylation reactions [1–3]. The diverse biological effects of c^3Ado have been associated with interactions with a number of primary cellular targets, while the effects of c^3Ari have not been ascribed to interaction with any cellular component apart from AdoHcyase [3]. Thus, c^3Ado and c^3Ari emerge as examples of non-selective and selective transmethylation inhibitors, respectively.

We have previously investigated the capacity of c^3Ado and c^3Ari as inducers of differentiation of leukemia cell lines *in vitro* [4, 5] and the effects of c^3Ado on viability and growth of human cells potentially involved in differentiation induction *in vivo* in man [6]. Herein, we present differential effects of c^3Ado and c^3Ari on cell cycle.

MATERIALS AND METHODS

Cells. Four different cell lines were tested: HL-60 (a human promyelocytic leukemia cell line), U937 (a monocytelike cell line derived from a patient with histiocytic lymphoma), K562 (a human myeloid leukemia-derived cell line) and NIH/3T3 (a murine fibroblast cell line). Cells were generous gifts from M.S. Hersfield, Duke University, U.S.A. (HL-60), S.O. Kolset, University of Tromsø (U937, K562) and I.W. Nilsen, University of Tromsø (NIH/3T3). Cells were grown in RPMI 1640 (Gibco Ltd, Paisley, U.K.) supplemented with 10% heat inactivated horse serum (HL-60), fetal calf serum (U937) or newborn calf serum (K562). NIH/3T3 cells were grown in Dulbecco's (Gibco) medium supplemented with 10% heat inactivated fetal calf serum. All cells were grown in a fully humidified atmosphere of 5% CO_2. 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 or medium added to a final volume of 10 mL. After each incubation interval, one set of flasks was harvested and two 100 μL samples were obtained for determination of cell concentration. The remaining cells were used for DNA-analysis.

NIH/3T3 cells were harvested by trypsination and resuspended in 10 mL of medium followed by the same procedure as for the other cultures.

HL-60 cells exposed to 25 μM c^3Ari were washed once in drug-free medium before adjustment of cell concentration and reincubation in drug-free medium.

DNA-analysis. Cells were centrifuged for 5 min at 200 *g*, washed in phosphate buffered saline (Dulbecco), resuspended in saline, fixed in 70% ethanol and kept refrigerated until analysis. Twenty-four hours before analysis, cells were heated at 45° for 30 min [7], centrifuged, and stained with 50 μg/mL Mithramycin (Pfizer, NY, U.S.A.) in 25% ethanol-solution containing 15 mM $MgCl_2$. Cells were subsequently kept refrigerated until analysed on an Argus Flowcytometer (Skatron, Lierbyen, Norway) equipped with an Ahrens FDAS data system (O.Ahrens, Bargteheide, F.R.G.). The number of cells in different cell cycle phases were calculated from the DNA-histograms by means of the peak reflect method [8]. The results are given as percentage of total histogram cell count. Calculations for statistical significance by Student's *t*-test were done on a microcomputer with the Microstat (Ecosoft, INC., U.S.A.) program.

RESULTS

After more than 36 hr incubation, 25 μM c^3Ado decreased the number of viable HL-60 cells compared to controls (Fig. 1). In cells incubated with

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† Abbreviations: c^3Ado, 3-deazaadenosine; c^3Ari, 3-deaza-(±)-aristeromycin; AdoHcyase, *S*-adenosyl homocysteine hydrolase; Adomet, *S*-adenosyl homocysteine.

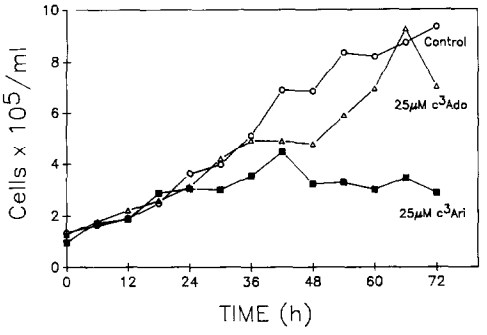

Fig. 1. Growth of HL-60 cells in the presence of 3-deazaadenosine and 3-deaza-(±)-aristeromycin.

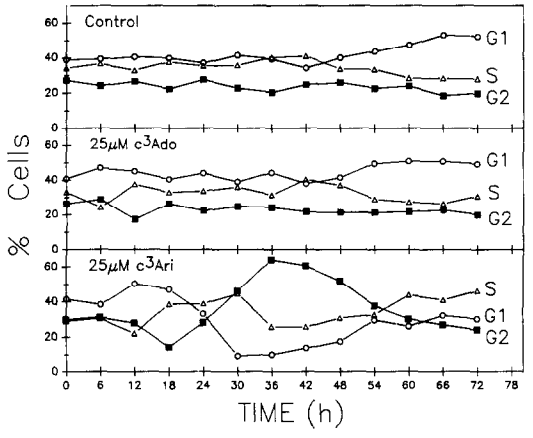

Fig. 2. Flow cytometric analysis of cell cycle distribution of HL-60 cells during exposure to 3-deazaadenosine and 3-deaza-(±)-aristeromycin.

25 μM c³Ari, growth was inhibited after 24 hr incubation.

Cells incubated with 25 μM c³Ari showed a different pattern of cell cycle distribution as compared to control cells, and c³Ado-exposed cells incubated for more than 18 hr (Fig. 2). The most striking effect of c³Ari was a rise in the percentage of cells in G_2+M, which reached a maximum of more than 50% of total after 36 hr (Fig. 3) and decreased to control values after 72 hr of incubation. Notably, the effect was also reflected in an increase in the absolute number of cells in G_2+M. c³Ari induced a decrease in the percentage of cells in G_0/G_1 after 18 hr with a trough value of about 10% at 30 hr, followed by a slow subsequent increase. The percentage of cells in S increased slightly between 18 and 30 hr of incubation, approaching control levels at 54 hr, with a slow increase from 36 hr onwards. The endpoint distribution represents a shift in percentage of cells in S and G_0/G_1 compared to control cells.

Table 1 summarizes the results from five experiments with HL-60 cells incubated for 36 hr. Cells incubated with 25 μM c³Ari demonstrates a G_2+M arrest while the most striking effect of 25 μM c³Ado after 36 hr was an increase in the percentage of cells in G_0/G_1.

After 36 hr incubation with c³Ari, effects were seen on cell cycle distribution at concentration of 5 μM, with a dose-dependent redistribution reaching a plateau at 40 μM (Fig. 4).

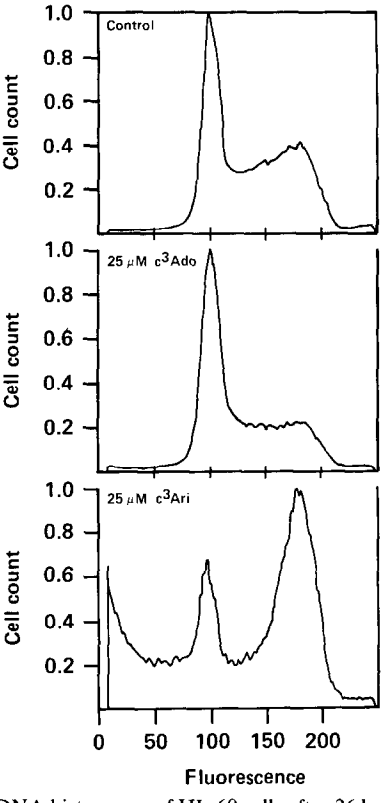

Fig. 3. DNA histograms of HL-60 cells after 36 hr exposure to 3-deazaadenosine and 3-deaza-(±)-aristeromycin. Cells were fixed in 70% ethanol and stained with mithramycin.

HL-60 cells exposed to 25 μM c³Ari for 36 hr regained control distribution after approximately 18 hr incubation in drug-free medium (Fig. 5). Compared to control cells, growth was slightly reduced in the pre-exposed cells after 24 hr of drug-free incubation (Data not shown).

A limited number of cell lines were investigated to see if the cell cycle effects of 25 μM c³Ari for 36 hr in HL-60 cells could be reproduced using other target cells. Only NIH/3T3 cells showed a significant increase in the percentage of cells in G_2+M and a reduction in G_0/G_1 (Table 2).

DISCUSSION

The data presented herein are the first demonstration of cell cycle effects of transmethylation inhibitors. The effects of c³Ari are particularly interesting because the compound apperas to be a selective inhibitor of AdoHcyase. Most known inducers of leukemia cell differentiation cause cell arrest in G_0/G_1. Dimethylsulphoxide and phorbolesters induce HL-60 arrest in G_0/G_1 after one doubling [9, 10]. Isotretinoin treatment causes a similar arrest after two divisions [9]; while sodium butyrate blocks cells in late G_1 [11]. Among agents known to induce differentiation *in vitro*, the G_2+M arrest seems to be confined to inhibitors of mitosis [12].

The present data show that a proposed selective transmethylation inhibitor, c³Ari, also reversibly arrests HL-60 cells in G_2+M. At the dose schedule

Table 1. Cell cycle distribution of HL-60 cells incubated with 25 μM 3-deazaadenosine and 3-deaza-(±)-aristeromycin for 36 hr

	% Cells in G_0/G_1	% Cells in S	% Cells in G_2+M	Growth as % of control
Control	34.3 ± 1.3	36.0 ± 1.2	29.7 ± 0.4	—
25 μM c^3Ado	47.5 ± 1.9*	32.3 ± 1.3*	20.2 ± 0.7*	90.9 ± 11.2
25 μM c^3Ari	21.9 ± 1.5*	26.6 ± 1.8*	51.6 ± 0.8*	60.7 ± 5.2*

Results are given as mean ±SD from five experiments.
* $P < 0.05$ by Student's *t*-test.

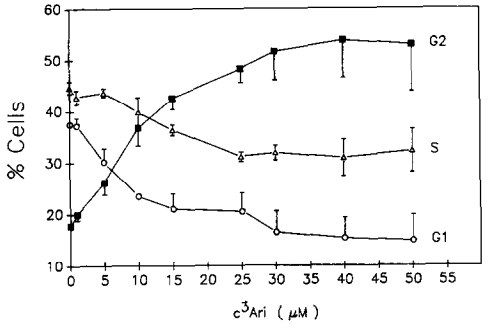

Fig. 4. Dose–response relationship for HL-60 cell cycle distribution after 36 hr exposure to 3-deaza-(±)-aristeromycin.

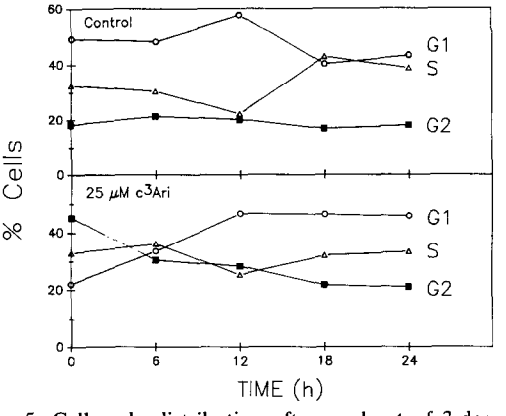

Fig. 5. Cell cycle distribution after washout of 3-deaza-(±)-aristeromycin. HL-60 cells were incubated with either medium alone (control) or with 3-deaza-(±)-aristeromycin for 36 hr, which is time zero on the figure. At this point medium was removed and cells reincubated in drug-free medium.

employed, this effect of c^3Ari could not be demonstrated in two other human hemopoietic cell lines. However, this effect of c^3Ari is not confined to human hemopoietic cell lines undergoing differentiation, as demonstrated by the G_2+M arrest in the murin fibroblast cell line NIH/3T3.

The G_2+M arrest in HL-60 was reversible both after continuous exposure to c^3Ari and after washout of c^3Ari. We are presently investigating how these effects are interrelated.

Differential effects on cell cycle can be included on the list of differences between the AdoHcyase inhibitors c^3Ado and c^3Ari. Only c^3Ado has been shown to affect cyclic AMP levels [13, 14] and microfilaments [15]. While both c^3Ado [16] and c^3Ari [17] are phosphorylated to the monophosphate in intact cells, only c^3Ado has been detected as the triphosphate [16, 17]. In addition, only c^3Ado commits HL-60 cells to differentiation after a short time (6 hr) of exposure [18], while continuous presence of c^3Ari is necessary to induce differentiation [5], and only c^3Ado modulates cellular glutathione levels [19]. The observation that cell cycle effects of c^3Ari are exerted at concentrations known to effectively inhibit HL-60 AdoHcyase [6], is consistent with the concept of c^3Ari as a selective transmethylation inhibitor. The G_2+M arrest is reversible upon removal of c^3Ari consistent with the c^3Ari effects reported for growth inhibition [6] and HL-60 cell differentiation [18]. Based on the present knowledge, it may seem reasonable to assume that c^3Ari induces G_2+M arrest by a mechanism related to inhibition of AdoHcyase.

Three possible relations have been partly explored. Firstly, c^3Ari could block increased activity of AdoHcyase needed for traverse through G_2+M. Although the activity of the enzyme in mouse mastocytoma P-815 cells and its c-AMP binding capacity were at a maximum in G_2+M [20], the activity of AdoHcyase showed no cell cycle dependency in untreated HL-60 cells [21].

Secondly, enzyme activities and cellular metabolites of general importance for transmethylation reactions could show variation through the cell cycle, and cells could be particularly vulnerable to perturbation of AdoHcyase inhibition in G_2+M. The

Table 2. Cell cycle distribution and growth of cells incubated with 25 μM 3-deaza-(±)-aristeromycin for 36 hr

	% Cells in G_0/G_1	% Cells in S	% Cells in G_2+M	Growth as % of control
3T3 Control	32.9 ± 6.2	39.6 ± 5.9	27.5 ± 0.7	—
3T3 25 μM c^3Ari	14.3 ± 0.5*	36.4 ± 4.3	49.3 ± 4.1*	53.3 ± 10.8*
K562 Control	37.0 ± 5.4	36.8 ± 5.7	26.1 ± 6.1	—
K562 25 μM c^3Ari	34.5 ± 6.3	39.9 ± 5.9	25.6 ± 8.2	84.5 ± 17.8
U937 Control	38.3 ± 5.4	37.6 ± 3.5	24.1 ± 6.1	—
U937 25 μM c^3Ari	42.5 ± 3.4	36.8 ± 1.1	20.7 ± 2.3	58.0 ± 22.8*

Results are given as mean ±SD from three experiments.
* $P < 0.05$ by Student's *t*-test.

activity of S-adenosyl methionine synthase did not, however, show cell cycle dependency in HL-60 cells, nor did AdoMet and AdoHcy concentrations, or the AdoMet/AdoHcy ratio [22].

Thirdly, c^3Ari could inhibit specific methylation reactions, like DNA methylation. Although c^3Ari did not change overall DNA methylation [23], a comprehensive exploration of the concept that c^3Ari may act on cell cycle distribution through transmethylation inhibition, should include the possibility that c^3Ari could alter sequence specific promoter methylation of genes [24], e.g. inhibitory genes involved at a restriction point in late G_2 [25].

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