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Lack of cross-resistance between cytosine arabinoside and a new halogenated nucleoside analogue, 2-bromo-2'-deoxyadenosine in human acute myeloid leukaemia cells*

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Summary. 2-Bromo-2'-deoxyadenosine (BdA) is one of a group of recently synthesised halogenated deoxyadenosine analogues that are relatively resistant to inactivation by adenosine deaminase (ADA). Its activity has been studied in human acute myeloid leukemia (AML) in vitro. In these studies BdA behaved as a cycle-active, phase-active agent that blocked cells at the G_1-S transition. It did not exhibit significant cross-resistance with cytosine arabinoside (Ara-C) in either clinical AML samples (from patients who exhibited Ara-C resistance in vivo) or in HL60 in which Ara-C resistance had been induced in vitro. Deoxycytidine kinase levels were not reduced in resistant lines. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase (ADA) inhibitor, with BdA produced a simple additive response without the dramatic synergism reported when it is used with deoxyadenosine. This is consistent with the idea that BdA is a poor substrate for ADA. This group of compounds warrants further investigation to determine their suitability for clinical use, especially in situations where Ara-C resistance is likely to be a problem.

Introduction

Recently, a new class of halogenated analogues of deoxyadenosine has been synthesised. Of these compounds the nucleoside most cytotoxic towards CCRF-CEM cells in vitro were 2-chloro-2'-deoxyadenosine (CdA) and 2-bromo-2'-deoxyadenosine (BdA), possessing IC_{50} values of 0.045 and 0.068 μM, respectively [14]. These analogues are also highly toxic to other T-lymphoblastoid lines and some B-lymphoblastoid and myeloid cell lines [3, 4, 8, 10, 14]. It has been reported that CdA is selectively toxic towards both dividing and resting lymphocytes [4, 9]. CdA inhibited the proliferation and survival of malignant T, non-T and non-B lymphocytes at concentrations that spare normal bone marrow cells. These compounds have demonstrated activity against L1210 murine leukaemia in vivo [10]. In addition, a phase I clinical trial with CdA demonstrated activity against a number of advanced haematopoietic malignancies, including three cases of myeloid leukaemia [16]. The mechanism of action of these compounds is thought to involve phosphorylation by deoxycytidine kinase and entry into a rapidly turning over nucleotide pool. Dividing lymphocytes incorporate CdA into DNA [3, 4].

Cytosine arabinoside (Ara-C) is commonly used clinically for AML induction and maintenance therapy. It is incorporated into DNA and is a potent inhibitor of de novo DNA synthesis, affecting S-phase cells and exhibiting proliferation-dependent cytotoxicity [5]. However, development of resistance to Ara-C in the clinical setting is common. Mechanisms of resistance to Ara-C include reduced drug uptake by the cell, decreased activation by deoxycytidine kinase and increased inactivation by deamination. BdA and CdA are resistant to metabolism by adenosine deaminase [18] but require activation by deoxycytidine kinase [3]. Hence, where Ara-C resistance is mediated by decreased activity of deoxycytidine kinase or by decreased nucleoside uptake then a degree of cross-resistance between Ara-C and BdA would be expected. If resistance to Ara-C had developed through increased activity of cytidine deaminase, then cross-resistance would not be expected to occur.

Initially experiments were performed to determine whether there was cross-resistance between Ara-C and BdA in clinically derived AML cells. Once it had been demonstrated that this was not the case, the human myeloid leukemia cell line, HL60, was used to elucidate the site of action of BdA in the cell cycle and the role of ADA blockade in the activity of this compound.

Methods and materials

Patient cells. Samples from 16 patients with a diagnosis of AML were studied. Patients were classified and treated as previously described [11, 12]. In brief, they received induction treatment with an anthracycline and Ara-C administered as part of the TAD (thioguanine, Ara-C and daunorubicin) regimen. Complete remission was defined as the disappearance of disease, with normal bone marrow and peripheral blood smear. Patients who did not enter remission were classified as non-responders. Patients who entered complete remission after one or two courses of treatment were classified as responders. Bone marrow and peripheral blood were collected in preservative-free heparin (CSL, Australia; 50 units/ml). Samples were obtained from patients with AML as part of the diagnostic procedure at presentation or relapse prior to treatment.

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Clinical and in vitro responses of 16 AML patients to Ara-C are given in Table 1. The in vitro response to Ara-C was quantitated as previously described [12]. Bone marrow cells from AML patients were exposed to clinically relevant AraC concentrations for 24 h prior to plating in semisolid agar. The number of colonies formed after drug exposure was compared with that of untreated control cells and expressed as a percentage of this number. These percentages were then plotted against the exposure dose. It has been found that the area under these dose-response curves (AUC) [15] is the parameter of sensitivity which best correlates with petient response in vivo [11, 12]. Because the absolute value of AUC is dependent on the maximum dose, the values are standardized by expressing them as percentages of no response, i.e. the value of AUC obtained if no cell kill is achieved at the highest dose administered. In this study clinical non-response correlated well with the in vitro response to Ara-C; 87% (14/16) of the patients were correctly classified as sensitive or resistant.

Diluted peripheral blood (1:1 with Roswell Park Memorial Institute medium, RPMI 1640) was centrifuged on Ficoll-paque (specific gravity, 1.077, Pharmacia Uppsala) at 400 *g* for 30 min, after which interface cells were collected, washed twice and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (RPMI 1640 10%). Bone marrow was centrifuged once at 150 *g* for 10 min and also resuspended in RPMI 1640 10%. The fresh patient samples were plated in agar at 5×10^3 to 5×10^5/ml with 10% GCT-conditioned medium (Gibco Laboratories) to determine cloning efficiency. Remaining cells were suspended in RPMI 1640 10% with 5% dimethyl sulphoxide (DMSO), frozen and stored in liquid nitrogen. When required they were thawed rapidly to the point of phase transition, then kept on ice, diluted slowly and washed with ice-cold RPMI 1640 10%.

Cell line. The AML cell line HL60 was grown as a suspension in RPMI 1640 10%. HL60 has a doubling time of approximately 24 h, and studies were performed when cells were in the log phase of growth. Viable cell counts were performed manually using the Trypan Blue exclusion technique. The cells were mycoplasma-negative when tested at regular intervals.

Colony assay. The agar colony-forming assay was used to study the sensitivity of AML colony-forming cells and HL60 to BdA. Agar (0.8% Bacto-Agar, Difco Laboratories USA) was boiled for 2 min, then cooled to 37° C and mixed with one part hypertonic medium (37° C). The hypertonic medium used was as previously described [11]. Cells were suspended in agar at 37° C to give a final concentration of 2×10^2/ml for HL60 and 5×10^3 to 5×10^5/ml for AML patient samples. Aliquots (1 ml) of this suspension were plated in triplicate in 35-mm petri dishes (Kayline Plastics, Sth Australia). Plates were then incubated at 37° C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2. After 7 days, colonies with >40 cells were scored.

Concentration × time. A cell suspension in RPMI 1640 10% was prepared and 450-µl aliquots were pipetted into 24-well tissue culture trays (16-mm well diameter; Costar, Cambridge, Mass). At each time point, 50 µl BdA dissolved in RPMI 1640 at 10× the final concentration, or RPMI 1640 alone in the case of the controls, was added to the wells. Since no clinically relevant pharmacokinetic data on BdA were available, the concentration × time (C × T) values of BdA used were 1.0, 0.5, 0.25, and 0.125 times the molar values calculated from published pharmacokinetic data for Ara-C [1]. Hence, the BdA C × T value of 1.0 is equivalent to a 24-h incubation with 3.2 µ*M* of drug. As duration of exposure to drug (T) increased, concentration (C) was decreased proportionally, thus maintaining five constant values of C × T. The importance of the concentration × time product (C × T) in determining pharmacologically relevant values of C and T is discussed by Alberts [1] and Mellet [13]. Stock solutions were prepared by dilution on day 1 and stored frozen until use.

The plates were incubated for 48 h, with additions at 0, 24, 32, 40 and 44 h; thus incubations with the drug were of 48, 24, 16, 8 and 4 h duration, respectively. Cells were washed and fresh BdA added after every 8 h of incubation, because FACS experiments showed decreased drug activity after this time. After 48 h incubation the cells were washed twice with 10 ml RPMI 1640 and finally resuspended in 4 ml agar medium for plating in triplicate (as described above).

Deaminase inhibition. Colony assays were performed as described, with the addition of 5, 7.5 or 10 µ*M* of erythro-9(3-(2-hydroxynonyl))adenine (EHNA was a gift from Drs R. Kefford and G. Mann, Ludwig Institute for Cancer Research, Sydney) 2 h before the addition of BdA. BdA was added at four different concentrations (3.2, 1.6, 0.8 and 0.4 µ*M*) and incubated for 24 h. The cells were then washed twice in RPMI 1640 and plated.

Cross resistance. HL60 were made resistant to either Ara-C or BdA by chronic exposure to progressively increasing low doses of drug. The cell lines produced were called HL60 (Ara-C) or HL60 (BdA), depending on the drug with which they were treated. To test for drug resistance and cross-resistance colony assays were performed on these two resistant cell lines. The drug-resistant cells, along with controls, were exposed to four different concentrations of both Ara-C and BdA (1.6, 0.8, 0.4 and 0.2 µ*M*) for 24 h before being plated in triplicate in agar.

Deoxycytidine kinase assay. The method used was based on one previously reported [19]. Briefly, cells were suspended at 10^8 cells/ml after two washes in imidazole-buffered saline. Cells were centrifuged and resuspended in 50 m*M* Tris buffer/2 m*M* mercaptoethanol, pH 8.0. Cells were lysed by freeze-thawing. Cell debris was removed by centrifugation (8000 *g*) for 15 min and the supernatant reserved for measurement of enzyme activity and protein content. The assay mixture contained 10 m*M* ATP, 50 m*M* Tris (pH 8.0), 10 m*M* $MgCl_2$, 15 m*M* NaF, 1 m*M* tetrahydrouridine (THU), 2 m*M* mercaptoethanol and 50 µ*M* [^{14}C]-deoxycytidine (dCyd). Then 30 µl cell-free extract was added to make a total assay volume of 120 µl. The reaction was started by addition of labelled deoxycytidine to the pre-warmed mixture of reagents and cell-free extract. Samples (5 µl) were taken at 0.25, 2, 4, 6, 8, and 10 min and spotted onto 6-cm lanes of PEI cellulose pre-spotted with 5 µl 5 m*M* dCyd, 5 m*M* dCMP. The reaction was terminated at the time of spotting. The PEI-cellulose was developed in water by ascending chromatography with all

phosphorylated product remaining at the origin and the unused substrate running with the solvent front. Pieces of PEI (2 cm) were cut corresponding to the origin and the solvent front. These were dried and put into counting vials with scintillant and counted. The product was calculated as a percentage of substrate plus product for each time point. The percentage product formed per minute was converted to picomoles per minute per 10^7 cells.

Cytidine deaminase assay. Cell extract prepared as above was used in these experiments, 30 µl cell-free extract being warmed to 37° C, after which the reaction was started by the addition of 30 µl 400 µ*M* [^{14}C]cytidine. [^{14}C]cytidine was diluted with unlabelled cytidine to produce a 400 µ*M* solution with a relative specific activity of 15 cpm/pmol. Samples of 5 µl were taken at 0.25, 3, 6, 9, 12 and 15 min and immediately spotted onto PEI cellulose. The PEI had been previously spotted with 5 µl 10 m*M* unlabelled cytidine and 10 m*M* unlabelled uridine. The PEI was developed in 86% butanol:water by ascending chromatography. Nucleosides were visualized under short-wave UV light and marked. The marked areas were cut out and counted in liquid scintillation vials. Samples of the cell-free extract were also reserved for protein assay (Biorad assay) using BSA as standard. Each assay contained 0.5×10^6 cells and allowed calculation of mean protein content.

Flow cytometry. A cell suspension (1×10^5 cells/ml) was prepared and 900-µl aliquots were pipetted into 24-well plates. At time zero, 100 µl BdA at 10× the final concentration, or in the case of controls RPMI 1640, was added. BdA was added at the four concentrations 3.2, 1.6, 0.8 and 0.4 µ*M*. The plates were incubated and cells removed at 2, 4, 6, 8, 10, 12, 24, 32 and 48 h.

The DNA content of cells was measured using a Becton Dickinson FACS IV. At the appropriate time point, 1 ml cell suspension was taken, fixed with 25% (v/v) cold ethanol, resuspended in 200 µl phosphate-buffered saline (PBS), and then stained with 50 µl of a solution consisting of 1.0% Triton X100, 0.5% RNAse, and 0.025% propidium iodide. Approximately 1×10^4 cells were analysed at 488 nm for each DNA content histogram. Calculations of percentages of cells in various phases of the cell cycle were made using a curve-fitting analysis [6].

A synchronized cell population was produced by incubation with 5 µl/ml Colcemid (demecolcine, Gibco Laboratories; 10 µg/ml stock in PBS), for 12 h. Cells were then washed twice with 10 ml RPMI 1640 and resuspended in complete medium to remove the Colcemid and thus the mitotic block. Aliquots of cells (900 µl) at 1×10^5/ml were plated in 24-well plates. At 8, 11, 14, and 24 h after synchronization, the cells were incubated with 0.8 µ*M* BdA for 3 h. Cells were then fixed and stained and DNA profiles determined.

Results

Patient samples

Samples from patients with AML were studied to determine whether cross-resistance between Ara-C and BdA occurred in clinically obtained material. The cells were incubated with Ara-C (0.4–3.2 µ*M*) and BdA (0.1–1.0 µ*M*). The area under the dose-response curve (AUC) was calculated (see "Materials and methods" section), and these data are listed in Table 1. Clinical non-response to Ara-C correlated well with in vitro AUC, with 14/16 patients' samples being correctly classified on this basis. In previous studies it has been shown with these techniques that AUC correlates with clinical response, producing a similar percentage of correct predictions [11, 12]. The results in Table 1 were ranked from most sensitive to least sensitive for each drug and a Spearman-Rank correlation coefficient determined. The correlation between response to the two drugs was not significantly different from zero, demonstrating no evidence of cross-resistance between Ara-C and BdA. In addition, as indicated in Table 1, some of the samples that were most sensitive to BdA came from patients who showed clinical Ara-C resistance as defined previously [12].

C × T experiments

A dose-response curve of HL60 colony formation as a function of concentration (C), (four values) was obtained for five values of exposure duration (T). The product of concentration times duration of exposure, i.e. C × T, was kept constant for each of the five points per curve (see "Materials and methods" and Fig. 1). The magnitude of response to BdA increased with increasing T. This apparent increase in sensitivity with increasing T occurs despite a proportional decrease in drug concentration. Short drug exposure times of 4 and 8 h for a given C × T result in approximately a 60% reduction in colony formation, while with 48-h incubation at consequently lower BdA concentrations, colony formation was reduced to virtually zero for all C × T values. Using a statistical method described by Moon [15], the AUCs of Fig. 1 were calculated and plotted against incubation time. A negative correlation is seen (Fig. 2), which is characteristic of cycle-specific, phase-specific drugs [11].

Table 1. Area under the dose-response curves from AML patient samples, treated for 24 h with BdA (0.1–1.0 µ*M*), and Ara-C (0.04–0.4 µg/ml)

Patient identification	AUC Ara-C	Rank order	AUC BdA	Rank order
A[a]	104.8	1	65.4	4
B[a]	91.8	2	72.66	3
C[a]	79.2	3	59.1	5
D[a]	78.2	4	19.5	13
E[a]	68.7	5	27.1	10
F[a]	61.4	6	80.5	2
G	57.9	7	16.5	14
H	56.8	8	28.2	9
I	56.7	9	26.2	12
J	49.0	10	43.7	7
K[a]	47.9	11	92.6	1
L	43.5	12	26.9	11
M	42.4	13	57.0	6
N[a]	40.8	14	29.4	8
O	40.6	15	13.3	15
P	24.2	16	5.0	16

Spearman rank correlation coefficient $r_s = 0.574$ signifies a lack of cross resistance between Ara-C and BdA when used on patient material

[a] These samples correspond to clinical non-response to Ara-C treatment

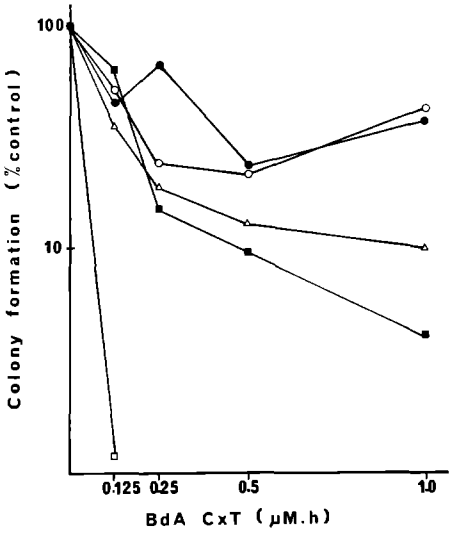

Fig. 1. Inhibition of HL60 colony formation by BdA. Five dose-response curves were obtained with five different time periods: 4 (●), 8 (○), 16 (△), 24 (■), and 48 (□) h. The concentration range at each time point (4 concentrations) was such that the C × T values studied were constant for each agent at each of the five time points. Each *point* represents the mean of triplicate determinations (SD < 10%). Where fewer than four points are given on the curve, colony inhibition was greater than can be represented on the scale used

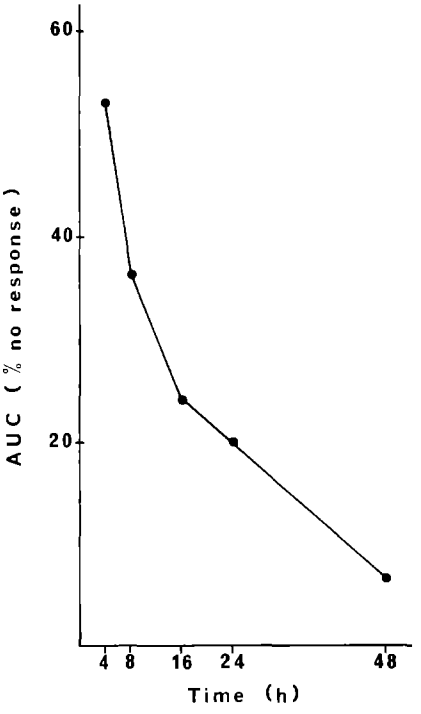

Fig. 2. Area under each dose-response curve (AUC) from Fig. 1 against duration of drug exposure

Cross-resistance in Ara-C- and BdA-resistant HL60

Colony inhibition assays were performed on control HL60, HL60 (Ara-C), and HL60 (BdA). The results obtained when these three groups were exposed to Ara-C and BdA are presented in Fig. 3. As an index of the development of drug resistance, D_{37} values were calculated. D_{37} represents the drug dose required to reduce colony formation to 37% of control. The control HL60 have D_{37} to Ara-C of 0.084 μ*M* and D_{37} BdA of 0.935 μ*M*. HL60 (BdA) displayed both an increased D_{37} Ara-C of 0.131 μ*M* and a D_{37} BdA of > 1.6 μ*M*. HL60 (Ara-C)-resistant cells had D_{37} of 0.248 μ*M* and 0.849 μ*M* to Ara-C and BdA, respectively (Table 2). Thus, in vitro induction of BdA resistance induced some degree of resistance to Ara-C. However, induction of Ara-C resistance, which is a clinical problem, did not induce significant resistance to BdA.

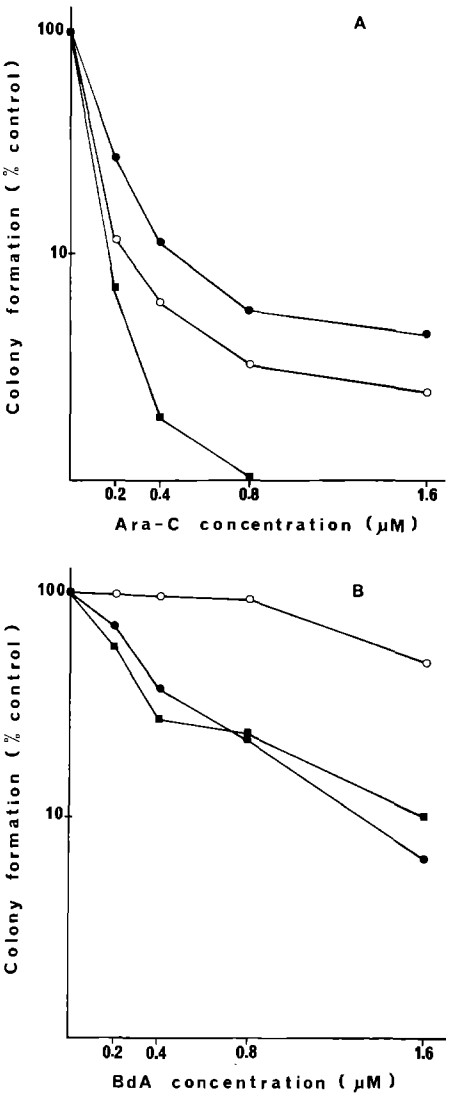

Fig. 3A, B. Inhibition of colony formation by HL60 control cells (■), HL60 (Ara-C) (●), HL60 (BdA) (○), after a 24-h incubation with four different concentrations of Ara-C **(A)** and BdA **(B)**. *Points* represent the mean of triplicate determinations (SD < 10%)

Table 2. D_{37} values obtained from dose-response curves of the three AML cell lines: HL60 control, HL60 (Ara-C), and HL60 (BdA), after exposure to BdA and Ara-C (3.2, 1.6, 0.8 and 0.4 μ*M*)

Cell line	D_{37} Ara-C	D_{37} BdA
HL60 control	0.084 μ*M*	0.935 μ*M*
HL60 (BdA)	0.131 μ*M*	> 1.6 μ*M*
HL60 (Ara-C)	0.248 μ*M*	0.849 μ*M*

Deoxycytidine kinase and cytidine deaminase assays

HL60 control, HL60 (Ara-C) and HL60 (BdA) were tested for deoxycytidine kinase levels. The relative specific activities were 936, 2078 and 956 pmol/min per mg protein, respectively. The deaminase activity was 509, 2108 and 488 pmol/min per mg protein for the same three cell lines. These results demonstrate that HL60 (Ara-C) resistance to Ara-C, and HL60 (BdA) resistance to Ara-C and BdA are not due to reduced activity of deoxycytidine kinase. Cytidine deaminase levels are not altered in HL60 (BdA), indicating little involvement of this enzyme in the induction of BdA resistance. In HL60 (Ara-C) the cytidine deaminase levels are elevated four-fold compared with HL60 control. Although there is a concomitant two-fold increase in the deoxycytidine kinase level of HL60 (Ara-C), the elevated cytidine deaminase could contribute to the induced Ara-C resistance in this cell line.

When EHNA, a potent adenosine deaminase inhibitor, was used in the absence of added nucleosides at 5, 7.5 and 10 μ*M*, it produced a proportional linear decrease in colony formation to 24%. When the cells were incubated with BdA and EHNA together there was a two-fold reduction in colony formation compared with that produced by either EHNA or BdA alone (Fig. 4). However, this combined effect was simply additive and not synergistic. This lack of synergism suggests that BdA is resistant to ADA degradation.

FACS results

Trypan Blue exclusion tests at each time point showed that some of the BdA concentrations used were cytostatic and not cytotoxic. After 4 h incubation with a cytostatic concentration of BdA, the FACS profile revealed evidence of a decrease in the proportion of cells in the S and G_2 phases as a result of a drug-induced G_1/S phase block. After 8 h of drug exposure this block appeared to be overcome (Fig. 5). If fresh drug was added prior to this time the block persisted, indicating that degradation of the drug

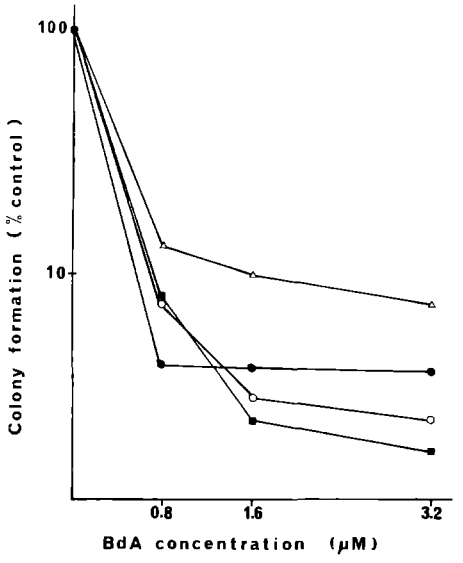

Fig. 4. Inhibition of colony formation by HL60 cells after a 24-h exposure to BdA. △, BdA alone; ●, BdA +5 μ*M* EHNA; ○, BdA +7.5 μ*M* EHNA; ■, BdA +10 μ*M* EHNA. *Points* represent means of triplicate determinations (SD < 10%)

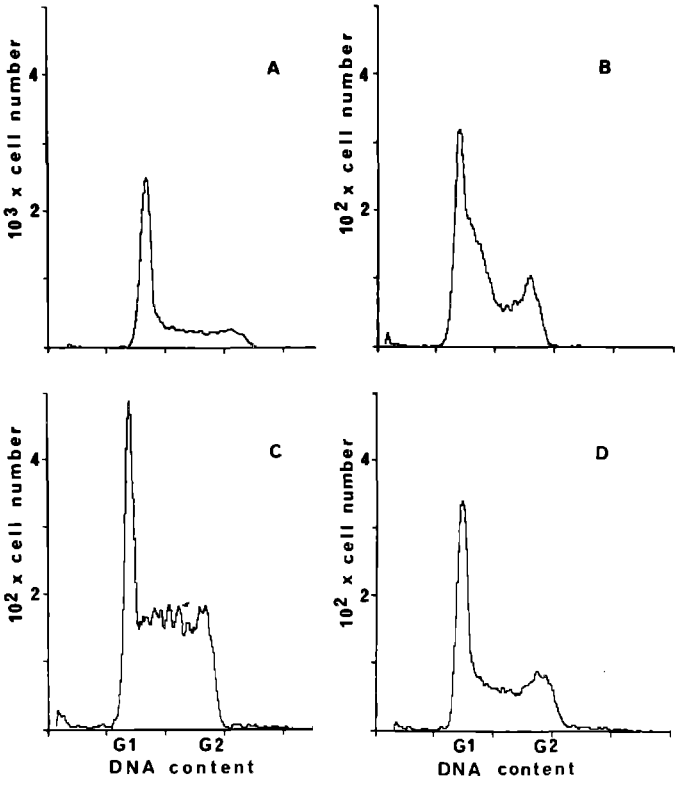

Fig. 5A–D. Changes in DNA distribution of HL60 cells with time of exposure to 1.6 μ*M* BdA. DNA content corresponds to relative fluorescence intensity. *Ordinate*, number of cells. **A** 4 h; **B** 8 h; **C** 12 h; **D** 24 h

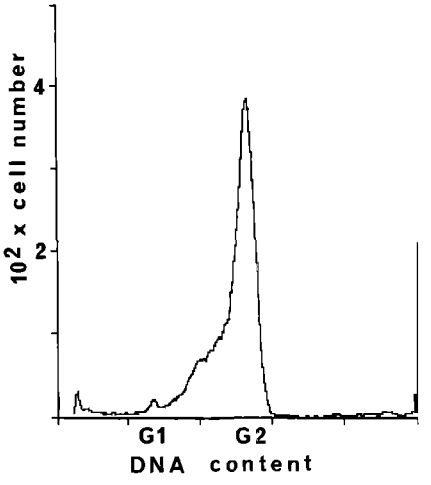

Fig. 6. DNA profile of HL60 cells after a 12-h incubation with Colcemid (5 μl/ml). DNA content corresponds to relative fluorescence intensity. *Ordinate*, number of cells

Table 3. The DNA content of HL60 cells after blockade with the mitotic inhibitor Colcemid (5 μl/ml for 12 h)

Time after Colcemid removal	Stage of cell cycle		
	G_1	S	G_2
8 h	90%	8%	–
11 h	66%	24%	10%
14 h	27%	60%	13%
24 h	41%	42%	15%

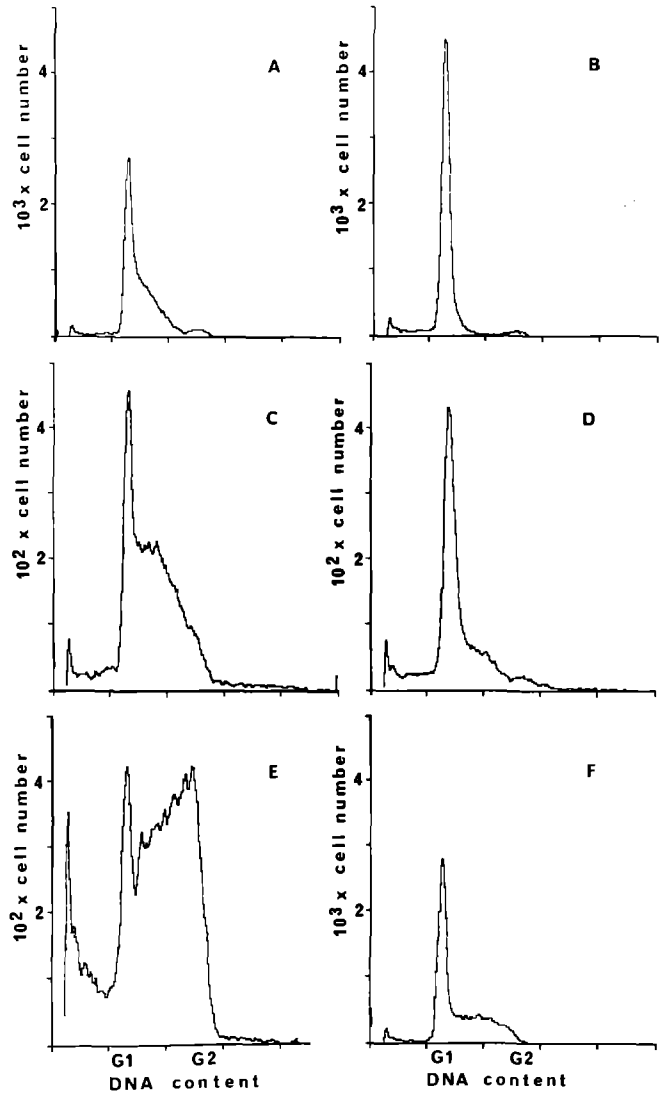

Fig. 7. Changes in the DNA distribution of Colcemid synchronized cells with and without 3-h exposure to 3.2 μ*M* BdA at 8-h after Colcemid removal: **A** Control, **B** BdA 11 h after: **C** control, **D** BdA; and 14 h after: **E** control, **F** BdA

within this 8-h period was responsible for the cells' overcoming the G_1/S phase block. Hence, in experiments requiring longer drug exposure the cells were washed and suspended in fresh medium containing fresh drug every 8 h (as for C × T experiments).

A 12-h incubation with the mitotic inhibitor Colcemid resulted in the accumulation of a synchronized population of cells in mitosis (Fig. 6). Upon removal of the Colcemid the block was released, resulting in a gradual progression of the synchronized population through the cell cycle. At 8 h after Colcemid removal 90% of the cells were in G_1; at 11 h 66% were in G_1 and 24% in S phase; and at 14 h 27% of cells were in G_1 and 60% in S phase (Table 3). By 24 h all evidence of cell synchrony had disappeared, with the proportions of cells in various stages of the cell cycle not differing from those of control cells that had not been treated with Colcemid.

The DNA histograms in Fig. 7 depict the effect produced by BdA addition at 8, 11 and 14 h after Colcemid; these times correspond to synchronized peaks in G_1, G_1/S phase and S phase, respectively. Starting at 8 h the cells were treated with BdA for 3 h. At the end of this 3-h incubation the cells displayed 92.1% in G_1. Cells treated with Colcemid alone displayed progression from G_1 into S phase within this 3 h (G_1 = 66%, S = 45%). Similarly, at 11 h after Colcemid, when BdA was added for 3 h, the FACS profiles reveal that BdA prevented the progression of cells through the cell cycle. In the presence of BdA, there are 60% in G_1 and 35% in S phase, as against 27% and 60% for the cells treated with Colcemid alone. At 14 h after Colcemid incubation there was a massive migration of cells moving as a synchronized group in S phase (69%), with a small residual population in G_1 (10%). The BdA profile for this same time period had 48% in G_1 and 44% in S phase. These FACS profiles confirm that BdA is producing a G_1/S transition blockade (Table 4).

Table 4. DNA content of HL60 cells after blockade with the mitotic inhibitor Colcemid (5 μL/ml for 12 h) then incubated with BdA for 3 h at the times shown

Time after Colcemid removal	Colcemid alone			Colcemid + 3 h BdA incubation		
	G_1	S	G_2	G_1	S	G_2
11 h	66%	24%	–	92%	–	–
14 h	27%	60%	13%	60%	35%	5%
27 h	10%	69%	21%	48%	44%	8%

Discussion

Ara-C, an anti-metabolite analogue of deoxycytidine, is one of the principal drugs used in remission induction and in maintenance therapy of AML. Unfortunately, Ara-C resistance is a common clinical problem, so that it is important to search for new drugs which will be effective in this situation. BdA, a halogenated deoxyadenosine derivative, is under investigation because of promising reports of effectiveness against both resting and proliferating lymphocytes, cell lines, and L1210 leukaemia [3, 4, 8, 10, 14]. In a phase I study CdA appeared to have some activity against myeloid leukaemia [16].

BdA may share common mechanisms of activation with Ara-C. The initial experiments were designed to determine whether there was significant cross-resistance between Ara-C and BdA in AML samples from patients (some of whom were known to be clinically Ara-C-resistant) and HL60 cells in which resistance to Ara-C and BdA was induced in vitro. In these studies it became clear that there was not a high degree of cross-resistance between Ara-C and BdA. Some patient samples, which were resistant to Ara-C both clinically and in vitro, were at the more sensitive end of the BdA-sensitivity spectrum. A human AML cell line which had AraC resistance induced in vitro, HL60 (Ara-C), did not show increased resistance to BdA. The mechanism of this differential sensitivity could be explained by increased activity of cytidine deaminase induced in the Ara-C-resistant cells. While reduced activity of deoxycytidine kinase might have explained the dual resistance of HL60 (BdA) to Ara-C and BdA, this was shown not to be the case when enzyme levels were measured.

Possible mechanisms for the induction of drug resistance include decreased intracellular drug accumulation or differences in enzyme activity further along the pathway of drug activation. Clearly more detailed studies are required for full elucidation of the mechanisms that explain the differential sensitivity of cells to Ara-C and BdA, but they are beyond the scope of this study. Whatever the underlying mechanisms, these findings suggest that the halogenated deoxyadenosine derivatives may be of clinical relevance in situations of Ara-C resistance, since BdA did not show cross-resistance with Ara-C in the AML patient samples or the HL60 cell line. The results of the reported phase 1 study support this finding [16].

In the present study, EHNA enhanced the cytotoxic effect of BdA two-fold with no evidence of synergism. This is in marked contrast to the study in Tattersall and Fox [17], who reported that when EHNA was included in a culture with T-lymphocytes the sensitivity to deoxyadenosine was increased by more than 10-fold. The most likely explanation for this is that the halogenated deoxyadenosine derivatives are poor substrates for ADA and thus ADA is not an important enzyme in the degradation of BdA [3].

Once it had been demonstrated that induction of Ara-C resistance did not necessarily induce BdA resistance, the question of the site of action of this agent in the cell cycle was addressed. This is an important issue, since the chloro-analogue of deoxyadenosine was reported to have activity against both cycling and resting lymphoid cells. The $C \times T$ experiments demonstrate that BdA in myeloid cells has a the profile of a cycle-specific agent with increasing cell kill as T increases and $C \times T$ is kept constant.

The flow-cytometric studies with both unsynchronized cells and cells synchronized with Colecmid show BdA to be phase-specific producing a G_1/S phase block. A recent study with a T-lymphoblastoid line, CCRF-CEM, showed both CdA and BdA to be cycle-active, phase-active agents producing a block in either S or G_1-S, depending upon the drug concentration used [14]. Similarly, our studies with BdA show that with myeloid cells this drug behaves as a cycle- and phase-specific agent. In contrast to this, the finding of activity against non-cycling lymphocytes [3, 10, 16] is probably a consequence of the fact that a cytotoxic agent can have different mechanisms of action when studied in different cell types [7].

Our studies on human AML cells suggest that BdA is a cycle-active, phase-active agent which blocks cells at the G_1/S transition and does not exhibit significant cross-resistance with Ara-C. This is particularly relevant to the clinically important situation where Ara-C resistance has occurred or is likely to occur. The halogenated deoxyadenosine derivatives warrant further investigation to elucidate their mechanism of action and to determine their suitability for clinical use. Assays have been developed to allow prediction of response to this class of compounds in AML [11, 12], and they may aid in appropriate selection of nucleoside analogues for clinical use in the future.

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