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TOXICITY OF COMBINATIONS OF ARABINOSYLCYTOSINE AND 3-DEAZAURIDINE TOWARD NEOPLASTIC CELLS IN CULTURE*

CECILY MILLS-YAMAMOTO,† GILLES J. LAUZON and ALAN R. P. PATERSON

Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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Abstract—The antiproliferative effects of 3-deazauridine and arabinosylcytosine toward cells in culture (HeLa and RPMI 6410 lymphoblastoid cells) were potentiated when the agents were present together. The conversion of arabinosylcytosine to nucleotide metabolites in RPMI 6410 cells was greatly enhanced in the presence of deazauridine; this enhancement is thought to be the basis of the potentiation in antiproliferative activity. These drug combination effects did not occur in two RPMI 6410 sublines which were resistant to deazauridine and deficient in uridine kinase activity.

The uridine analog, 3-deazauridine (DU)‡ [1], inhibits the proliferation of micro-organisms and cultured tumor cells [2], and of transplantable mouse neoplasms *in vivo* [2, 3]. A major metabolite of DU is the 5′-triphosphate; formation of the triphosphate in extracts of Ehrlich ascites carcinoma cells was shown by Wang and Bloch [4] and in intact leukemia L1210 cells by McPartland *et al.* [5]. DU was not incorporated into RNA or DNA by Ehrlich ascites carcinoma cells *in vivo* [4]. DU 5′-triphosphate is a potent, competitive inhibitor of CTP synthetase [5]; partial reversals of the DU inhibition of proliferation *in vitro* of leukemia L1210 cells by cytidine, deoxycytidine or uridine [6] are consistent with the idea that inhibition of CTP synthetase is involved in DU effects on cell growth. The marked depletion of cytidine and deoxycytidine phosphates in DU-treated cells reported by Brockman *et al.* [6, 7] is also consistent with this mechanism of DU action. The latter investigators have also shown that DU 5′-diphosphate inhibited the ribonucleotide reductase-catalyzed conversion of pyrimidine ribonucleoside diphosphates to the corresponding deoxyribonucleotides [6].

The present study investigated the possibility that the DU-induced perturbations of the metabolism of the cytidine-deoxycytidine phosphates might influence the growth inhibitory effects of araC. It was found that DU potentiated the cytotoxicity of araC toward cultured human lymphoblastoid cells, apparently through enhancement of araC anabolism.

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‡ Abbreviations used are: DU: 4-hydroxy-1-(β-D-ribopentofuranosyl)-2-pyridone; araC: arabinosylcytosine or 1-β-D-arabinofuranosylcytosine; araCMP, araCDP and araCTP: the 5′-mono-, di- and triphosphate esters of araC; araU: arabinosyluracil or 1-β-D-arabinofuranosyluracil; araUMP: araU 5′-monophosphate; araCDPcholine: the choline ester of araCDP; PEI: polyethyleneimine; and HEPES: *N*-2′-hydroxethylpiperazine-*N*′-2-ethanesulfonic acid.

MATERIALS AND METHODS

HeLa cells were maintained as monolayers in loosely capped flint glass prescription bottles (8 oz) at 37° in a 5% CO_2 atmosphere. Bottles were inoculated with 2×10^5 cells in 25 ml of MEM medium [Eagle's minimal essential medium containing 2 mM HEPES buffer (pH 7.4) and 10% calf serum]; culture media were replaced every 2 or 3 days and cultures were restarted every 7 days with cells from trypsinized monolayers.

In determining the effects of DU and araC on HeLa cell viability, 60 × 15 mm culture dishes (Falcon Plastics) were first seeded with 100–200 cells in 4.0 ml MEM and 24 hr later, 1.0-ml portions of MEM containing appropriate concentrations of the agents were added; after specified intervals at 37°, agent-containing medium was removed, culture dishes were washed with 5.0 ml of warmed MEM and 5.0 ml of fresh MEM was added to each. After incubation for 9–11 days (with one medium change), cultures were rinsed with 0.9% NaCl and the cell colonies then stained with crystal violet. Colonies of 50 cells or more were counted; ten replicate cultures were included for each drug concentration tested.

Human lymphoblastoid cells, RPMI 6410 [8], were cultured in RPMI 1640 medium containing 10% dialyzed fetal calf serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin. Initial cell concentrations were 0.5 to 1.0×10^5 cells/ml and cultures were diluted every 2 or 3 days to keep cell numbers below 5×10^5/ml, thereby ensuring exponential proliferation. When experiments involved exposure to drugs, heat-treated (56° for 18 hr) dialyzed fetal calf serum was used except as noted. After exposure to drugs,

Table 1. Deazauridine-arabinosylcytosine synergism in cytotoxicity toward HeLa cells*

Exposure (hr)	DU (μM)	AraC (μM)	Cell viability (% of control) Additive effects† (calculated)	Actual
24	43	0		76
	0	12		28
	43	12	21	11
	0	7		43
	43	7	32	12
	0	2		45
	43	2	34	24
18	19	0		95
	0	12		52
	19	12	49	24
	0	5		63
	19	5	60	36
	0	2		71
	19	2	67	40

* Effects of drug treatment on HeLa cell viability were determined by exposing attached cells (100–200/culture dish) to the indicated concentrations of drugs for 18 or 24 hr. Surviving cells were then allowed to form macroscopic colonies which were counted after 9–11 days; each value is the mean of ten replicates, expressed as a percentage of the mean number of colonies in culture dishes receiving no drugs.

† Effect of the combined agents, calculated from the observed effects of the individual agents, assuming independence of the latter.

cells were washed once in warmed, drug-free medium containing 10% dialyzed fetal calf serum and resuspended in the latter.

Cell numbers were determined with an electronic particle counter. Media and sera were purchased from the Grand Island Biological Co.

A line of 6410 cells lacking hypoxanthine guanine phosphoribosyltransferase activity (designated 6410/MP) was established by culturing cells in media containing 6-mercaptopurine to select a resistant cell population from which a clone was established.* Uncloned DU-resistant sublines, designated 6410/DU and 6410/MP/DU, were established from the 6410 and 6410/MP lines by serial passage of cultures which were: (a) exposed to 50 μM DU for two intervals of 23 hr with each exposure followed by recovery intervals, and then (b) cultured continuously in media containing DU at concentrations between 20 and 50 μM. The DU-resistant lines so selected were found to be deficient in uridine kinase activity.

The assay of uridine kinase employed extracts of 6410 cells prepared as follows: 5–10 × 10^6 cells were washed in 0.9% NaCl, resuspended in 1.0 ml cold 0.05 M Tris buffer (pH 8.0) and then disrupted with two 15-sec bursts of 20 kc ultrasound (Branson Sonifier S-75 with microtip; Heat Systems-Ultrasonics, Plainview, NJ). The sonicates were centrifuged (12,000 *g*, 20 min, 5°) and the supernatants assayed at once for uridine kinase activity. The assay system contained 5 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 50 mM Tris–HCl (pH 7.4) and 0.1 mM [2-^{14}C]uridine (Amersham/Searle) in a final volume of 1.0 ml. Samples (0.1 ml) removed from the assay mixtures at specified intervals were heated for 2 min at 100° and 0.02-ml portions then were chromatographed on paper with carrier UMP using this solvent: isopropanol–14 M ammonium hydroxide–water–0.1 M EDTA–toluene (320:4:44:6:40, v/v); nucleotide mixtures were not resolved in this system. To determine uridine phosphate formation, the nucleotide areas of these chromatograms were assayed for ^{14}C-activity by direct liquid scintillation counting using a toluene-based fluor solution. The time course of the assay reaction was linear over the intervals employed.

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To determine the conversion of [5-^{3}H]araC (Amersham/Searle) into cellular acid-soluble nucleotides, exponentially growing cultures of 6410 cells were incubated at 37° with the labeled substrate, and after two washes with cold 0.9% NaCl, cell pellets were extracted first with 0.1 ml of 0.4 M perchloric acid and then with 0.05 ml of 0.2 M perchloric acid. The extracts were combined, neutralized with KOH and portions therefrom were chromatographed with appropriate carriers on Whatman 3 MM paper with ethyl acetate–isopropanol–water (65:22.5:12.5, v/v) as the solvent; nucleotide mixtures were not resolved in this system. In assessing conversion of [5-^{3}H]araC into "total" (i.e. unresolved) araC nucleotides, the nucleotide areas of such chromatograms were assayed for radioactivity by a combustion–liquid scintillation procedure using a Packard model 305 Sample Oxidizer. The identity of araC metabolites in the perchloric acid extracts was also examined by thin-layer chromatography. Portions of the neutralized extracts were chromatographed, along with appropriate markers, on thin layers of PEI-impregnated cellulose (MN 300 thin-layer sheets; Macherey-Nagel & Co.). The thin-layer chromatograms were developed in either of two solvent systems. In system 1, the follow-

* C. Mills-Yamamoto and A. R. P. Paterson, unpublished results.

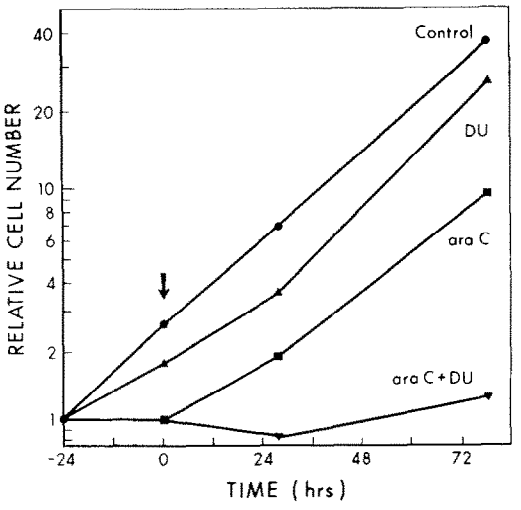

Fig. 1. Synergism in antiproliferative effects of deazauridine (9 μM) and arabinosylcytosine (3 μM). DU and araC were added to culture media at −24 hr; cells were resuspended in drug-free media at 0 hr (arrow).

ing three solvents were used in succession without drying between transfers from one solvent to another; the front was run to these distances beyond the origin: (a) 1 M acetic acid, 2 cm; (b) 0.33 M lithium chloride in 0.67 M acetic acid, 8 cm; (c) 0.67 M lithium chloride in 0.67 M acetic acid, 16 cm. In solvent system 2, PEI cellulose chromatograms were developed in ethyl acetate–isopropanol–water (65:22.5:12.5, v/v). After development, markers were located under ultraviolet light and the entire chromatographic medium of each lane was scraped off in sections for assay of [^{3}H]activity by the combustion–liquid scintillation procedure.

RESULTS

The experiment of Table 1 examined the effects on HeLa cell viability (as measured by the ability of individual cells to form macroscopic colonies) of incubation in culture media containing DU or araC, singly or present together. It is apparent in these data that the cytotoxicity of DU–araC combinations was somewhat greater than that expected from a simple combination of the effects of the individual agents, assuming their independence ("additive" effects, Table 1). Synergism in the antiproliferative effects of these agents resulting from their presence together was clearly apparent when 6410 cells were cultured in medium containing DU and araC. It is seen in Fig. 1 that, following culture in medium containing 3 μM araC or 9 μM DU, proliferation rates of 6410 cells were close to those in untreated control cultures; in cultures exposed to these agents in combination, cells did not proliferate during exposure, nor did cell numbers increase until about 48 hr after the drug treatment ended.

The enhancement of toxicity resulting from the combination of DU and araC was also apparent in experiments with normal mice. When male BDF$_1$ mice (in groups of six) received five intraperitoneal doses of either agent at 24-hr intervals, DU at 100 mg/kg, and araC at 10 mg/kg, no weight loss and

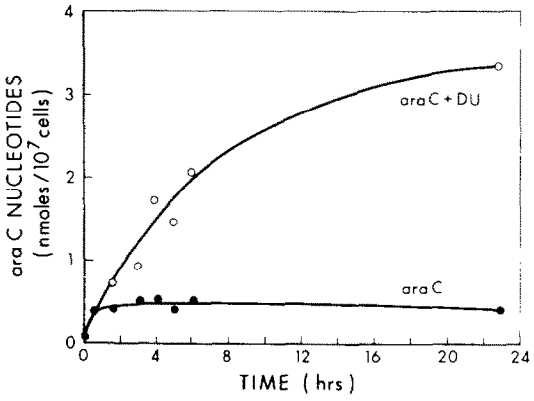

Fig. 2. Enhancement of arabinosylcytosine anabolism in 6410 cells by pretreatment with deazuridine. Duplicate cultures with 3.5 × 10^5 cells/ml were prepared and to one DU was added; 50 min later, [5-^{3}H]araC was added to both. Final concentrations were: DU, 23 μM and araC, 5 μM. At the times indicated, cell samples were assayed for their content of acid-soluble araC nucleotides which were isolated by paper chromatography and determined by a combustion–scintillation counting technique.

Table 2. Deazauridine resistance in RPMI 6410 cells*

Cell line	DU concn (μM) during the 0- to 23-hr interval	Cell numbers at 48 hr relative to those at 0 hr
6410	0	6.48
	45	1.00
6410/DU	0	6.40
	45	4.20
6410/MP	0	6.02
	180	1.03
	270	1.02
6410/MP/DU	0	5.04
	180	4.97
	270	5.63

* RPMI 6410 cells, initially at 1 × 10^5/ml, were cultured in medium containing the indicated concentrations of DU for 23 hr at which time cells were resuspended in fresh, DU-free medium and culture continued until 47 hr. Proliferation rates were exponential in the absence of DU.

Table 3. Uridine kinase activities in extracts of deazauridine-resistant lines of RPMI 6410 cells

Cell line	Uridine kinase activity*
6410	160
6410/DU	15
6410/MP	23
6410/MP/DU	21

* Arbitrary units: pmoles of uridine phosphates formed in 60 min by cell-free extract from 10^7 cells under assay conditions given in Materials and Methods.

no deaths occurred; however, when the agents were administered together at the above dosages, the weight loss was 20 per cent by the last treatment day and all mice were dead by the fourth day after treatment ended.

Figure 2 indicates that, in the presence of DU, the anabolism of araC by 6410 cells was markedly enhanced. In this experiment, cells incubated in medium containing 5 μM [5-^{3}H]araC formed araC metabolites, evidently nucleotides, achieving within 1 hr of incubation, intracellular concentrations representing about 0.4 nmole araC/10^7 cells. In the presence of 23 μM DU, the cellular accumulation of araC nucleotides was progressive, so that after 6 and 22 hr of incubation, cells contained, respectively, 2 and 3.3 nmoles of araC nucleotides/10^7 cells. The possibility was considered that the enhancement by DU of araC nucleotide formation might be due to a DU-induced reduction of araC degradation. However, a chromatographic study showed that in media and in acid-soluble extracts of 6410 cells cultured with 5 μM [5-^{3}H]araC, araU/araC ratios were essentially unchanged in the presence of 23 μM DU. During 24 hr of culture, about 10 per cent of the araC was converted to araU in the presence or absence of DU.

The selection of two lines of 6410 cells with deficiencies in uridine kinase activity afforded an opportunity to test the hypothesis that DU phosphates, rather than DU *per se*, were responsible for the enhancement of araC anabolism (and, presumably, for synergism with araC). Sublines of 6410 and 6410/MP cells, designated 6410/DU and 6410/MP/DU, respectively, were selected for resistance to DU as described in Materials and Methods. The DU resistance of these lines is illustrated in Table 2; both cell lines were found to be deficient in uridine kinase activity (Table 3). It would appear that uridine kinase is responsible for the first step in the conversion of DU into metabolically active forms. The differences seen in Table 4 between DU-sensitive and resistant 6410 cells in respect to their uptake of [2-^{14}C]uridine into the acid-soluble fraction are consistent with the uridine kinase data of Table 3. DU failed to enhance the uptake of araC in the DU-resistant cells and, as well, the antiproliferative effects of araC toward these cells were not enhanced by DU (data not shown). Similarly, DU did not enhance araC anabolism or synergize with araC in experiments with an araC-resistant line of 6410 cells.*

The experiment of Fig. 3 showed that the extent to which the araC nucleotide pool of 6410 cells was expanded in the presence of DU was a saturable function of the DU concentration in the culture medium. In this experiment, DU and araC were present together throughout the period of incubation and the araC nucleotide content of the cells was assayed after the indicated intervals.

The experiment of Table 5 examined the identity of the metabolites of araC formed in 6410 cells under the circumstances of the DU enhancement of araC anabolism. Cells were cultured for 24 hr in medium containing 9 μM [5-^{3}H]araC and neutralized perchloric acid extracts of those cells were analyzed by multidirectional chromatography on PEI cellulose. The data

Table 4. Uridine uptake by deazauridine-resistant 6410 cells*

Cell line	Uridine in acid-soluble fraction (nmoles 10^7 cells·hr)
6410	28.1
6410/DU	4.0
6410/MP	30.0
6410/MP/DU	0.2

* Cells (3×10^5/ml) were incubated for 60 min in culture medium containing 9 μM [2-^{14}C]uridine and washed; perchloric acid extracts of the cells, prepared as specified in Materials and Methods, were assayed directly for ^{14}C content.

* C. Mills-Yamamoto and A. R. P. Paterson, unpublished results.

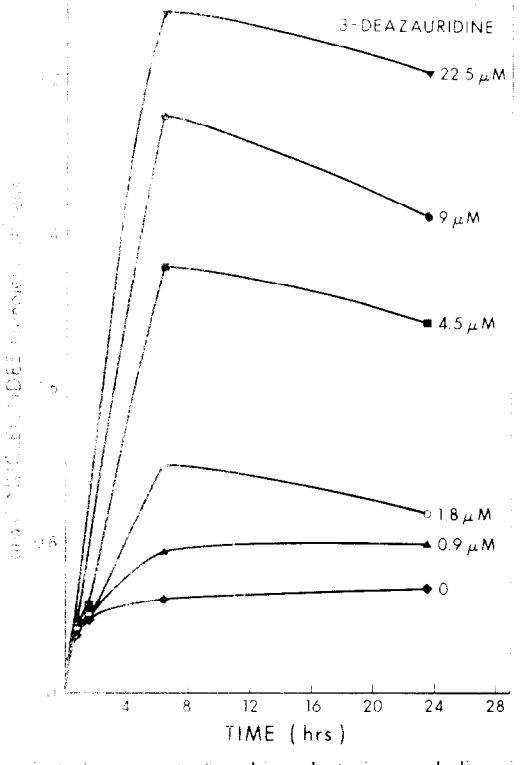

Fig. 3. Enhancement of arabinosylcytosine anabolism in 6410 cells by pretreatment with deazauridine. DU was added to replicate cultures of 6410 cells (2.5×10^5 cells/ml) to the final concentrations noted; 30 min later, [5-^{3}H]araC was added (5 μM, final concentration). At the times indicated, cell samples were assayed for their content of araC nucleotides, as in Fig. 2.

Table 5. Metabolites of arabinosylcytosine formed by RPMI 6410 cells in the presence and absence of deazauridine*

Metabolite	R_f	Cellular concn (nmoles/10^9 cells) − DU	+ DU
Chromatography solvent I			
AraCTP	0.05	12.8	35.6
AraCDP	0.23	0.5	2.8
AraCMP	0.63	0.3	0.8
AraCDPcholine†	0.72	14.3	111.6
Chromatography solvent II			
AraC	0.22	2.8	6.8
AraU	0.58	0.8	1.0
Unresolved nucleotides	0	29.5	154.7

* Cells were cultured for 24 hr in medium containing 3 μM [5-^{3}H]araC and 10% dialyzed fetal calf serum (unheated) with and without 9 μM DU. Perchloric acid extracts of the cells were analyzed by chromatography on thin layers of PEI cellulose using solvent systems 1 and 2, as specified in Methods. Similar distributions of araC metabolites were found in several confirmatory experiments; in these, the metabolites listed accounted for 95–105 per cent of the [^{3}H]activity applied in the chromatographic analysis.

† Provisional identification (see text).

of Table 5 show that the mono-, di- and triphosphates of araC together represented only a portion of the cellular araC metabolites and that a compound provisionally identified as araCDPcholine was a major araC metabolite, accounting for over 70 per cent of the cellular araC derivatives formed in the presence of DU. The putative araCDPcholine co-migrated with chemically synthesized araCDPcholine in several chromatographic systems; the phosphorous content and hydrolysis products of the compound were consistent with the proposed identity.* The enhancement in anabolism of araC in the presence of DU was due principally to increases in the cellular content of araCDPcholine (8-fold) and of araCTP (3-fold).

DISCUSSION

The deficiency of uridine kinase activity in the two DU-resistant lines of RPMI 6410 cells presently described indicates that the activity of this enzyme is essential to the conversion of DU into toxic metabolites, presumably phosphate esters. The presence of DU phosphates in several types of animal cells has the effect of depleting cellular pools of cytidine and deoxycytidine phosphates, evidently through inhibition of CTP synthetase [4–7]. This study reports the observations that: (a) in 6410 cells possessing uridine kinase activity, the presence of DU caused a profound increase in the cellular uptake of araC and in conversion of araC to nucleotide metabolites, and (b) the antiproliferative effects of araC and DU toward cultured cells were potentiated when these agents were present together. This potentiation by DU of the uptake and growth inhibitory effects of and did not occur in experiments with DU-resistant 6410 cell lines deficient in uridine kinase. It would appear that the DU effect on araC anabolism is the basis of the potentiation of antiproliferative effects. The presumed reduction in cellular concentrations of deoxycytidine phosphates in the presence of DU would be expected to decrease competition between dCTP and araCTP for DNA polymerase and so would tend to enhance inhibition of the latter, an effect which would probably contribute to araC cytoxicity [9].

The progressive increase with time of the cellular content of araC metabolites which occurred in the presence of DU is in marked contrast to the much smaller, steady-state cellular concentrations of araC metabolites found in the absence of DU (Fig. 2). The DU-induced enhancement of araC metabolite formation was due to increases in cellular concentrations of araCTP and of a metabolite, provisionally identified as araCDPcholine on the basis of chromatographic behavior, phosphorous content and hydrolysis products. AraCDP choline was a major metabolite of araC under the circumstances of these experiments, whether in the presence or absence of DU; the characterization of this hitherto unrecognized compound will be reported elsewhere.* It is not known whether araCDPcholine is involved in the cytotoxic effects of araC.

Under the circumstances of these experiments (Table 5), conversion to araU phosphates was a minor fate of araC in the presence or absence of DU; hence, decreases in the activity of dCMP deaminase were evidently not involved in the enhancement of araC anabolism. As a basis for the enhancement by DU of araC anabolism, we consider it possible that DU-induced reductions in cytosine nucleotide concentrations might have released feedback constraints on one or more steps in araC anabolism. For example, deoxycytidine kinase, which catalyses the first step in araC anabolism, is subject to allosteric regulation and is inhibited by dCTP [10]. Another step from which control might be lost is that of araC transport across the plasma membrane; araC transport has been shown to be a mediated process in some cell types [11, 12], but the possibility of metabolite regulation at this step is speculative.

* G. J. Lauzon and A. R. P. Paterson, unpublished results.

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