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1-β-D-Arabinosyl-5-azacytosine

Cytocidal Activity and Effects on the Synthesis and Methylation of DNA in Human Colon Carcinoma Cells

ROBERT I. GLAZER AND MARIAN C. KNODE

Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205

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SUMMARY

The cytocidal activity of arabinosyl-5-azacytosine (araAC) and its effect on the synthesis and methylation of DNA in the human colon carcinoma cell line HT-29 was examined and compared with three other cytidine analogues. Treatment for 2 hr with 10^{-6} M arabinosylcytosine (araC), araAC, 5-azacytidine (AZC), or 2′-deoxy-5-azacytidine (dAZC) produced a 7–30% reduction in cell viability. Prolongation of drug exposure to 24 hr significantly enhanced the cytotoxicity of all analogues, and particularly dAZC. AZC and dAZC were potent inhibitors of DNA methylation in the absence of inhibition of DNA synthesis, whereas araC and araAC primarily affected DNA synthesis. RNA synthesis was not affected by any of the analogues. dAZC and AZC were incorporated into DNA to a greater extent than were araC or araAC upon short- and long-term drug exposure, whereas only AZC was incorporated into RNA. These data indicate that araAC appears to behave more as an analogue of araC rather than of dAZC or AZC, wherein it produces rapid inhibition of DNA synthesis and is incorporated into DNA.

INTRODUCTION

The cytidine antimetabolites, AZC[1] and araC, are effective anticancer drugs for the treatment of acute myelogenous leukemia (1–4). dAZC is currently being evaluated in clinical trials of patients with pediatric malignancies (5). More recently, araAC was developed in this laboratory as a bioisostere of AZC which encompassed the structural properties of araC and AZC (6, 7). This analogue was found to possess activity equivalent to that of araC and AZC against murine L1210 leukemia and far greater efficacy than AZC, dAZC, or araC against the human colon carcinoma xenograft,[2] of which cell line HT-29 is the tissue culture counterpart. Although araAC appears to be therapeutically more akin to araC in terms of its schedule dependency (6, 7), little is known of its mode of action.

AZC has generated interest as a result of its ability to activate the latent fetal globin gene in β-thalassemia patients (8) and anemic baboons (9). These studies stemmed from the earlier observations of Constantinides *et al.* (10, 11), who demonstrated that AZC and dAZC could induce the expression of a muscle phenotype in mouse embryo cells in culture. Jones and Taylor (12, 13) further showed a link between the phenotypic changes and hypomethylation of DNA resulting from the incorporation of AZC and dAZC into nascent DNA. These results suggest that AZC and its analogues may have additional therapeutic modes of action apart from their cytocidal activities.

The present investigation was initiated to examine the antiproliferative activity of araAC and to compare it with that of the structurally related cytidine analogues, AZC, dAZC, and araC. In addition, we examined their effects on DNA synthesis and methylation, as well as their incorporation into DNA and RNA in order to ascertain whether any similarities existed in their mechanisms of action.

EXPERIMENTAL PROCEDURES

Materials. [5-^{3}H]Uridine (30 Ci/mmol), [*methyl*-^{14}C]dThd (53 mCi/mmol), and [*methyl*-^{3}H]methionine (12 Ci/mmol) were obtained from New England Nuclear Corporation (Boston, Mass.). [5-^{3}H]araC (11 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, Ill.). [6-^{3}H]AZC (15 Ci/mmol), [6-^{3}H]araAC (2.8 Ci/mmol), and [6-^{3}H]dAZC (9 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, Calif.). Unlabeled AZC, dAZC, araAC, and araC were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. All drugs were chemically and radiochemically pure

[1] The abbreviations used are: AZC, 5-azacytidine; dAZC, 2′-deoxy-5-azacytidine; araC, 1-β-D-arabinosylcytosine; araAC, 1-β-D-arabinosyl-5-azacytosine; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; m^{5}dCyd, 5-methyl-2′-deoxycytidine.

[2] Drug Evaluation Branch, Developmental Therapeutics Program, National Cancer Institute, unpublished data.

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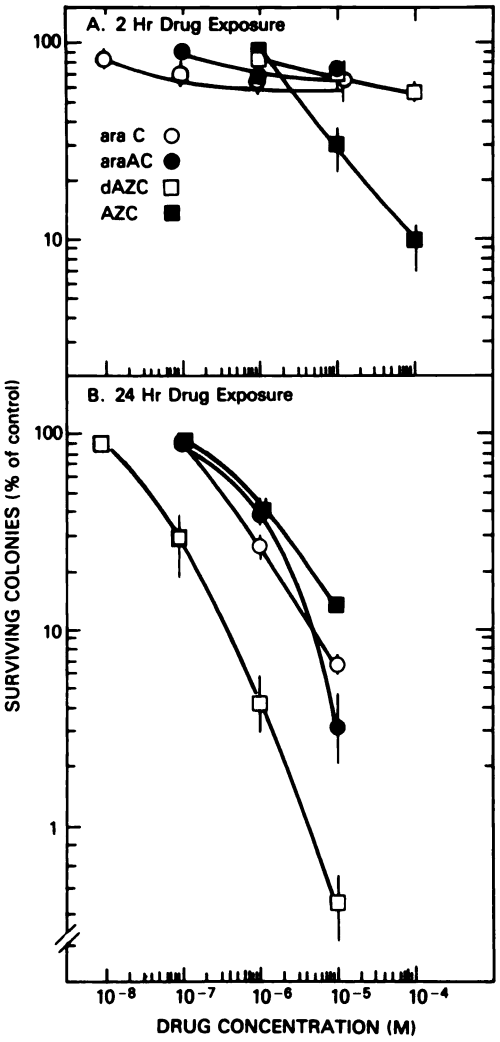

FIG. 1. *Cell viability following drug treatment*

Cells were exposed to varying concentrations of drug for 2 hr (A) or 24 hr (B), and colony formation was measured by soft agar cloning as described under Experimental Procedures. Each point represents the mean ± standard error of five to eight determinations. The cloning efficiency of control cells was 45%.

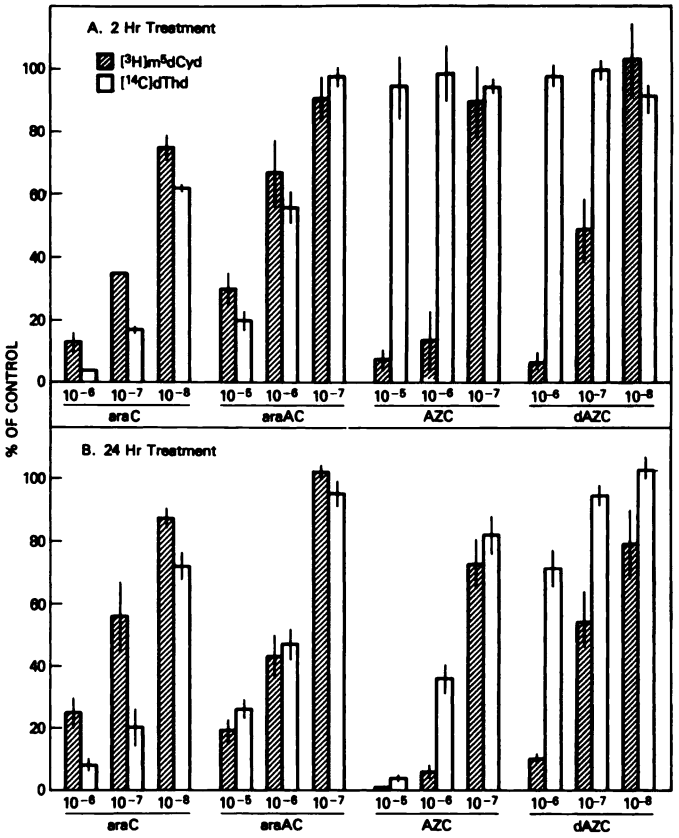

FIG. 2. *Methylation and synthesis of DNA during drug treatment*

Cells were exposed to varying concentrations of drug for 2 hr (A) or 24 hr (B) and pulse-labeled during the last hour with [*methyl*-^{3}H] methionine and [^{14}C]dThd. DNA was extracted, digested enzymatically to deoxyribonucleosides, and [^{3}H]m^{5}dCyd and [^{14}C]dThd were measured by reversed-phase HPLC as described under Experimental Procedures. Each value is the mean ± standard error of three or four determinations. Control values (disintegrations per minute per nanomole) were as follows: 2 hr, m^{5}dCyd = 870 ± 110, dThd = 2290 ± 80; 24 hr, m^{5}dCyd = 1180 ± 330, dThd = 2500 ± 270 for four determinations.

with the exception of [^{3}H]araC, which was 98.4% pure, as assessed by HPLC using a Brownlee RP-18 column with 20 mM KH_2PO_4 (pH 3.8) at a flow rate of 2 ml/min and monitoring absorbance at 242 nm. RNase A (Type I-A), RNase T_2 (Grade V), RNase T_1 (Grade IV), DNAase I (ribonuclease-free), and bacterial alkaline phosphatase (Grade III-R) were obtained from Sigma Chemical Company (St. Louis, Mo.). Snake venom phosphodiesterase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Tissue culture. HT-29 cells were grown under air at 37° in Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, 40 mM Hepes (pH 7.4), and gentamicin (50 μg/ml). Cells were plated at an initial concentration of 10^4 cells/ml in 10 ml of medium per 25 cm^2 plastic flask. Logarithmically growing (3-day) cells were incubated with AZC, araAC, dAZC, or araC prepared fresh in sterile water for 2 hr or 24 hr. Alternatively, 25 μCi of ^{3}H-labeled drugs were added at a final concentration of 10^{-6} M (5550 dpm/pmol) or 10^{-5} M (555 dpm/pmol).

Cell viability. Colony formation in soft agar medium was performed as previously described except that Hepes-buffered medium was used and cells were grown in 25 cm^2 plastic flasks (14).

DNA methylation. Log phase cells in 75 cm^2 plastic flasks were labeled during the last hour of drug treatment with 150 μCi of [*methyl*-^{3}H]methionine (50 mCi/mmol) and 1 μCi of [^{14}C]dThd (53 mCi/mmol). DNA and RNA were extracted with 2 ml of 1% SDS/0.1 M Tris-HCl (pH 8.0)/0.01 M EDTA and 0.5 volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 0.5 volume of chloroform (15). Following RNase digestion, DNA was hydrolyzed with DNase I, snake venom phosphodiesterase, and bacterial alkaline phosphatase, and the deoxyribonucleosides were separated by reversed-phase HPLC with 20 mM KH_2PO_4 (pH 3.8) for 2 min, 20 mM KH_2PO_4 (pH 3.8)/2% methanol for 3.0 min, and 20 mM KH_2PO_4 (pH 3.8)/15% methanol for 3 min and monitored by their absorbance at 268 nm. The retention of dCyd, m^{5}dCyd, dGuo, dThd, and dAdo were 2.0, 4.5, 5.2, 6.0, and 7.8 min, respectively.

Cs_2SO_4 Density gradient centrifugation. The incorporation of ^{3}H-labeled drug into total cellular DNA and RNA from 1 to 2 × 10^6 cells was measured by density gradient centrifugation in Cs_2SO_4 by dissolving the DNA and RNA isolated by SDS/phenol/chloroform extraction described above in 0.2 ml of 50% formamide at 80° for 15 min. The samples were immediately cooled to 4°, diluted with 5 ml of 5 mM EDTA (pH 7), and mixed with an equal volume of saturated Cs_2SO_4. Samples were centrifuged at 200,000 × *g* in a Beckman 50Ti rotor for 60–65 hr at 20°. Tubes were punctured from the bottom, and fractions were collected and counted.

Digestion of DNA and RNA. DNA and RNA were isolated from 1 to 2 × 10^6 cells by SDS/phenol extraction as described above. DNA was

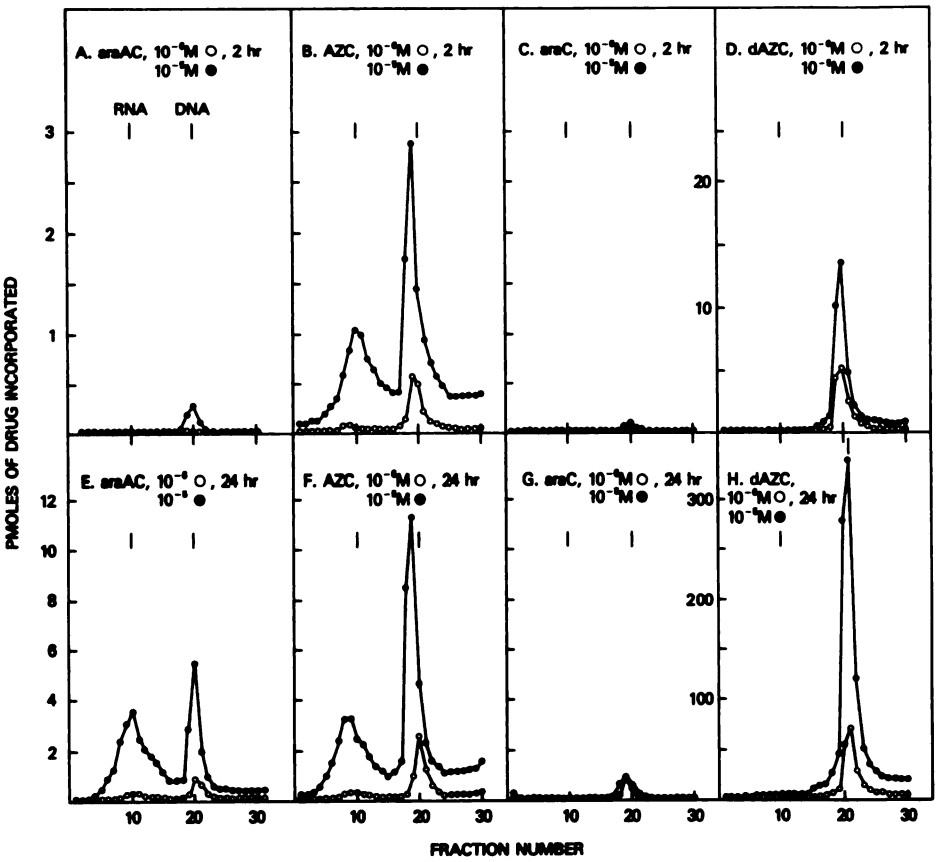

FIG. 3. *Cs_2SO_4density gradient centrifugation of RNA and DNA after 2 and 24 hr of treatment with drug*

Cells were exposed to varying concentrations of drug for 2 hr (A–D) or for 24 hr (E–H). The incorporation of radiolabeled drug into nucleic acids was measured as described under Experimental Procedures, and each gradient represents the incorporation of drug into 1 to 2 × 10^6 cells. Gradients are not normalized for cell number.

isolated after digestion with 20 μg of RNase A, 20 units of RNase T_1, and 20 units of RNase T_2 and precipitated with 2 volumes of 2% potassium acetate in 95% ethanol; RNA then was isolated by digestion with 100 μg of DNase I followed by ethanol precipitation. RNA and DNA were each digested with snake venom phosphodiesterase to obtain nucleoside monophosphates and further hydrolyzed with bacterial alkaline phosphatase to obtain nucleosides (15). Deoxyribonucleosides were separated by reversed-phase HPLC as described above for m^5dCyd, and ribonucleosides were separated similarly with 20 mM KH_2PO_4 (pH 3.8) for 2 min and 20 mM KH_2PO_4 (pH 3.8)/5% methanol for 6 min.

Agarose gel electrophoresis of DNA. Cells were prelabeled with [^{14}C] dThd for 2 days followed by incubation in [^{14}C]dThd-free medium for 1 day, and treated for 24 hr with the indicated concentration of drug. Cells were then pulse-labeled during the last hour of treatment with 10 μCi of [^{3}H]dThd, washed with cold phosphate-buffered saline (6.3 mM Na_2HPO_4/0.8 mM KH_2PO_4/0.154 M NaCl, pH 7.4), and trypsinized. The cells were transferred to an Eppendorf tube and lysed in 50 μl of 30 mM NaOH/2 mM EDTA (pH 12.2)/5% glycerol/0.05% bromophenol blue at 100° for 15 min. Samples containing 1 to 2 × 10^6 cells were applied to 0.7% agarose gels containing 30 mM NaOH/2 mM EDTA (pH 12.2) and electrophoresed at 50 V in the same buffer (16).

RESULTS

Cell viability. The effect of short- and long-term drug exposure on the viability of HT-29 cells was assessed by colony formation (Fig. 1). Treatment of cells for 2 hr with 10^{-6} M AZC, araAC, dAZC, and araC decreased viability by 7, 31, 17, and 30%, respectively (Fig. 1A). Only AZC achieved a 90% reduction in cell viability, but 10^{-4} M drug was required. In contrast, long-term drug exposure of cells to 10^{-6} M AZC, araAC, dAZC, and araC reduced cell viability by 60, 63, 96, and 74%, respectively (Fig. 1B). Of particular note was the marked increase in cell lethality produced by dAZC following 24 hr of drug exposure.

DNA synthesis and methylation. Since both AZC and dAZC have been reported to be effective inhibitors of DNA methylation, this parameter was measured using [*methyl*-^{3}H]methionine and [^{14}C]dThd to pulse-label newly synthesized DNA (Fig. 2). The four drugs could be categorized into two groups according to their relative activities for inhibiting the methylation and synthesis of DNA. AZC and dAZC produced a rapid and concentration-dependent inhibition of methylation without causing a major suppression of DNA synthesis, whereas araC and araAC inhibited DNA methylation to an equal or lesser extent than DNA synthesis.

Drug incorporation into DNA and RNA. The incorporation of radiolabeled AZC, dAZC, araAC, and araC into DNA and RNA was evaluated by Cs_2SO_4 density gradient centrifugation (Fig. 3; Table 1). AZC and dAZC were readily incorporated into DNA after 2 hr, whereas araAC and araC were utilized to a lesser degree (Fig. 3A–D); however, upon incubation for a further 24 hr in drug-free medium, greater amounts of araAC, and particularly

TABLE 1

Incorporation of cytidine analogues into DNA and RNA

See Fig. 4 for experimental details. Each value is the mean of two or three determinations. The standard error or range was 10–207.

Drug concentration	Drug incorporated				% Surviving colonies
	During drug treatment		After a further 24 hr in drug-free medium		
	RNA	DNA	RNA	DNA	
M	*pmol/10⁶ cells*				
2-hr treatment					
araC					
10^{-6}	0	1	0	8	70
10^{-5}	0	2	0	12	70
araAC					
10^{-6}	0	1	0	4	69
10^{-5}	0	3	0	11	72
AZC					
10^{-6}	1	5	1.4	8	93
10^{-5}	16	29	11	59	30
dAZC					
10^{-6}	0	16	0	16	83
10^{-5}	0	29	0	90	68
24-hr treatment					
araC					
10^{-6}	0	6	0	5	26
10^{-5}	0	4	0	16	6
araAC					
10^{-6}	5	6	2	7	37
10^{-5}	52	48	12	32	3
AZC					
10^{-6}	6	15	1.8	8	40
10^{-5}	36	39	20	82	13
dAZC					
10^{-6}	0	107	0	73	4
10^{-5}	0	481	0	214	0.6

araC, were incorporated into DNA (Table 1). Following 24 hr of drug treatment, all compounds were incorporated into DNA, and in particular, dAZC, where its level reached 7–12 times that of AZC (Fig. 3E–H). Radioactivity from araAC was incorporated into RNA as well as DNA in a fashion similar to that of AZC (Fig. 3E and F).

The radiolabeled drug metabolite incorporated into DNA was identified by analysis by HPLC of deoxynucleosides from DNA digests (Fig. 4). Virtually all of the labeled araC, AZC, and dAZC eluted as the parent nucleoside or the hydrolytically unstable ring-open form (17) (denoted with an *asterisk*), eluting just before the parent nucleoside upon reversed-phase HPLC (Fig. 4B–D). A small percentage of the radioactivity derived from [^{3}H]AZC was present in dGuo and dAdo, a result reflecting utilization of [^{3}H]formate produced by ring hydrolysis and deformylation of [6-^{3}H]AZC (17). Approximately 50% of araAC was present as the parent or ring-open form of the nucleoside (Fig. 4B), with the remaining radioactivity being present in dGuo and dAdo. The greater amount of tritium derived [^{3}H]araAC found in purines indicates a greater lability of this drug in comparison to AZC and dAZC.

Similar HPLC analyses of the ribonucleosides derived

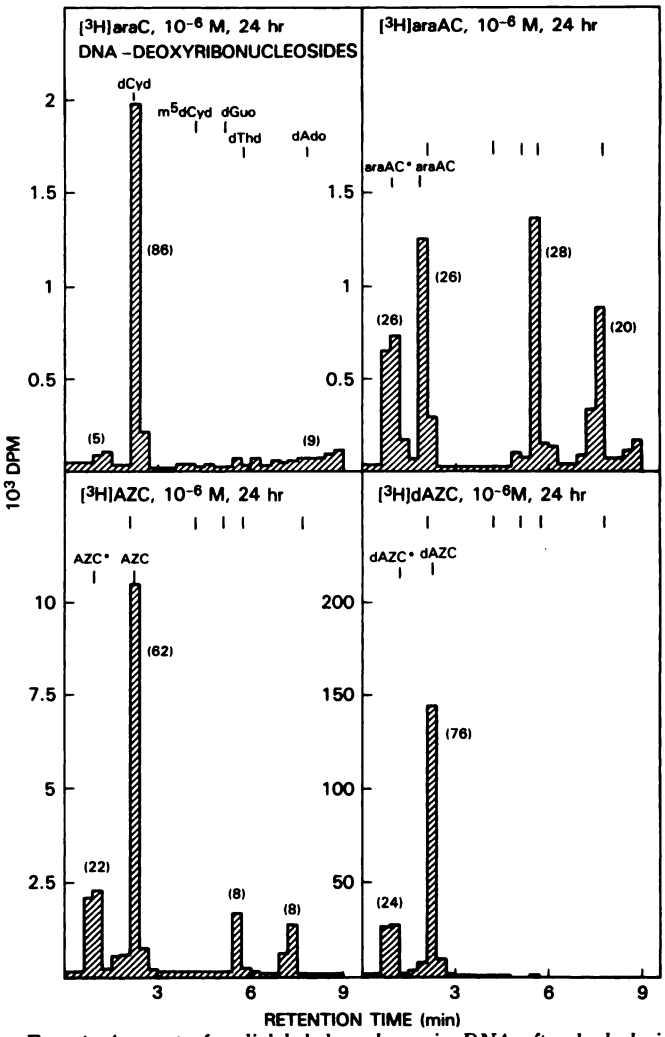

FIG. 4. *Amount of radiolabeled analogue in DNA after hydrolysis with DNase I, phosphodiesterase, and alkaline phosphatase*

Cells were treated for 24 hr with the indicated concentrations of radiolabeled drug, and equal amounts of DNA were hydrolyzed to deoxynucleosides and separated by reversed-phase HPLC as described under Experimental Procedures. The retention time of the ring-open metabolite (17) of either AZC, dAZC, or araAC is indicated by an *asterisk*.

from RNA labeled with [^{3}H]AZC or [^{3}H]araAC were performed (Fig. 5). Approximately 40–50% of the radioactivity derived from [^{3}H]AZC was found in the parent nucleoside or its hydrolysis product, whereas the remaining radioactivity was present in Guo and Ado. In contrast, 70–90% of the radioactivity in RNA derived from [^{3}H]araAC was found to be present in the two purines, and none of the radioactivity present in RNA coeluted with the parent nucleoside. These results suggest that virtually all of the radioactivity in RNA after incubation of cells with [^{3}H]araAC is a result of ring hydrolysis with subsequent incorporation of the hydrolysis product, [^{3}H]formate. It should also be noted that cytidine deaminase did not appear to be limiting the activity of the analogues in HT-29 cells. Preincubation of cells for 10 min with 10^{-4} M 1-β-D-ribofuranosyl-1,3,4,7-tetrahydro-2*H*-1,3-diazepin-2-one, a highly potent inhibitor of this enzyme (18), before addition of araC or araAC did not affect the

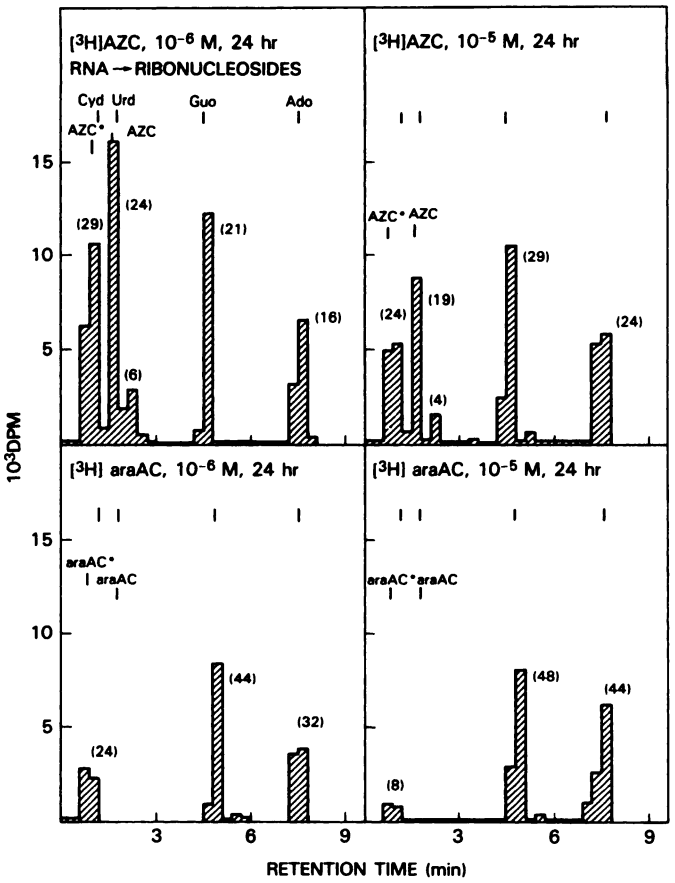

FIG. 5. *Amount of radiolabeled analogue in RNA after hydrolysis with RNases, phosphodiesterase, and alkaline phosphatase*

Cells were treated as in Fig. 4, and equal amounts of the isolated RNA were hydrolyzed to ribonucleosides and separated by HPLC as described under Experimental Procedures. The retention time of the ring-open metabolite of either AZC or araAC is indicated by an *asterisk*.

extent of inhibition of DNA synthesis (results not shown).

DNA size. The high level of incorporation of dAZC into DNA suggested that DNA damage might be the reason for the cell lethality produced by this drug. To assess this possibility, cells were prelabeled with [^{14}C] dThd before drug treatment to label parental DNA and were pulse-labeled with [^{3}H]dThd during the last hour of drug treatment to label newly synthesized DNA. The size of newly and previously synthesized DNA was analyzed by alkaline agarose gel electrophoresis (Fig. 6). Following 24-hr exposure to varying concentrations of dAZC, newly synthesized DNA appeared as lower molecular weight DNA, but prelabeled parental DNA remained intact (Fig. 6B–D); at 10^{-6} M AZC, araAC (Fig. 6E and F), and araC (data not shown) or 10^{-7} M dAZC, little or no change occurred in the size of parental or newly synthesized DNA. Higher concentrations of AZC, araAC, and araC could not be evaluated because of their inhibitory effects on the 1-hr pulse-labeling of DNA with [^{3}H] dThd. In no instance was degradation of previously synthesized DNA observed with any of the drugs at concentrations up to 10^{-5} M, indicating an absence of single- or double-strand breakage of DNA by drug treatment. Alkaline elution of prelabeled DNA following incubation of HT-29 cells for 24 hr with 10^{-5} M dAZC or araC did not reveal any change in single-strand break frequency.[3]

DISCUSSION

The initial objective of this study was to compare the cytotoxicity produced by araAC with structurally similar analogues of cytidine, and to determine whether there was any common biochemical basis for their mechanism of action. Among the analogues with the 5-azacytosine moiety, a close relationship was observed between their incorporation into DNA and cell lethality. A similar pattern was observed for the incorporation of AZC into RNA. The incorporation of AZC into RNA (19–21) and DNA (11, 13), as well as dAZC into DNA (22) of mouse ascites and embryo cells, has been previously reported, but the association between this phenomenon and cell viability was not established. The degree of DNA substitution for all AZC analogues approached 50 pmol/10^{6} cells for a 1 log reduction in cell viability. This represents approximately a 1% substitution of dCyd residues in DNA. The greatest degree of cell lethality was produced by 10^{-5} M dAZC after 24 hr of treatment and represented 10% substitution of dCyd in DNA and more than a 2-log reduction in cell viability. These levels of drug incorporation are far in excess of that which is required for inhibition of DNA methylation (13, 23–25), as shown by the 90% or greater inhibition of DNA methylation by 10^{-6} M AZC or dAZC in 2 hr, when 0.1% and 0.3% substitution occurred, respectively. Moreover, under the latter conditions, no cell lethality was observed. Thus, the reduction in DNA methylation by AZC and dAZC suggests that inhibition of this process precedes cell lethality and is not responsible for antitumor activity. That hypomethylated DNA is not sufficient in itself to produce a loss of cell viability was also demonstrated by Adams *et al.* (26), who found no relationship between cell lethality in Chinese hamster ovary cells and inhibition of DNA methylation by dAZC. In contrast to AZC and dAZC, araC and araAC did not *preferentially* reduce DNA methylation in excess of their ability to impair DNA synthesis. It was previously observed that araC may enhance DNA methylation in mouse P815 mastocytoma cells (27). However, under the latter conditions, cells were exposed for two to six generation cycles, in contrast to a maximum of one generation in the present study. Therefore, under the present experimental conditions, DNA hypermethylation does not appear to be a significant feature of the action of araC.

AraC is a potent and rapid inhibitor of DNA synthesis by competitive inhibition via its metabolite araCTP (28, 29). On the basis of dThd incorporation into DNA, araAC was 50 to 100-fold less potent than araC; however, this parameter per se did not correlate with loss of cell viability by these drugs. The lack of correspondence between inhibition of DNA synthesis and cell lethality was further confirmed by the rapid reversal of inhibition of DNA synthesis upon removal of drug for 24 hr (data not shown). The incorporation of araAC and araC into DNA during the treatment interval also did not correlate with cell lethality; however, upon incubation of cells in

[3] J. Minford, D. Kerrigan, and L. A. Zwelling, unpublished results.

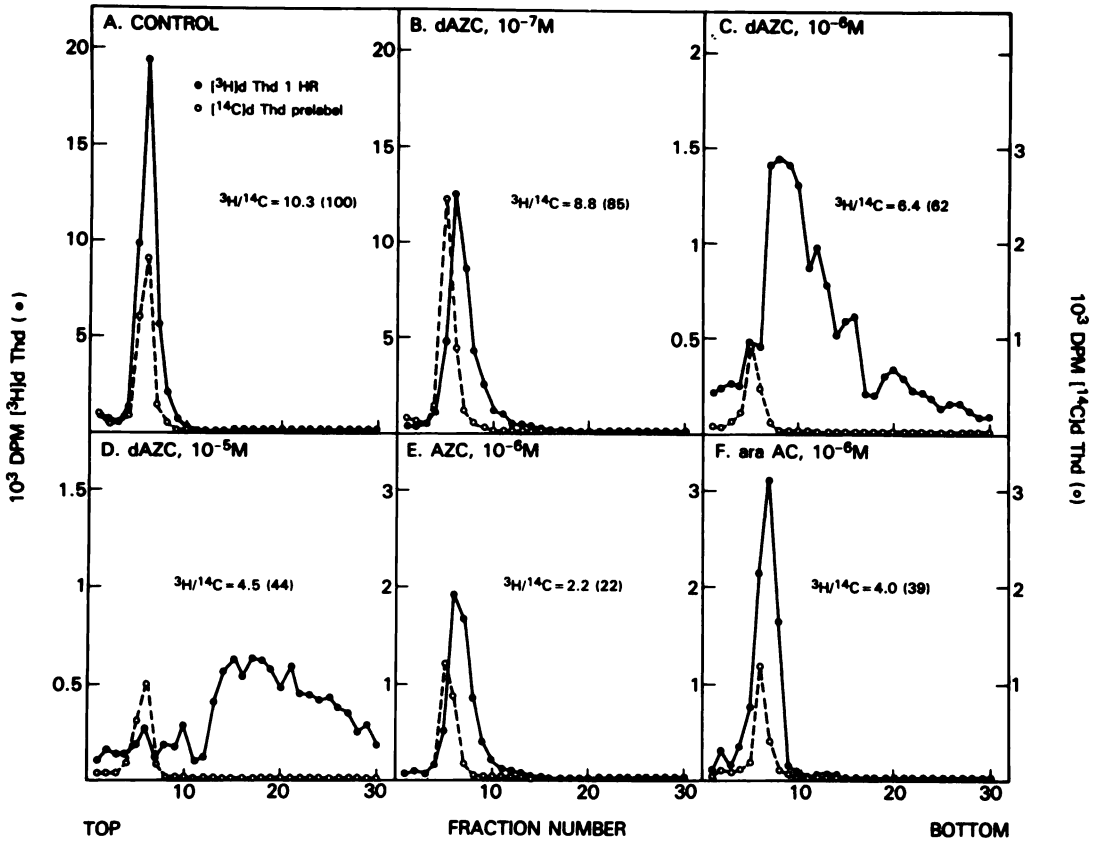

FIG. 6. *Alkaline agarose gel electrophoresis of DNA from drug-treated cells*

Cells were prelabeled with [^{14}C]dThd, exposed to varying concentrations of drug for 24 hr, and pulse-labeled during the last hour with [^{3}H]dThd. Cells were lysed directly in alkaline buffer, and DNA was separated by agarose gel electrophoresis as described under Experimental Procedures.

drug-free medium (Table 1), significant amounts of araC were chased into DNA, a result reflecting a reduction in araCTP levels and a partial release of DNA polymerase inhibition. Previous studies have noted a correlation between colony formation (30) and the incorporation of araC into DNA. The apparent time-dependent utilization of araAC and araC for DNA synthesis in our studies suggests that this effect is probably responsible for their cytocidal activity.

One consequence of dAZC incorporation into DNA was the formation of low molecular weight newly synthesized DNA, a result indicating that DNA elongation was inhibited. The amount of low molecular weight nascent DNA was directly associated with the level of drug substitution and its cytocidal activity. This effect was not apparent with AZC, araAC, and araC at 10^{-6} M concentrations, but may have resulted at more lethal levels of drug where DNA substitution was greater. The marked inhibition of dThd incorporation into DNA at higher concentrations of these drugs did not allow us to assess this possibility. The lack of effect of these drugs on prelabeled parental DNA indicates that DNA strand breakage does not result from either drug treatment or the subsequent loss of cell viability during this exposure interval. Contrary to our results, araC was reported to enhance the alkaline elution of previously synthesized DNA in L1210 cells (31), although this effect was not dose-related to cell lethality (30, 31). We have not observed this effect in HT-29 cells with either similar methodology[3] or using alkaline agarose gel electrophoresis. These dissimilarities may reflect either differences in DNA repair and drug sensitivity between L1210 and HT-29 cell lines or the enormous concentrations (10^{-3} M) of araC used in these experiments (31).

The mode of action of AZC may also involve an RNA-dependent mechanism such as inhibition of tRNA methylation (32–34), rRNA synthesis (21, 32, 35), and tRNA acceptor activity (33, 36), but these effects have not been unequivocally related to cell lethality. In contrast, the cytotoxicity of araAC does not appear to be RNA-dependent. The radioactivity derived from [^{3}H]araAC that was incorporated into RNA appeared to result predominantly from [^{3}H]formate generated by ring hydrolysis of the drug (17). This also occurred to a lesser extent with [^{3}H]AZC and should serve as a caution in interpreting drug incorporation data with hydrolytically labile analogues. This conclusion is further supported by the fact that araAC does not affect rRNA processing, in contrast to the effect of AZC, which results in a buildup of 32 S rRNA precursor.[4]

In summary, araAC appears pharmacologically more akin to araC than to AZC and dAZC. Although it is a less potent inhibitor of DNA synthesis than araC, it is

[4] M. B. Cohen and R. I. Glazer, unpublished results.

incorporated into DNA to an equal or greater extent and is equally cytotoxic to HT-29 cells *in vitro*.

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Send reprint requests to: Dr. Robert I. Glazer, Building 37, Room 6D28, National Cancer Institute, Bethesda, Md. 20205.