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Studies on the mechanism of the synergistic interaction between 2′-deoxy-5-azacytidine and cisplatin*

James L. Abbruzzese[1] and Philip Frost[1, 2]

Departments of [1] Medical Oncology and [2] Cell Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcome Blvd., Houston, Texas 77030, USA

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Summary. 2′-Deoxy-5-azacytidine (5-aza-CdR) and cisplatin interact to produce synergistic cytotoxicity against many human tumor cell lines. Preliminary experiments designed to explore the mechanism of this synergy suggested a poor correlation between synergy and the degree of genomic hypomethylation measured following exposure to 5-aza-CdR. Subsequent studies using plasmid DNA suggested that rather than DNA hypomethylation, incorporation of 5-aza-CdR into DNA mediated increased cisplatin binding to DNA and could therefore be essential to the synergistic interaction between these two agents. In this series of experiments, we evaluated the degree of synergy with cisplatin produced against two human melanoma cell lines by two additional antimetabolites that were chosen on the basis of their biochemical properties. In addition, we investigated the synergy between 5-aza-CdR and cisplatin in parental and 5-aza-CdR-resistant murine cell lines, which differed in their sensitivity to 5-aza-CdR and DNA methylation status but incorporated similar amounts of 5-aza-CdR into DNA when exposed to this antimetabolite. In the studies testing additional antimetabolites, cytosine arabinoside, which is incorporated into DNA but does not hypomethylate it, produced synergy with cisplatin that was similar or superior to that obtained using 5-aza-CdR. With 3-deaza-adenosine, which is not incorporated into DNA but produces DNA hypomethylation through inhibition of S-adenosylhomocysteine hydrolase, a primarily antagonistic interaction was observed in the two cell lines studied. In the 5-aza-CdR-sensitive and -resistant cell lines, a very similar synergistic interaction was documented for 5-aza-CdR and cisplatin despite the significant difference observed in DNA methylation levels. Taken as a whole, these data suggest that DNA hypomethylation was not critical to the synergistic cytotoxicity produced by 5-aza-CdR and cisplatin. This finding suggests additional strategies that could further modulate this interaction.

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Offprint requests to: J. L. Abbruzzese, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 78, Houston, 77030, USA

Introduction

The antitumor antimetabolite 2′-deoxy-5-azacytidine (5-aza-CdR) is metabolized by deoxycytidine kinase to the triphosphate (2′-deoxy-5-azacytidine triphosphate, 5-aza-CTP) and incorporated into DNA [22, 27]. It is known that 5-aza-CdR hypomethylates eukarotic DNA through inhibition of DNA methyltransferases [10, 17] and that hypomethylation of cellular DNA is an important activator of transcription [20, 24]; however, the contribution of genomic DNA hypomethylation to the cytotoxicity of 5-aza-CdR is not known. This issue is complicated by the observation that both hypomethylation and cytotoxicity probably require incorporation of the fraudulent nucleotide 5-aza-CTP into DNA and may account for the correlation between antitumor activity and inhibition of DNA methylation observed in some studies [29].

Recently, progress toward understanding the relative roles of incorporation and hypomethylation has been made through biochemical analysis of cells rendered resistant by stepwise exposure to 5-aza-CdR. This study showed that as compared with the parental cell line (C3H 10T1/2 C18), 5-aza-CdR-resistant cells (56-41) incorporated similar amounts of 5-aza-CdR into DNA, were profoundly hypomethylated, and contained levels of methylcytosine that could not be further reduced by exposure to 5-aza-CdR [13]. These authors presented evidence supporting the hypothesis that there is a complex interaction between incorporation of 5-aza-CdR into DNA, subsequent generation of hemimethylated sites in newly synthesized DNA, and cytotoxicity [21].

Previous studies in our laboratory showed that a synergistic cytotoxic interaction could be demonstrated be-

tween 5-aza-CdR and cisplatin or 4-hydroperoxycyclophosphamide (4-HC) [15]. However, in our preliminary experiments, there was a poor correlation between synergy and the degree of hypomethylation measured following exposure to 5-aza-CdR in the six human tumor cell lines tested. Subsequent studies using plasmid DNA were designed to analyze the molecular events underlying the interaction between 5-aza-CdR and cisplatin and to assess specifically the importance of DNA hypomethylation in the synergy. This study suggested that rather than DNA hypomethylation, incorporation of the fraudulent nucleotide 5-aza-CTP into DNA mediated increased cisplatin binding to plasmid DNA and could therefore be essential to the synergistic interaction between these two agents (Ellerhorst et al., manuscript in preparation). However, the role of 5-aza-CdR-induced DNA hypomethylation in the synergistic cytotoxicity observed in cellular systems remained unclear.

A structurally similar antimetabolite, cytosine arabinoside (ara-C), is also capable of interacting with cisplatin against L1210 mouse leukemia cells and human colon cancer cells to enhance cytotoxicity [4, 14, 26]. Experiments designed to elucidate the mechanism of this interaction have also suggested that synergy between ara-C and cisplatin may be dependent on the incorporation of 5-ara-CTP into DNA [14]. However, in recent studies evaluating the role of DNA methylation in drug-induced resistance, ara-C, unlike 5-aza-CdR, seemed to lead to hypermethylation in human tumor cell lines [23].

A separate class of antimetabolites has been shown to induce hypomethylation of cellular DNA. These agents are not phosphorylated to nucleotides and are not incorporated into cellular DNA but function through the competitive inhibition of S-adenosylhomocysteine hydrolase (AdoHcy hydrolase), the enzyme responsible for the catabolism of the endogenous transmethylase inhibitor S-adenosylhomocysteine (AdoHcy) [11]. Inhibition of AdoHcy catabolism results in its accumulation, which acts as a strong inhibitor of the numerous S-adenosylmethionine (AdoMet)-dependent transmethylation reactions. An example of this class of antimetabolite is 3-deaza-adenosine (C^3Ado), which causes an increase in intracellular levels of AdoHcy and a correlative decrease in S-adenosylmethionine (SAM) production, leading to inhibition of all methyltransferase reactions [19].

Using these three antimetabolites and the 5-aza-CdR-sensitive and -resistant murine cell lines $10T^1/_2$ and 56-41 (described above), we sought to clarify whether 5-aza-CdR-induced hypomethylation was an important event in the synergistic cytotoxic interaction of 5-aza-CdR with cisplatin.

Materials and methods

Tumor cell lines. We used two human melanoma cell lines, A375 [18] and DX-3 [2], in which similar degrees of synergy between 5-aza-CdR and cisplatin were previously documented [15]. The cloning efficiency for these cell lines is approximately 40%. Murine cell lines C3H $10T^1/_2$ C18 ($10T^1/_2$) and 56-41 are 5-aza-CdR-sensitive and -resistant, respectively. The 56-46 line was derived by repetitive treatment of the $10T^1/_2$ parent line with increasing concentrations of 5-aza-CdR. The cloning efficiency for these cell lines is approximately 10%. Both murine lines were generously provided by Dr. P. A. Jones of the Department of Biochemistry, University of Southern California Cancer Center (Los Angeles, Calif.).

Cells were maintained in minimal essential medium or Eagle's basal medium (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 50,000 IU penicillin G, 50,000 IU streptomycin, 150 mg L-glutamine, 20 μM HEPES, 375 mg sodium bicarbonate, and 10% fetal bovine serum per 500 ml medium. Cell cultures were grown as monolayers at 37° C in an atmosphere containing 5% CO_2 at 80% – 100% humidity. Cell detachment was achieved by incubation for 5 min at 37° C with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Cells were routinely screened for *Mycoplasma* using the Gen-Probe assay (Gen-Probe, San Diego, Calif.); they were *Mycoplasma*-free

Drugs. 5-Aza-CdR was provided by Pharmachemie B. V. (Haarlem, The Netherlands). Ara-C was obtained from Sigma Chemical Co. (St. Lois, Mo.). C^3Ado was supplied by the Southern Research Institute (Birmingham, Ala.). Cisplatin was obtained from Dr. R. Davis of the National Institutes of Health (NIH). All drugs were prepared immediately before use.

Drug treatment. Subconfluent cultures were trypsinized and replated at 2×10^6 cells in 5 ml medium in a T-25 flask. For the single-agent experiments, cells were exposed to increasing concentrations of 5-aza-CdR, ara-C, or C^3Ado for 18 – 20 h. The duration of exposure to cisplatin was 1 h. Following drug exposure, the cells were washed three times in PBS. Combination treatments were always performed using sequential exposure to the two agents as follows. Cells (2×10^6) were plated in a T-25 flask. The appropriate antimetabolite (5-aza-CdR, ara-C, or C^3Ado) was added at increasing concentrations and incubated for 18 – 20 h. The cells were then washed, exposed to the appropriate concentration of cisplatin for 1 h, and washed three times with PBS.

Colony-forming ability and concentration-effect curves. The colony-forming ability (CFA) of each cell line was assessed after single- or double-agent treatment in the following manner. After treatment, 200 – 500 cells (depending on the cell line) were dispersed in 60-mm tissue-culture dishes and incubated for 7 – 14 days. Because of the high reproducibility of the assay, five replicate dishes were used per concentration. The plates were then stained with 1% methylene blue and dried, and the number of colonies were counted with the aid of a Bellco hand-held colony counter. A colony was defined as comprising more than 50 cells. The CFA, or the fraction of cells unaffected (F_u), was calculated by dividing the number of colonies counted in drug-treated dishes by the number recorded in untreated control dishes. A control consisting of untreated cells was included in each experiment.

Concentration-effect curves were generated for each agent alone and in combination, plotted as the fraction affected ($F_a = 1 - F_u$) versus concentration. The concentration effective in 50% of the cell population (ED_{50}) was defined as the drug concentration required to produce an F_a value of 0.5 as compared with untreated controls.

Assessment of synergy. We used the median-effect principle [6] to determine the concentration ratio of one agent to another that would allow calculation of the interaction between both drugs. The ratios used were based on the ED_{50} concentration established from the concentration-effect relationship. Linear correlation coefficients were generated for each curve to determine the applicability of the data to this method of analysis. In all cases, these coefficients (r) for the synergy experiments were greater than 0.8.

Quantitation of the interaction of two independent drugs was analyzed using a computerized program [5] to calculate the combination index (CI) based on the equations of Chou and Talalay [7, 8]. Data were plotted as CI versus F_a, whereby a CI value of less than 1 indicated synergy, a value of greater than 1 indicated antagonism, and a value of 1 indicated additivity. For comparison of synergy curves, the degree of synergy was estimated by calculation of the CI at an F_a value of 0.5. Each

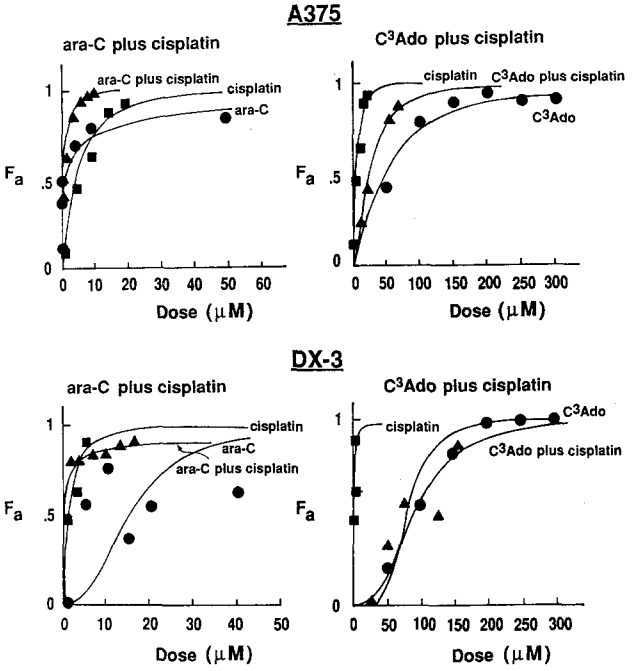

Fig. 1. Concentration-effect curves for ara-C, C³Ado, cisplatin, ara-C plus cisplatin, and C³Ado plus cisplatin against the A375 and DX-3 human melanoma cell lines. Cells were exposed to ara-C or C³Ado for 18–20 h or to cisplatin for 1 h. Combination studies were performed by exposing the cells to the antimetabolite for 18–20 h, washing them, and then exposing them to cisplatin for 1 h

experiment was repeated at least three times and representative data are presented.

Assessment of DNA methylation. We used a modification of the procedure described by Flatau et al. [12] for assessment of DNA methylation. Cells were plated at 2×10^5/60-mm petri dish in medium containing either 5-aza-CdR, ara-C, or C³Ado along with 5 μCi [6-³H]-uridine and were incubated for 18–20 h. Thereafter, the cells were washed with PBS, lyzed with 1 ml 0.1% sodium dodecyl sulfate in 0.3 *N* NaOH, and incubated for 1 h at 65° C. The pellet was then prepared for HPLC analysis as previously described [15]. The percentage of 5-methylcytosine (MC) relative to cytosine (C) was calculated as follows:

$$\% \text{ MC} = \frac{\text{cpm MC}}{\text{cpm C} + \text{cpm MC}} \times 100.$$

Statistical analysis. The differences between samples were assessed using Student's T-test.

Results

Synergistic cytotoxicity observed for cisplatin with 5-aza-CdR and ara-CdR but not with C³Ado

Our previous studies using 5-aza-CdR and cisplatin demonstrated a similar degree of synergistic cytotoxicity against both A375 and DX-3 cells [15]. We repeated these experiments, substituting ara-C and C³Ado for 5-aza-CdR, against both cell lines. First, concentration-effect curves were generated for ara-C and C³Ado against A375 and DX-3 cells (Fig. 1). From these curves, the ED_{50} value (F_a = 0.5) was calculated for each agent and cell line. These ED_{50} concentrations were then used to establish the appropriate ratios for the evaluation of synergy produced by the combinations ara-C plus cisplatin and C³Ado plus cisplatin. For example, in the DX-3 cell line, the ED_{50} concentration was 1 μM for both 5-aza-CdR and cisplatin; thus, the ratio of 5-aza-CdR to cisplatin for the combination studies was maintained at 1 : 1. The ratios used for the combinations ara-C plus cisplatin and C³Ado plus cisplatin as well as the previously published ratios for 5-aza-CdR plus cisplatin against A375 and DX-3 cells are indicated in Table 1.

Figure 2 summarizes the interaction of 5-aza-CdR plus cisplatin, ara-C plus cisplatin, and C³Ado plus cisplatin against A375 and DX-3 cells. As in the case of 5-aza-CdR and cisplatin, these graphs suggest a synergistic interaction for ara-C and cisplatin; in contrast, a primarily antagonistic effect was observed for C³Ado and cisplatin. A compari-

Table 1. Quantitative interaction of 5-aza-CdR plus cisplatin, ara-CdR plus cisplatin, and C³Ado plus cisplatin against A375 and DX-3 cells [a,e]

Cell line	Antimetabolite: cisplatin ratio		Concentration (μM) of single agent[d]		Concentration (μM) for combination[d]		
	5-Aza-CdR: cisplatin	F_a[b]	5-Aza-CdR	Cisplatin	5-Aza-CdR	Cisplatin	CI (F_a = 0.5) [c]
A375	1:6	0.9	37.9	21.2	1.4	8.2	0.45
DX-3	1:1	0.9	52.6	6.8	6.8	2.9	0.44
	Ara-C: cisplatin		Ara-C	Cisplatin	Ara-C	Cisplatin	
A375	1.5:5	0.9	74.7	21.2	1.2	3.9	0.45
DX-3	1.5:0.1	0.9	36.1	6.8	31.9	2.1	0.01
	C³Ado: cisplatin		C³Ado	Cisplatin	C³Ado	Cisplatin	
A375	49:5	0.9	225.0	21.2	81.1	8.3	1.1
DX-3	100:1.3	0.9	138.1	6.8	198.9	2.6	3.0

[a] All drug concentrations are micromolor. Drug exposures were carried out as described in Materials and methods

[b] Fraction of cells affected

[c] CI (F_a = 0.5) is defined as the combination index (CI) for the drug combination measured at an F_a value of 0.5. CI <1.0 indicates synergism, CI = 1.0 indicates additivicity, and CI >1.0 indicates antagonism

[d] Values represent the concentrations of 5-aza-CdR, ara-C, and cisplatin needed to achieve the ED_{90} for each drug as a single agent or in combination

[e] Data for 5-aza-CdR and cisplatin have previously been published elsewhere [15]

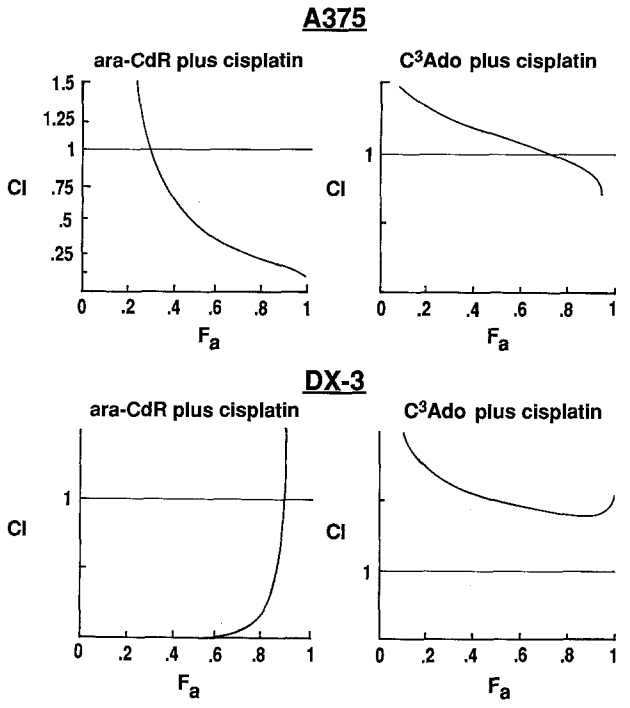

Fig. 2. Computer-generated synergy curves for ara-C plus cisplatin and C^3Ado plus cisplatin against the A375 and DX-3 human melanoma cell lines. CI <1, synergy; CI >1, antagonism; C = 1, additivity

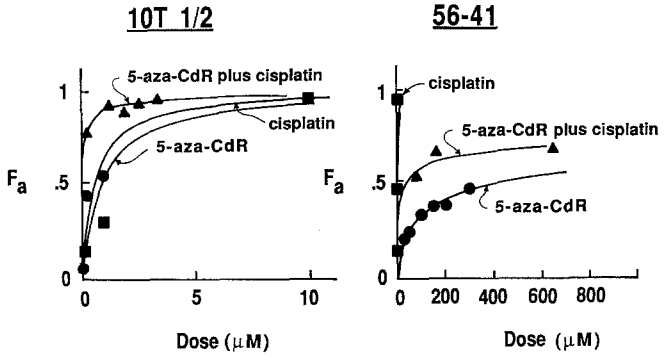

Fig. 3. Concentration-effect curves for 5-aza-CdR, cisplatin, and the combination against parental $10T^1/_2$ cells and 5-aza-CdR-resistant 56-41 cells. Cells were exposed to 5-aza-CdR for 18–20 h or to cisplatin for 1 h. Combination studies were performed by exposing the cells to 5-aza-CdR for 18–20 h, washing them, and then exposing them to cisplatin for 1 h

son of these curves is presented quantitatively in Table 1; the CI at an F_a value of 0.5 was calculated from each synergy curve to facilitate comparison. At an F_a value of 0.5, the CI for C^3Ado: cisplatin against A375 was 1.14 and that for C^3Ado: cisplatin against DX-3 was 2.99, indicating a lack of synergy between these two agents for these two cell lines.

Table 2. DNA hypomethylation following exposure of A375 and DX-3 cells to 5-aza-CdR, ara-C, or C^3Ado

Drug	Concentration (μM)	% Methylcytosine	*P* value[a]
A375 cells:			
None	–	2.02±0.01	–
5-Aza-CdR	0.3	0.35±0.02	<0.001
Ara-C	5.0	1.91±0.01	NS
C^3Ado	300	1.60±0.18	0.02
DX-3 cells:			
None	–	1.92±0.08	–
5-Aza-CdR	0.73	0.29±0.02	<0.001
Ara-C	15.0	1.65±0.24	0.26
C^3Ado	300.0	1.45±0.17	0.003

[a] Determined by Student's T-test
NS, Not significant

Lack of correlation between measurable cellular DNA hypomethylation and the degree of synergy observed

To determine whether DNA hypomethylation played a role in the cytotoxic synergy observed, we calculated the percentage of methylcytosine present in genomic DNA at the time at which cisplatin was added (Table 2). The 5-aza-CdR and ara-C concentrations used corresponded to the ED_{50} values for each drug against the cell line in question. For C^3Ado, the ED_{50} (A375) and ED_{90} (A375 and DX-3) concentrations were used to explore the range of hypomethylating effects achieved with this agent.

As can be seen in Table 2, 5-aza-CdR produced profound hypomethylation of newly synthesized DNA in both A375 and DX-3 cells. Ara-C produced no statistically significant DNA hypomethylation, although it was highly synergistic with cisplatin. The lack of correlation between hypomethylation and cytotoxicity was more obvious in the case of C^3Ado, whereby statistically significant hypomethylation of genomic DNA could be demonstrated following the exposure of A375 and DX-3 cells to a concentration of 300 μM, but only minimal synergistic cytotoxicity (A375) or frank antagonism (DX-3) was encountered.

Studies using 5-aza-CdR-sensitive and -resistant murine cell lines

As outlined in the Introduction, the murine $10T^1/_2$ (5-aza-CdR-sensitive) and 56-41 (5-aza-CdR-resistant) cell lines were used for further analysis of the role of DNA hypomethylation in 5-aza-CdR-induced synergy. It has previously been established that despite the resistance of the 56-41 cell line to 5-aza-CdR, the levels of 5-aza-CdR incorporated into 56-41 DNA are similar to those incorporated into DNA of the parent $10T^1/_2$ cells [21]. However, the basal percentage of methylated cytosines in the resistant 56-41 cells is much lower (methylcytosine content, 3.33% ± 0.11% in $T10^1/_2$ cells vs 1.27% ± 0.03% in 56-41 cells). Moreover exposure of 56–41 cells to 5-aza-CdR does not change the level of DNA methylation (P. Jones, personal communication), whereas exposure of

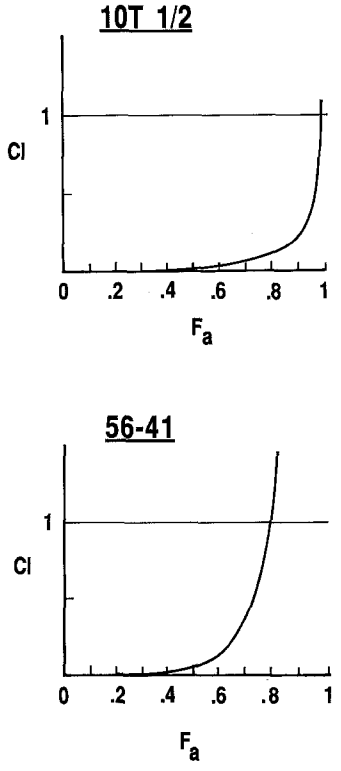

Fig. 4. Computer-generated synergy curves for 5-aza-CdR and cisplatin against parental $10T^1/_2$ cells and 5-aza-CdR-resistant 56-41 cells. CI <1, synergy; CI >1, antagonism; C = 1, additivity

$10T^1/_2$ cells to 0.09 μM 5-aza-CdR reduces genomic methylation levels to 0.34% ± 0.14%. On the basis of these biochemical characteristics, we reasoned that if the DNA methylation levels prior to or following treatment with 5-aza-CdR were critical to synergy, different patterns of drug synergy would be encountered for the $10T^1/_2$ and 56-41 lines. However, if the incorporation of 5-aza-CdR into DNA constituted a more critical event, a similar degree of synergy would be expected for the two lines.

The dose-response curves obtained for the $10T^1/_2$ and 56-41 lines are presented in Fig. 3. As anticipated, the 56-41 cell line was highly resistant to 5-aza-CdR; the ED_{50} value was 470.5 μM. The ED_{50} values for cisplatin were 0.8 and 3.0 μM for the $10T^1/_2$ and 56-41 lines, respectively. The results of the synergy experiments using 5-aza-CdR and cisplatin are shown in Fig. 4. The curves suggest that a broad range of concentrations of 5-aza-CdR and cisplatin are synergistic in both cell lines. Thus, these data would again argue against the role of DNA hypomethylation and suggest that the incorporation of 5-aza-CdR into DNA is important to this interaction.

Discussion

Many studies have described synergistic interactions between cytotoxic agents [3, 4, 14, 26], between cytotoxic agents and biologic compounds [28], and between two biologic agents [1, 25]. However, relatively few studies have evaluated the mechanism underlying the synergistic interaction. An understanding of this mechanism may help us to anticipate the selectivity of a given combination of drugs against neoplastic cells relative to normal tissues (therapeutic ratio) prior to the initiation of clinical trials or to develop additional strategies designed to enhance synergy through the addition of other agents or the implementation of changes in the schedule of drug administration.

Our initial studies that analyzed the mechanism of interaction between 5-aza-CdR and cisplatin suggested that synergy was a result of the incorporation of 5-aza-CTP into DNA, since 5-aza-CdR-substituted plasmid DNA bound greater amounts of cisplatin than did nonsubstituted plasmid DNA and methylated plasmid DNA bound greater amounts of cisplatin than did nonmethylated plasmid. In the present study, we extended our observations in cellular systems to support the hypothesis that the synergistic cytotoxic interaction between these two drugs is not dependent on the hypomethylation of cellular DNA induced by 5-aza-CdR. This view is supported by (a) the lack of significant synergy between C^3Ado and cisplatin, despite the demonstrable cellular hypomethylation induced by C^3Ado at the time at which cisplatin was added; (b) the observation of marked synergy between ara-C and cisplatin in the absence of significant DNA hypomethylation; and (c) the observation that synergy was similar for the 5-aza-CdR-sensitive and -resistant murine cell lines despite profound differences in their basal or posttreatment methylation levels.

These data, like the plasmid DNA studies discussed above, suggest but do not prove that a potential factor in the synergy between 5-aza-CdR and cisplatin is the incorporation of 5-aza-CdR into DNA. This view is supported by the data presented because C^3Ado is not incorporated into DNA, whereas both 5-aza-CdR and ara-C are known to be incorporated. Similarly, it has been established that similar amounts of 5-aza-CdR are incorporated into DNA in the murine 5-aza-CdR-sensitive and -resistant lines [21]. However, it must be emphasized that other mechanisms, such as inhibition of DNA repair by 5-aza-CdR and ara-C, were not examined in these studies and may also play an important role. The likelihood that additional mechanisms might be operative is suggested by the striking differences observed in the shape of the synergy curves for the A375 and DX-3 melanoma lines.

These observations suggest other ways in which the interaction between 5-aza-CdR and cisplatin could be enhanced and studied. If incorporation of 5-aza-CdR into DNA is indeed critical to the synergy, agents that enhance the former should increase the interaction with cisplatin. Thus, inhibitors of ribonucleotide reductase [9] and inhibitors of cytidine triphosphate synthetase, such as cyclopentenyl cytosine [16], may be capable of modulating the synergy between 5-aza-CdR and cisplatin, thus providing further insight into the mechanism of this potentially useful synergistic interaction.

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