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Evaluation of the antineoplastic activity of Adozelesin alone and in combination with 5-aza-2′-deoxycytidine and cytosine arabinoside on DLD-1 human colon carcinoma cells

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Adozelesin (Ado), a CC-1065 analog, shows significant antineoplastic activity *in vivo* against several types of murine tumors and human tumor xenografts. Ado is a DNA alkylating agent. One objective of this study was to investigate the cytotoxic action of Ado against the human colon (HT-29, DLD-1) and the lung (SK) carcinoma cell lines. The concentrations of Ado that produced 50% cell kill for a 4 and 24 h exposure were in the range of 0.001–0.02 ng/ml for both colon and lung carcinoma cells, indicating that this analog was a very potent cytotoxic agent. Since most clinical regimens for tumor therapy consist of several drugs, we investigated the antineoplastic action of Ado in combination with 5-aza-2′-deoxycytidine (5-Aza-CdR), a potent inhibitor of DNA methylation or cytosine arabinoside (Ara-C), a potent inhibitor of DNA synthesis. The Ado plus 5-Aza-CdR combination showed a synergistic effect on cytotoxicity of DLD-1 colon carcinoma cells for both a 6 and 24 h exposure. However, combination of Ado and Ara-C for a 6 h exposure showed an antagonistic effect, whereas a 24 h exposure showed a synergistic effect. These preclinical results provide some preliminary data on possible drugs that can be selected for use in combination with Ado in future clinical trials in patients with cancer.

Key words: Adozelesin, 5-aza-2′-deoxycytidine, cytosine arabinoside, chemotherapy, colon carcinoma, lung carcinoma.

Introduction

CC-1065, a cyclopropylpyrroloindole, is an antitumor antibiotic produced by *Steptomyces zelensis* which was discovered and characterized in the mid-1970s in the research laboratories of the Upjohn Company.[1,2] *In vitro* studies showed that this antibiotic is one of the most potent cytotoxic antitumor agents known.[3] The development of CC-1065 as an anticancer drug was stopped when it was later found to cause irreversible organ toxicity and lethality in animals at therapeutic doses.[4] A large number of analogs of CC-1065 were synthesized with less host toxicity.[5,6] One of the analogs was Adozelesin (Ado).

Ado (Figure 1) is a potent alkylating agent which binds to double-stranded DNA at A–T-rich regions forming a covalent bond with the N-3 adenine.[7,8] Based on its high potency against several experimental murine tumors and human tumor xenografts,[9] Ado was chosen for investigation and is currently undergoing phase I clinical trials.[10,11] In this report we compared the *in vitro* cytotoxic activity of Ado against human colon and lung carcinoma cells since these types of tumors respond poorly to chemotherapy when metastatic.

Since preliminary clinical results indicated that after an initial response with Ado tumor regrowth eventually occurred,[9] the full chemotherapeutic potential of this agent will be realized only when used in combination with other antineoplastic drugs. In addition, drug combinations are used in cancer therapy to avoid the problem of drug resistance. In this study we have investigated the combination of Ado with the deoxycytidine analogs, 5-Aza-2′-deoxycytidine (5-Aza-CdR) and cytosine arabinoside (Ara-C). We have chosen these drugs since their mechanism of action is completely different from Ado.

5-Aza-CdR is a potent inhibitor of DNA methylation[12] which can lead to the activation of specific genes and induction of cellular differentiation.[13-15] Ara-C is a potent inhibitor of DNA synthesis[16] and blocks cell cycle progression through the S phase.[17]

Our results indicate that Ado is a very potent *in vitro* cytotoxic agent against human colon and lung carcinoma cells. Also, a synergistic interaction against the human colon carcinoma cells was observed when this agent was used in combination

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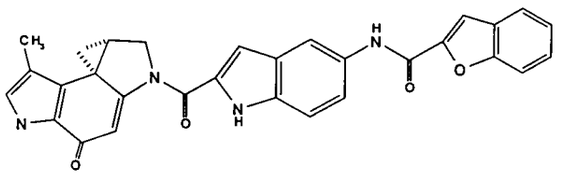

Adozelesin (U-73,975)

Figure 1. Chemical structure of Ado.

with 5-Aza-CdR for both short and long exposure times, but only at longer exposure time with Ara-C.

Materials and methods

Materials

The CPI analog, Ado, was provided by the Upjohn Company (Kalamazoo, MI) as a 1 mg/ml solution in *N*,*N*-dimethylacetamide (DMA) and stored at −20°C. Dilutions of Ado were made in glass bottles in 0.45% NaCl containing 10 mM potassium phosphate, pH 7.0. Dilutions were made immediately prior to use and were protected from light. 5-Aza-CdR was obtained from Mack Co. (Germany). Ara-C was obtained from the Upjohn Company of Canada. These two latter drugs were dissolved in 0.45% NaCl containing 10 mM sodium phosphate, pH 6.8, and stored at −70°C and −20°C, respectively. Minimal essential medium (MEM), non-essential amino acids and trypsin–EDTA were obtained from Gibco/BRL (Burlington, Ontario, Canada). Fetal calf serum (FCS) was obtained from Flow Laboratories (Mississauga, Ontario, Canada). [^{3}H-methyl]thymidine (20 Ci/mmol) was obtained from DuPont Canada (Mississauga, Ontario).

Cell culture

Human HT-29 and DLD-1 colon adenocarcinoma cells and SK-MES-1 lung squamous carcinoma cell lines were obtained from American Type Tissue Culture Collection (Rockville, MD). The tumor cell lines were maintained in culture in MEM containing non-essential amino acids and 10% heat-inactivated FCS in a 5% CO_2 incubator at 37°C. The doubling time for HT-29 and DLD-1 cell lines was between 26 and 30 h. The doubling time of SK-MES-1 lung cells was about 55 h. For subculture, the cells were placed in 0.05% trypsin–EDTA for 2 min at 37°C to obtain a suspension of cells.

Inhibition of cell growth assay

For the growth inhibition studies, 5 ml of DLD-1 tumor cells at 3000 cells/ml in MEM containing 10% heat-inactivated FCS were placed in a tissue culture flask. The flasks were gassed with 5% CO_2 and incubated for 48 h at 37°C in a 5% CO_2 incubator. Then, the indicated concentration of drugs (Ado/5-Aza-CdR) was added for 6 or 24 h. The cells were washed and incubated in MEM containing 10% heat-inactivated FCS for 5 days (120 h). Then, the medium was aspirated and 2 ml of 0.05% trypsin–EDTA were added for 6 min at 37°C to suspend cells. Then 0.5 ml cells were counted with a Model ZM Coulter Counter. The growth inhibition values are expressed as percentage relative to control.

Cytotoxicity assay

For cytotoxicity experiments, a 5 ml sample at 30 cells/ml (HT-29, DLD-1) and 50 cells/ml (SK-MES-1) were suspended in MEM containing 10% heat-inactivated FCS and placed in a tissue culture flask. The flasks were gassed with 5% CO_2 and incubated for 48 h at 37°C in a 5% CO_2 incubator. Then, the indicated concentration of drugs (Ado, 5-Aza-CdR, Ara-C) was added for 6 or 24 h and the cells were then washed with MEM containing 10% FCS. After an incubation at 37°C in 5% CO_2 for 14–21 days, the colonies (more than 500 cells) were rinsed, stained with 0.5% methylene blue/50% methanol and counted. The cell kill values are expressed as percentage of survival relative to control. The concentration of drug that kills 50% of the cells (LC_{50}) was determined from a dose–response curve. The cloning efficiency of the control cells was in the range 50–75%.

DNA synthesis measurement

The rate of DNA synthesis was measured by the incorporation of [^{3}H]-methyl-thymidine into DNA. Samples of 5 ml at 3000 cells/ml were suspended in MEM containing 10% heat-inactivated FCS were placed in a tissue culture flask. The flasks were

gassed with 5% CO_2 and incubated for 48 h at 37°C in a 5% CO_2 incubator. Then, the indicated concentration of drug (Ado) was added for 24 h. The cells were then washed and placed in 2 ml medium containing 5% dialyzed serum and 1 μCi of [^{3}H]thymidine (20 Ci/mmol). After incubation for 4 h at 37°C, the cells were washed with 2 ml PBS (Ca^{2+} and Mg^{2+} free) and suspended in 2 ml of 0.25% trypsin–1 mM EDTA at 37°C for 15 min. A 0.1 ml aliquot of cells was counted with a Model ZM Coulter Counter. Cold 10% trichloroacetic acid was added to the cell suspension in a centrifuge tube. The tubes were placed on ice for 10 min and centrifuged at 2000 r.p.m. for 8 min. This step was repeated twice. The pellet was solubilized in 0.1 N NaOH at 37°C overnight. The radioactivity was counted in Biodegradable Counting Scintillant (Amersham Canada, Oakville, Ontario).

Results

The cytotoxic effects of Ado against HT-29 colon, DLD-1 colon and SK lung carcinoma cell lines after 4 and 24 h exposure are shown in Tables 1 and 2. As evaluated by a colony assay, a concentration of 0.005 ng/ml Ado for a 4 h exposure produced 12.9, 23.9 and 58.9% cell kill against HT-29, DLD-1 and SK carcinoma cells, respectively (Table 1). The same concentration of Ado produced a much greater cell kill for 24 h exposure (Table 2). These results indicate that the sensitivity of the carcinoma cells for Ado was SK > DLD-1 > HT-29.

From the dose–response curve from the data of Tables 1 and 2 we estimated the concentration of Ado that produced 50% cell kill (LC_{50}) for these tumor cell lines (Table 3). The LC_{50} values for 4 h exposure were in the range of 0.005–0.019 ng/ml. The LC_{50} values for 24 h exposure were in the range of 0.001–0.006 ng/ml. These data show that Ado is a very potent *in vitro* cytotoxic agent to the colon and lung carcinoma cell lines.

The inhibition of growth in DLD-1 colon carcinoma cells produced by different concentrations of Ado for 6 and 24 h exposure is shown in Figure 2. At concentrations of 0.005, 0.01 and 0.02 ng/ml Ado inhibited the growth of DLD-1 by 29, 38 and 61%, respectively, for a 6 h exposure. At concentrations of 0.0025, 0.005 and 0.01 ng/ml Ado inhibited the growth of cells by 26, 44 and 79%, respectively, for a 24 h exposure. For the longer exposure time a higher concentration of Ado was required to produce a 50% growth inhibition as compared with the LC_{50} (Table 3).

The inhibition of DNA synthesis in DLD-1 colon carcinoma cells produced by different concentrations of Ado for 24 h exposure is shown in Table 4. At concentrations of 0.002, 0.005, 0.01, 0.02 and 0.04 ng/ml, Ado decreased the level of DNA synthesis by 19, 41, 48, 53 and 73%,

Table 1. Cytotoxic action of Ado for a 4 h exposure on HT-29 colon, DLD-1 colon and SK lung carcinoma cell lines

Ado concentration (ng/ml)	Cell kill (%)		
	HT-29 colon	DLD-1 colon	SK lung
0.001	2.7 ± 1.3[a]	7.0 ± 2.3[a]	8.7 ± 6.8[a]
0.002	11.0 ± 5.4	7.0 ± 4.0	28.8 ± 5.4
0.005	12.9 ± 3.2	23.9 ± 4.4	58.9 ± 6.5
0.01	24.6 ± 3.4	42.4 ± 6.4	87.6 ± 5.1
0.02	51.3 ± 9.1	75.4 ± 7.5	ND

ND, not determined.
[a]Mean ± SE (n = 3–7).

Table 2. Cytotoxic action of Ado for a 24 h exposure on HT-29 colon, DLD-1 colon and SK lung carcinoma cell lines

Ado concentration (ng/ml)	Cell kill (%)		
	HT-29 colon	DLD-1 colon	SK lung
0.0005	2.0 ± 1.2[a]	18.7 ± 1.8[a]	17.0 ± 4.9[a]
0.002	8.3 ± 4.8	50.8 ± 4.7	88.8 ± 5.7
0.005	43.5 ± 5.2	94.3 ± 2.7	98.5 ± 0.9
0.01	96.0 ± 1.5	ND	ND

ND, not determined.
[a]Mean ± SE (n = 3–7).

Table 3. Summary of cytotoxic action of Adozelesin on HT-29 colon, DLD-1 colon and SK lung carcinoma cell lines

Tumor cell lines	Exposure time (h)	Cytotoxicity (LC_{50}) (ng/ml)
HT-29 colon	4	0.019
	24	0.006
DLD-1 colon	4	0.013
	24	0.002
SK lung	4	0.005
	24	0.001

LC_{50}, concentration which produces 50% cell kill; from data of Tables 1 and 2.

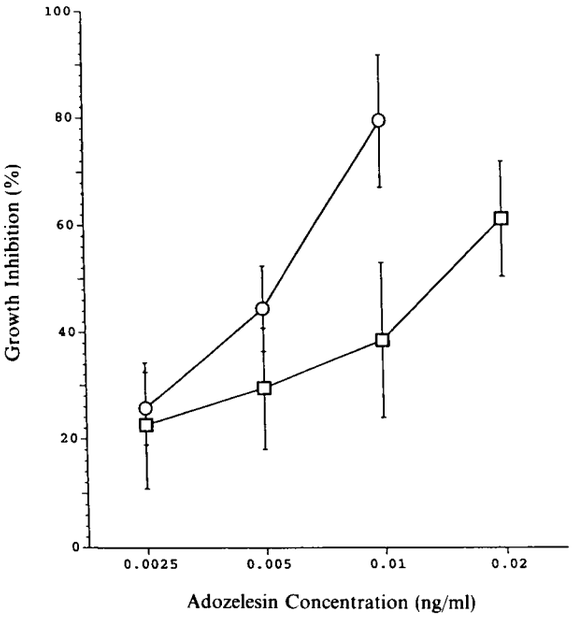

Figure 2. Effect of different concentrations of Ado for 6 h (□) and 24 h (○) exposure time on the growth of DLD-1 colon carcinoma cell line. Cells were incubated with indicated concentration of Ado for 6 h or 24 h and then placed in drug-free medium. Cell count was performed at 120 h as described in Materials and methods. Values are expressed as percentage of control. Means of three (24 h) to seven (6 h) experiments with average SE < 15%.

respectively. The concentration of Ado required to inhibit DNA synthesis by about 50% was greater than the LC_{50} (Table 3).

The second aspect of this study was to evaluate the cytotoxic action of Ado in combination with 5-Aza-CdR or Ara-C against DLD-1 colon carcinoma cells. Tables 5 and 6 show the cytotoxic action of Ado, 5-Aza-CdR and the combination of these agents for 6 h and 24 h, respectively. For a 6 h exposure, 5-Aza-CdR (1.0 μM) alone produced 15.6% cell kill, Ado alone at 0.005 ng/ml produced 17.0% cell kill whereas the combination of these agents produced 54.6% cell kill. At these concentrations of 5-Aza-CdR and Ado the interaction was synergistic as defined by Valeriote and Lin.[18] For a 24 h exposure, 5-Aza-CdR (0.5 μM) alone produced 18.7% cell kill and Ado alone at 0.005 ng/ml produced 39.0% cell kill (Table 6). The combination of these drugs produced 88.0% cell kill, a clearly synergistic effect.

In Tables 7 and 8 the cytotoxic action of Ado, Ara-C and the combination of these agents for a 6 h and 24 h treatment are presented. For a 6 h exposure, Ara-C (10 μM) alone produced 21.7% cell kill, Ado alone at 0.01 ng/ml produced 34.0% cell kill (Table 7). The combination of these drugs produced only 32.3% cell kill, an antagonistic interaction as defined by Valeriote and Lin.[18] However, for a 24 h exposure the interaction of these agents was synergistic (Table 8).

Table 4. Effect of Ado on DNA synthesis in DLD-1 colon carcinoma cells after a treatment of 24 h

Ado concentration (ng/ml)	DNA synthesis (c.p.m./10^5 cells)	Inhibition (%)
Control	14 295 ± 1429[a]	0
0.002	11 520 ± 1397	19
0.005	8460 ± 359	41
0.01	7366 ± 345	48
0.02	6784 ± 673	53
0.04	3821 ± 349	73

Cells were incubated for 24 h with the drug following by a 4 h incubation with [^{3}H-methyl]thymidine.
[a]Mean ± SE (n = 3).

Table 5. Cytotoxic action of Ado and/or 5-Aza-CdR for a 6 h exposure on DLD-1 colon carcinoma cell lines

5-Aza-CdR concentration (μM)	Ado concentration (ng/ml)	Cell kill (%)	Interaction[a]
1.0	0	15.6 ± 3.5[b]	
0	0.005	17.0 ± 3.7	
0	0.01	51.0 ± 5.1	
0	0.02	88.0 ± 2.4	
1.0	0.005	54.6 ± 2.5	synergistic
1.0	0.01	81.6 ± 4.9	synergistic
1.0	0.02	97.5 ± 1.5	synergistic

[a]Determined by the method of Valeriote and Lin.[18]
[b]Mean ± SE (n = 5).

Table 6. Cytotoxic action of Ado and/or 5-Aza-CdR for a 24 h exposure on DLD-1 colon carcinoma cell lines

5-Aza-CdR concentration (μM)	Ado concentration (ng/ml)	Cell kill (%)	Interaction[a]
0.5	0	18.7 ± 0.3[b]	
0	0.0025	19.0 ± 2.6	
0	0.005	39.0 ± 7.0	
0	0.01	91.0 ± 3.0	
0.5	0.0025	34.0 ± 4.6	additive
0.5	0.005	88.0 ± 3.8	synergistic
0.5	0.01	98.3 ± 1.6	synergistic

[a]Determined by the method of Valeriote and Lin.[18]
[b]Mean ± SE (n = 3).

Table 7. Cytotoxic action of Ado and/or Ara-C for a 6 h exposure on DLD-1 colon carcinoma cell lines

ARA-C concentration (μM)	Ado concentration (ng/ml)	Cell kill (%)	Interaction[a]
10	0	21.7 ± 4.4[b]	
0	0.005	20.0 ± 5.4	
0	0.01	34.0 ± 6.8	
0	0.02	74.5 ± 4.7	
10	0.005	24.7 ± 2.3	antagonistic
10	0.01	32.3 ± 7.1	antagonistic
10	0.02	63.8 ± 6.9	antagonistic

[a]Determined by the method of Valeriote and Lin.[18]
[b]Mean ± SE (n = 6).

Table 8. Cytotoxic action of Ado and/or Ara-C for a 24 h exposure on DLD-1 colon carcinoma cell lines

ARA-C concentration (μM)	Ado concentration (ng/ml)	Cell kill (%)	Interaction[a]
1.0	0	31.6 ± 2.2[b]	
0	0.001	10.4 ± 4.2	
0	0.0025	30.3 ± 8.3	
0	0.005	54.9 ± 8.7	
0	0.01	70.8 ± 8.9	
1.0	0.001	54.7 ± 4.5	synergistic
1.0	0.0025	65.9 ± 6.3	synergistic
1.0	0.005	75.8 ± 6.3	synergistic
1.0	0.01	90.4 ± 3.3	synergistic

[a]Determined by the method of Valeriote and Lin.[18]
[b]Mean ± SE (n = 9).

Discussion

Ado is an interesting experimental antineoplastic agent of the class CC-1065 which shows very potent antitumor activity both *in vitro*[5,19–21] and in mice.[9] In this study we have confirmed the very potent *in vitro* antineoplastic activity produced by Ado against human tumor cell lines. We have observed that Ado at concentrations of less than 0.01 ng/ml produced significant cytotoxicity against human DLD-1 and HT-29 colon adenocarcinoma cells and SK lung squamous carcinoma cells (Tables 1–3). These human tumor cell lines were chosen because the metastatic disease of these tumor types responds poorly to conventional chemotherapy. The longer exposure time (24 h) of Ado was more cytotoxic than the shorter exposure time (4 h), with LC_{50} values being about 3- to 6-fold lower for 24 h exposure as compared with the 4 h exposure (Table 3). The SK lung carcinoma cells were more sensitive to the cytotoxic effects of Ado than the colon adenocarcinoma cells. In comparison the *in vitro* cytotoxic potency of Ado is 100- to 10 000-fold greater than values reported for the antineoplastic agents from the class of anthracyclines[22] and also for the deoxycytidine analogs, 5-Aza-CdR and Ara-C (Tables 5–8). These data clearly indicate that Ado is a much more potent *in vitro* cytotoxic agent than most conventional and experimental antineoplastic agents. The current phase I and II clinical trials on Ado will provide data on its toxicity in patients with tumors, which is essential for the evaluation of its therapeutic index in comparison to conventional antineoplastic agents.

We also observed that Ado was a very potent inhibitor of the growth of DLD-1 colon carcinoma cells (Figure 2). The longer exposure time of Ado produced a greater growth inhibition than the shorter exposure time. It is interesting to note that the LC_{50} of Ado was about 3-fold greater than its LC_{50} of the colon carcinoma cells for the 24 h exposure. In addition, the concentration of Ado that produced a 50% inhibition of DNA synthesis (Table 4) was about 10-fold greater than its LC_{50} for a 24 h exposure. Similar results have been reported with different CC-1065 analogs by others workers.[21] These results suggest that the cytotoxic action of Ado is delayed with minimal growth inhibition and inhibition of DNA synthesis occurring during the initial hours of exposure.

Li *et al.*[9] have reported that Ado is very effective against human colon and lung carcinoma xenografts in mice, but tumor regrowth eventually occurs indicating that therapy in animal models with this agent is not curative. If Ado is approved for the clinical therapy of malignant disease, it will most likely be used in combination with other antineoplastic agents in an attempt to achieve curative therapy. Preliminary *in vitro* studies on human tumors may provide some data on which antineoplastic agents may interact favorably with Ado. Our laboratory has considerable experience of preclinical investigations with the deoxycytidine analogs, 5-Aza-CdR and Ara-C, both of which have a mechanism of action completely different from Ado. In this study we have investigated the antineoplastic activity of Ado on DLD-1 human colon carcinoma cells in combination with 5-Aza-CdR, an inhibitor of DNA methylation,[12] or Ara-C, an inhibitor of DNA replication.[16]

Smith *et al.*[23] reported that Ado in combination with 5-azacytidine, the ribose form of 5-azacytosine, produced a synergistic cytotoxic effect on tumor cells. We have observed similar results with

5-Aza-CdR, the deoxyribose form of 5-azacytosine. For both a 6 and 24 h exposure, the cytotoxic activity of Ado in combination with 5-Aza-CdR was additive to synergistic depending on the concentration of this CC-1065 analog (Tables 5 and 6). The method used for analysis of the interaction was that of Valeriote and Lin.[18] In this method, if the survival fraction (SF) of the drug combination is less than, equal to or greater than the product of the SF of each agent alone, the interactions are classified as synergistic, additive or antagonistic, respectively. For example, from the data in Table 5 for a 6 h exposure the SF of 1 μM 5-Aza-CdR alone was 0.844 (15.6% cell kill) and the SF of 0.005 ng/ml Ado alone was 0.83 (17.0% cell kill); in combination the SF was 0.454 (54.6% cell kill) which is significantly less than the calculated SF of 0.70 (SF of 5-Aza-CdR alone multiplied by SF of Ado alone).

The molecular mechanism responsible for the synergistic cytotoxic interaction of Ado in combination with 5-Aza-CdR on the DLD-1 colon carcinoma cells is unknown. One hypothesis to explain this interaction is that the alkylation of DNA by Ado may interfere with the methylation of DNA, which is in a hypomethylated state as the result of the action of 5-Aza-CdR. Repair enzymes probably have to remove Ado from DNA to permit DNA methylase to proceed along the DNA strand.

The type of interaction of Ara-C with Ado on the DLD-1 colon carcinoma cells depended markedly on the duration of exposure to these agents (Tables 7 and 8). For a 6 h exposure, the cytotoxic interaction of Ara-C with Ado was antagonistic since the SF of the combination was greater than the product of SF of each agent alone. However, for the 24 h exposure, the interaction between Ara-C and Ado was synergistic. How can one explain this dual action of Ara-C? There are several hypotheses that can be proposed. The inhibition of DNA synthesis produced by Ado (Table 4) will reduce the incorporation of Ara-C into DNA. Ara-C has to be incorporated into DNA to produce a chain termination effect which results in an inhibition of DNA synthesis.[16] In addition, the DNA synthesis inhibition produced by Ado may slow down the progression of cells from the G_1 to the S phase. Since Ara-C is an S phase specific agent,[17] this latter proposed mechanism of action of Ado on cell cycle progression could antagonize the cytotoxic action of Ara-C for the short exposure time. For the longer exposure (24 h), Ara-C will produce an accumulation of cells at the G_1–S phase.[17] Since CC-1065 analogs are most toxic to cells in late G_1 phase,[21] the Ara-C treatment should be more effective.

In conclusion, our results show that Ado is a very potent cytotoxic agent to human lung and colon carcinoma cells in tissue culture. Also, the interaction of Ado with the deoxycytidine analogs, 5-Aza-CdR and Ara-C, on the colon carcinoma cells was synergistic. However, for Ara-C the short exposure time was antagonistic. It will be interesting to test Ado in combination with 5-Aza-CdR or Ara-C in mice with tumors where drug metabolism and toxicity may influence the interaction of these agents. In addition, the mouse model will permit the verification of the *in vitro* antineoplastic activity and allow us to determine if these drug combinations merit phase I clinical test.

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