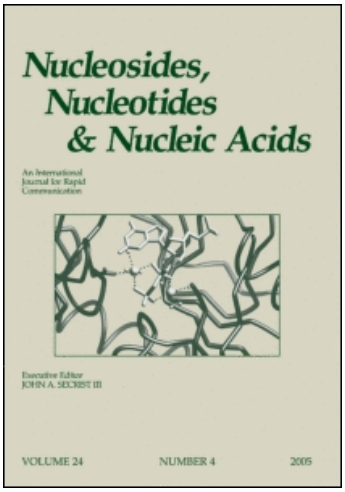

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Biologic Activity of a Nucleotide Conjugate Between Mitomycin C and Cytarabine Monophosphate

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BIOLOGIC ACTIVITY OF A NUCLEOTIDE CONJUGATE BETWEEN MITOMYCIN C AND CYTARABINE MONOPHOSPHATE

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ABSTRACT:

A dual prodrug conjugate between the antimetabolite cytarabine monophosphate and the alkylating agent 2,7-diaminomitosene (derived from mitomycin C), cytaramycin, was synthesized and tested for antileukemic activity in sensitive and resistant tumors. The compound was active against parental L-1210, CCRF-CEM, HL-60 and K-562 leukemia cells but did not overcome resistance in sublines developed for (1) multidrug resistance (L-1210/MDR and K-562-R) or (2) for cytarabine resistance (CCRF-CEM/ARA-C and HL-60/ARA-C). Alkaline DNA elution tests demonstrate a predominance of strand breaking activity due to the cytarabine moiety, and a lesser degree of DNA crosslinking, due to the mitosene moiety. The conjugate was active in mice bearing P-388 leukemia (80% increased lifespan), but was not more effective than mitomycin C alone in mice bearing a cytarabine-resistant L-1210 cell line (38% to 31% increased lifespan). These findings suggest that mitomycin nucleotide conjugates do not overcome resistance to the parent antimetabolites.

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INTRODUCTION:

Resistance to the antimetabolite cytarabine (ARA-C)+ is often associated with a loss of deoxycytidine kinase activity.[1,2,3,4] This defect blocks the initial phosphorylation of the nucleoside which is required for cytotoxic activity as the drug cannot be converted to the triphosphate form which inhibits DNA polymerization.[5] A deficiency in deoxycytidine kinase activity has also been observed in leukemic bone marrow cells harvested from patients with acute myelogenous leukemia refractory to ARA-C.[6] Another ARA-C resistant leukemic cell line was shown to be simultaneously deficient in deoxycytidine kinase activity and hypersensitive to alkylating agents.[7]

These findings suggested that resistance to ARA-C might be overcome by the conjugation of monophosphorylated cytarabine to an active DNA alkylating agent. The rationale for this design motif includes the observation that mitosene conjugates could be reduced to yield a nucleotide and a moiety with the capacity to alkylate DNA.[8] Several such conjugates with the alkylator mitomycin C have been synthesized and were previously shown to be active in non-resistant (parental) L-1210 murine leukemia cells in vitro.[8] One agent, a conjugate between 1-(β-D-arabinofuranosyl cytosine 5'monophosphate and 2,7-diaminomitosene (cytaramycin or CMC), was shown to be more potent than either ARA-C or mitomycin C in L-1210 cells (FIG. 1).[9] Another conjugate between mitomycin C and the non-cytotoxic moiety, 5'-uridylate monophosphate was shown to be taken up into L-1210 cells in vitro and inhibited leukemic cell growth at one-fifth the concentrations of mitomycin C. The uridylate conjugate was also active against P-388 leukemia in vivo.[8] These results suggested that mitomycin C-nucleotide conjugates were active and could possibly circumvent resistance to ARA-C in cells deficient in deoxycytidine kinase activity.

The purpose of the current study was to characterize the biologic activity of the ARA-C/MMC conjugate in leukemia cells resistant to ARA-C. In addition, the effects of the conjugate on the DNA of sensitive leukemic cells was also examined to determine whether active alkylating and/or strand breaking effects could be achieved with the dual prodrug conjugate.

+ Abbreviations: ARA-C, cytarabine; MMC, mitomycin C; CMC, cytaramycin; ILS, increased lifespan; MTT, microculture tetrazolium dye; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; SGOT, serum glutamate oxaloacetic transaminase; SGPT, serum glutamate pyruvate transaminase; LDH, lactic dehydrogenase; DMSO, dimethylsulfoxide; DPC, DNA-protein crosslinks.

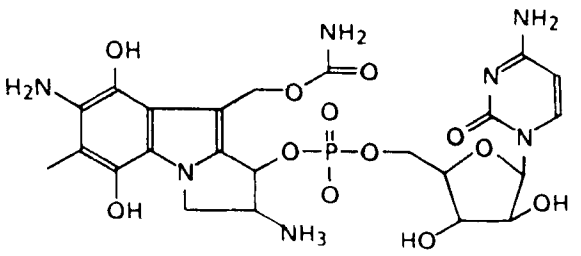

FIG. 1: Structure of the mitosene-nucleoside conjugate cytaramycin (cytosine-β-D-arabinofuranoside-5'-monophosphate).

METHODS:

Drugs and Reagents:

Cytosine-β-D-arabinofuranoside 5'monophosphate (ARA-C monophosphate) was obtained from Sigma Chemical, St. Louis and used without further purification. Mitomycin C (MMC) was obtained as the commercial formulation with mannitol (Mutamycin®) from Bristol Myers Oncology Division, Evansville, IN). It was purified by thin layer chromatography to > 99% prior to use in chemical syntheses. Cytarabine (ARA-C) was obtained as the commercial formulation (Cytosar®) from Upjohn Laboratories, Kalamazoo, MI.

Preparation of Cytaramycin (CMC) Conjugate:

The conjugate was prepared as described previously.[9] Briefly, this involved heating mitomycin C with 2 equivalents of ARA-C monophosphate in dry dimethylformamide at steam bath temperature for 60 minutes. This gave 2,7-diaminomitosene 1-β-D-arabinofuranosylcytosine 5'-monophosphate after chromatography as the neutral form in a 21% yield (FIG. 1). The material was purified by TLC and the structure was verified by proton NMR spectroscopy, mass spectrometry, and microanalysis. Specific NMR signals and analytic characteristics are described in a prior publication.[9]

Tumor Cell Growth Inhibition In Vitro:

Cell culture studies were performed using the microculture tetrazolium dye procedure[10] as modified for enhanced formazan solubilization in dimethylsulfoxide by Alley et al.[11] The colorless dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced to a formazan product by

mitochondrial reductase activity in viable cells. Tumor cells were plated at 50,000/well onto 96-well microfilter plates (Falcon Plastics, Oxnard, CA) and treated in vitro at several serial drug dilutions. These drug tests involved a continuous exposure method for 6-8 days at 37°C in an incubator delivering 95% air and 5% CO_2. The growth medium, RPMI 1640, was supplemented with 10% (v/v) heat-inactivated fetal bovine serum and containing 1% (v/v penicillin (60 μg/mL) and streptomycin (100 μg/mL) all from Gibco BRL. At the end of the incubation period (6 days for murine cells and 8 days for human cells), the formazan was solubilized with DMSO and viable cell numbers enumerated by automated spectrophotometry (BioMech, Becton Dickinson Company, Fullerton, CA) with light absorbance set at 540 nm. The percent growth inhibition was determined by comparison to untreated control wells (n = 6 wells per concentration, each exposure tested in 2 experiments).

Cell lines used in these experiments included murine L-1210 lymphocytic leukemia (CCL 219, American Type Culture Collection) and a multiple drug resistant subline, L-1210/MDR, developed by serial exposure to escalating concentrations of MMC.[12] The CCRF-CEM human lymphoblasts were obtained as a generous gift from Dr. Arnold Fridland, St. Judes Childrens Research Hospital, Memphis, TN. The ARA-C resistant sub-line of CCRF-CEM cells was originally selected for ARA-C resistance and is known to have only 10% activity of deoxycytidine kinase compared to the parental line.[13] The human promyelocytic leukemia cell lines HL-60 and HL-60/ARA-C were originally described by Bhalla et al.[14] and were tested in the laboratory of Dr. Alan List. The HL-60/ARA-C cells are over 1,000-fold resistant to ARA-C in colony-forming assays and have only 1.2% of the deoxycytidine kinase activity found in the parental cells.[14] The human K-562 myelomonocytic leukemia cells and a multiple drug resistant subline, K562-R, were tested by Dr. Alan List. The K562-R cells were developed by serial exposure to escalating concentrations of daunomycin.[15] Like the L-1210/MDR cells, K562-R cells are positive for membrane P-glycoprotein and have an enhanced capability to efflux natural product based anticancer agents.[15]

Antitumor Studies in Mice:

The murine P-388 lymphocytic leukemic cells were obtained from American Type Culture Collection (Rockville, MD). The ARA-C resistant subline of the murine L-1210 lymphocytic leukemia cell line was generously donated by Dr. Stephen Schmid, Southern Research Institute, Birmingham, AL. This stable

cell line is known to possess significantly[15] reduced levels of deoxycytidine kinase and is markedly resistant to ARA-C in vivo.[16] With each cell line, mice were innoculated with 10^6 cells intraperitoneally (IP) from logarithmic-growing cultures. Drug treatments were initiated IP one day later for 1-5 daily doses. Survival was assessed twice daily. The single mitomycin C dose of 3.2 mg/kg[17] and the ARA-C dose range, 15-30 mg/kg/day for 5 consecutive days, were based on prior studies in leukemic mice.[18]

Alkaline DNA Elution Assays:

The methodology for these studies followed the detailed protocol of Kohn.[19] Briefly, logarithmically growing murine L-1210 leukemia cells are labeled in vitro using [2-^{14}C]thymidine for 36 hours at a concentration of 0.1 μCi/mL (55 mCi/mmol, Research Products International Corporation, Mount Prospect, IL). This labels high molecular weight DNA without effecting cell viability or the doubling time of approximately 12.4 hours. Cytaramycin was added to such labeled cells for 1 hour then removed by serial washing in ice cold phosphate buffered saline. The cells, loaded in an ice-water bath were then exposed to ionizing x-rays and immediately lysed on 20 um polyvinyl chloride filters (Millipore, Bedford, MA) at 4°C with an alkaline detergent solution to remove cell structures other than large single-stranded DNA molecules. The DNA is then pumped through the filters using an alkaline solution at pH 12.1 to detect DNA-DNA crosslinks identified as DNA retention on filter following radiation and elution with a proteinase K alkaline solution. Conversely, DNA single strand breaks are identified as enhanced DNA elution from either non-radiated cells or from radiated cells. For assays assessing DNA-protein crosslinks, cells receive higher x-ray exposures (30 cGy) and proteinase K is omitted to allow drug-induced DNA-protein adducts to be retained on the filter.

RESULTS:

Cytotoxic Activity In Vitro:

Cytaramycin was more potent than both ARA-C and MMC in parental murine L-1210 leukemia cells in vitro (TABLE 1). It was also significantly more potent than MMC in human CCRF-CEM lymphoblastoid leukemia cells and in parental human K-562 leukemia cells. However, the conjugate was significantly less active than either MMC or ARA-C in human HL-60 promyelocytic leukemia cells. In cytarabine-resistant leukemia cells, it demonstrated a substantial degree of cross-resistance in each cell line. This ranged from an 8-fold level of

TABLE 1: Comparison of Growth Inhibitory Effects in Leukemia Cells Sensitive and Resistant to Cytarabine In Vitro

Leukemia Cell Line	Resistance Phenotype[2]	Mean Inhibitory Concentration (μM) in 50% of Cells[1] (SD) ARA-C	MMC	CMC
L-1210	P	.036 (.011)	.09 (.017)	.011 (.007)
L-1210/MDR	R	.036 (.009)	1.5 (.051)	.115 (.021)
	Fold R[3]	1.0	16.7	10.2
CCRF-CEM	P	.052 (.012)	1.29 (0.24)	0.14 (0.03)
CCRF-CEM/ARA-C	R	> 15.65	1.17 (0.31)	> 16.92
	Fold R	> 300	0.91	> 120
HL-60	P	.005 (.003)	0.14 (0.04)	1.0 (0.31)
HL-60/ARA-C	R	> 1.0	0.15 (0.03)	> 100
	Fold R	> 200	1.07	> 100
K-562	P	.0032 (.002)	1.0 (0.13)	.25 (0.03)
K562-R	R	0.1 (.021)	1.9 (0.22)	2.0 (0.19)
	Fold R	31.25	1.9	8.0

[1]MTT dye assay, continuous drug exposure for 6-8 days.[11]

[2]P - Parental (sensitive) cell line; R = drug resistant cell line.

[3]Fold resistance = IC_{50} (R)/IC_{50} (P).

resistance in K562-R cells to a 154-fold level of resistance in L-1210/MDR cells. This degree of cross resistance was significantly less than that to cytarabine (31- to 434-fold resistance) in these same cell lines. In contrast, MMC was not significantly cross-resistant in any of the ARA-C resistant leukemia cell lines. This shows that while MMC is not cross-resistant with cytarabine, the conjugate, CMC, demonstrates a partial but consistent loss of activity in ARA-C-resistant cell lines in vitro.

TABLE 2: Comparison of Antitumor Activity (Survival) for Leukemias Sensitive and Resistant to Cytarabine in DBA/2J Mice (n = 10/group)

Treatment (IP)			P-388 Sensitive		L-1210/ARA-C	
Drug	Dose (mg/kg)	Days	Median (Days)[3]	% ILS[1]	Median (Days)[3]	% ILS
Saline	0.1 mL/10 g	1-5	10	—	8	—
ARA-C	15	1-5	14.5	45%	8	0
	30	1-5	17.5	75%	8	0
MMC	3.2	1	13.5	35%	11	38%
CMC	7.5	1-5	13	30%	10.5	31%
	15	1-5	18	80%	8	0
	15	1-5 (twice daily)	ND[2]	ND	7	-13%
	20	1-6	ND	ND	10	25%
	25	1-5	ND	ND	8	0

[1]Percent increased lifespan = median survival days: $\left[\left(\frac{\text{Treated} - \text{Control}}{\text{Control}}\right) \times 100\right]$

[2]ND = Not Done due to limited drug supply.

[3]Days of survival after tumor implantation; range of survival varied 11-23% of median value.

Antileukemic Activity In Vivo:

TABLE 2 shows the survival effects of each agent tested in mice bearing P-388 leukemia. Cytarabine was particularly active in parental P-388 cells whereas MMC demonstrated significantly less activity. The conjugate CMC at a dose of 15 mg/kg, produced survival effects comparable to a cytarabine dose of 30 mg/kg. In the ARA-C resistant L-1210 cells, marginal MMC antitumor activity was maintained whereas cytarabine was inactive at all dose levels. The conjugate possessed little activity in the resistant cells with a maximal survival advantage of only 25% after a dose of 20 mg/kg for six consecutive days (TABLE 2).

Toxicity in Normal Mice:

A single 30 mg/kg dose of cytaramycin produced a significant degree of granulocytopenia and thrombocytopenia measured on day 4 after dosing (FIG. 2). The degree of myelosuppression with CMC was greater than that

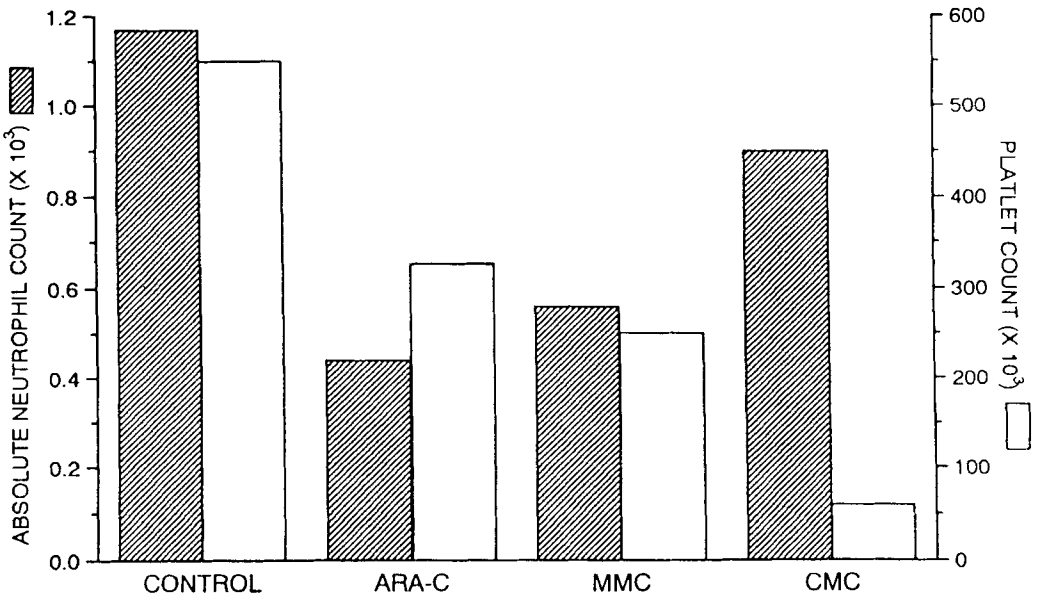

FIG. 2: Hematopoietic toxicity of cytarabine (ARA-C), mitomycin C (MMC) and cytaramycin (CMC) in CD-1 adult male mice. Drugs were administered intraperitoneally at a single dose of 100 mg/kg (ARA-C), 3 mg/kg (MMC) or 30 mg/kg (CMC). Blood was withdrawn four days later. Each bar is the mean of 2-3 mice.

produced by either single agent alone. As shown in FIG. 2, thrombocytopenia appears to be the major toxicity of this agent. There were no other abnormal toxicologic findings seen with CMC (data not shown). This includes the hepatic function tests, SGOT, SGPT, LDH and alkaline phosphatase, serum creatinine and BUN, serum glucose, total serum proteins and glucose. None of the agents affected erythrocytic (RBC) parameters.

Alkaline DNA Elution Results:

A one hour exposure to CMC resulted in the production of DNA-DNA interstrand crosslinks in L-1210 (parental) cells (FIG. 3). Surprisingly, the degree of DNA-DNA crosslink production was inversely proportional to the exposure concentration. This was true for all CMC exposures except the lowest concentration of 1.0 μg/mL. In other words, higher CMC concentrations produced lower frequencies of DNA-DNA crosslinks. This is evidenced by a more rapid elution of radiolabeled DNA from the irradiated cells following exposure to higher CMC concentrations. Overall, these findings suggest that the

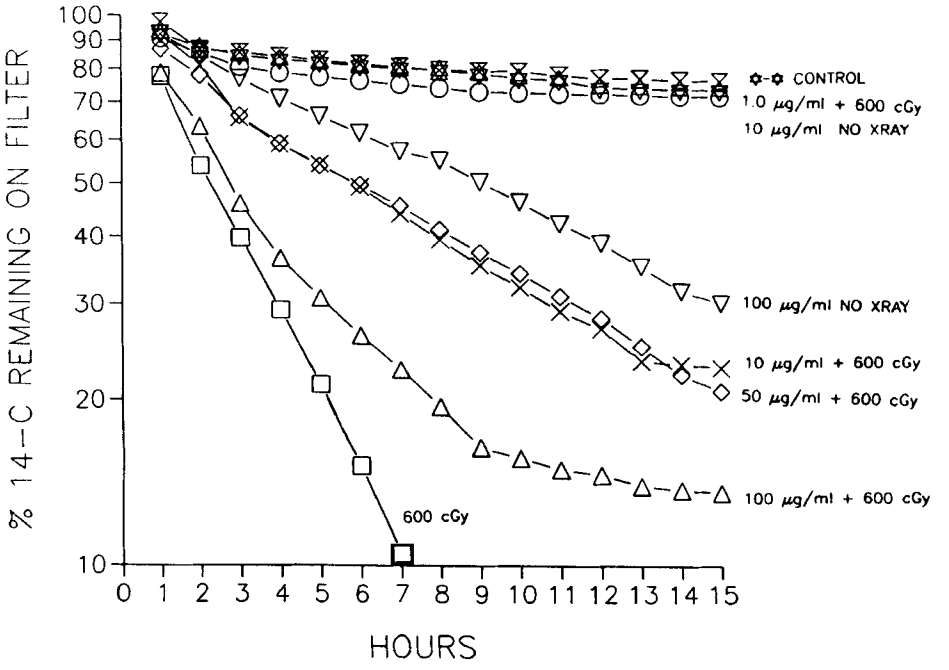

FIG. 3: An inverse correlation is shown between (1 hour) cytaramycin concentrations and the formation of DNA-DNA interstrand crosslinks in murine L-1210 leukemia cells. Increased retention of DNA over the 15 hour elution signifies DNA-DNA crosslinks.

predominant effect of CMC on DNA is to stabilize x-ray-induced strand breaks. Of note, DNA strand breakage is described following ARA-C,[20] but not with MMC[21]. Mitomycin C induces DNA-DNA crosslinks as the major nuclear effect.[21] However, since there is increased DNA retention seen with all CMC concentrations in the radiated cells, some degree of crosslinking consistently occurred following CMC exposure and this became more pronounced at low drug levels. Stated differently, the results in FIG. 3 show that CMC produces two effects simultaneously: (1) a predominant and dose-dependent retention of x-ray induced strand breaks, and (2) a lesser frequency of DNA-DNA crosslinks.

Further alkaline elution experiments were performed with CMC to detect the presence of an alternate form of DNA adducts, DNA-protein crosslinks. In these assays, cells receive a large dose of x-rays (30 Gy) following a 1-hour drug exposure. The x-rays produce extensive DNA strand breaks leaving very small fragments of DNA which are retained on the filter only if the DNA fragment is bound covalently to protein. In this analysis, CMC was shown to induce the

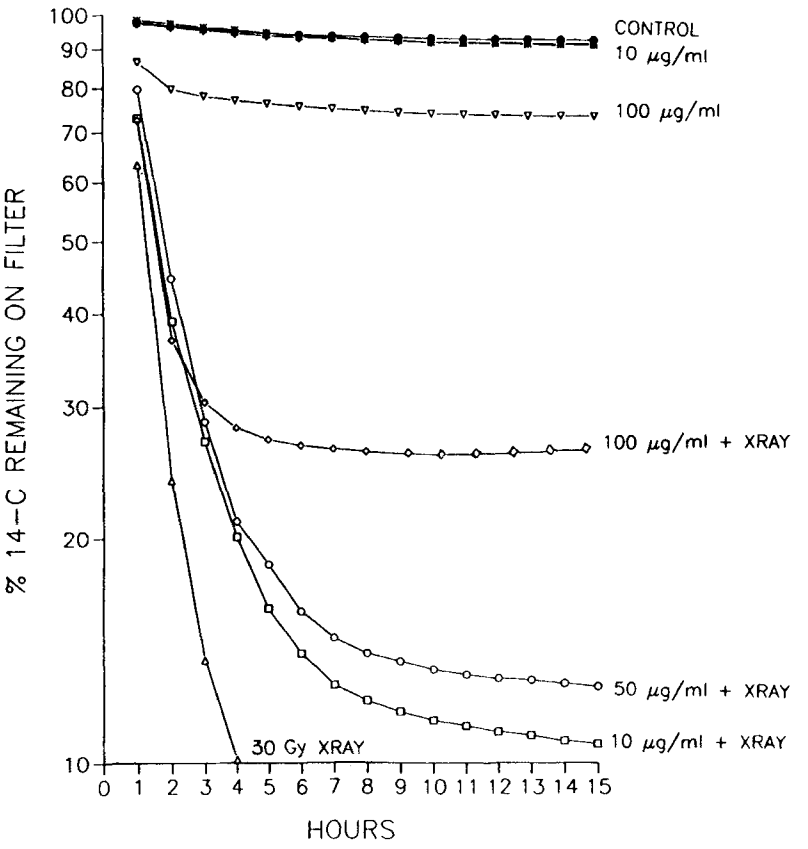

FIG. 4: Concentration-dependent formation of DNA-protein crosslinks is depicted in L-1210 cells following a 1 hour treatment with cytaramycin. This is noted by an increase in the retention of DNA at latter time points.

classic biphasic DNA retention pattern with a late plateau phase characteristic of DPC's formation. These DPC's occurred in a concentration-dependent fashion (FIG. 4). Conversely, a 100 μg/mL exposure to CMC without subsequent exposure to x-rays did show evidence of strand breakage. This reinforces the findings of predominant DNA breakage with CMC over DNA-DNA crosslinking.

Elution studies performed in HL-60/ARA-C cells detected no DNA strand breakage with CMC in the absence of x-rays, and no DNA crosslinks in cells irradiated after exposure to CMC concentrations up to 50 μg/mL (data not shown). This suggests that no active fragments (mitosene or nucleotide) are available intracellularly following CMC exposure in this deoxycytidine kinase-deficient, ARA-C resistant cell line.

DISCUSSION:

The results with this nucleotide conjugate between MMC and ARA-C demonstrate that while the agent is active in vitro and in vivo, it does not overcome tumor cell resistance to ARA-C in vitro. This includes 1) resistance mediated by overexpression of the membrane transport molecule, P-glycoprotein, which is associated with the multidrug resistance phenotype (L-1210/MDR and K562/MDR leukemia cells) and 2) resistance mediated by a decrease in deoxycytidine kinase (CCRF-CEM lymphoblasts and HL-60 promyelocytic leukemia cells). The CMC conjugate also lacked activity in an ARA-C resistant L-1210 leukemia model in vivo wherein the slight (31%) enhancement in the survival was probably due entirely to the MMC component. This is based on the observation that MMC alone, at a maximally tolerated dose of 3.2 mg/kg, produced a similar (38%) increase in survival, whereas ARA-C alone was inactive at all doses tested (TABLE 2). Since the two CMC component drugs (ARA-C monophosphate and MMC) have similar molecular weights, the 7.5 mg/kg dose of CMC would yield approximately 3.6 mg/kg of MMC equivalents. Furthermore, this assumption does not necessarily require a mechanism involving intracellular uptake of the conjugate since any mitosene liberated extracellularly would be available for cell uptake and for subsequent production of cytotoxic effects. However, prior liquid chromatography studies documented cellular uptake of a 5'-uridylate monophosphate conjugate to MMC in L-1210 cells in vitro.[8] The activity of this uridylate conjugate was significantly inferior to the antileukemic effects achieved by the CMC conjugate in the current studies. This suggests that the substitution of cytarabine monophosphate for the inactive uridylate monophosphate results in enhanced antileukemic activity in parental leukemia cells.

Unfortunately, the CMC conjugate was consistently inactive in resistant leukemia cells. These findings reinforce the conclusions of Bennett et al regarding the use of nucleotide derivatives to overcome resistance to purine and pyrimidine-based antitumor agents.[22] Numerous prior studies with pyrimidine nucleotide derivatives used to overcome activation-based resistance to antimetabolites have been similarly disappointing.[23] These attempts include oxazaphosphorinan derivatives of 5-fluorodeoxyuridylates,[24] n-butyl esters of cytarabine monophosphate,[25] palmitoyl derivatives of ARA-C monophosphate[16] and a diacylglycerol ester of ARA-C monophosphate.[22] Similar problems have been seen with nucleotide derivatives based on mercaptopurine.[26,27,28]

The reason for the inactivity of the nucleotide conjugates in resistant cells is probably multifactorial. It is quite possible that resistant cells simultaneously

express diverse types of resistance mechanisms including down-regulation of drug activation enzymes such as deoxycytidine kinase[1,2,3] and enhanced drug efflux activity due to up regulation of P-glycoprotein expression. In L-1210 cells, the alkaline elution experiments showed that some cytarabine and/or cytarabine monophosphate was delivered intracellularly following CMC exposure. This is based on the predominant strand breaking activity seen in these non-resistant cells. In contrast, fewer crosslinks were evident in these experiments and mitomycin C is known to produce only DNA-DNA and DNA-protein crosslinks in this system.[21] Similarly, in the HL-60/ARA-C cells there was no evidence of alkylating activity nor effects from the nucleotide component of CMC. This suggests that these deoxycytidine kinase resistant human leukemia cells 1) do not take up CMC due to membrane changes; 2) that CMC is taken up but not broken down intracellularly; or 3) that the conjugate is not broken down extracellularly to yield active mitosene or nucleotide fragments. Since all of the culture mediums were similar, explanation 3 is unlikely, and therefore a lack of uptake and/or active extrusion of active fragments are the most likely explanations for CMC resistance in this cell line. Active extrusion may be considered since multidrug resistance involving increased P-glycoprotein expression has been demonstrated with ARA-C exposure in HL-60 cells, even though ARA-C is not a classic substrate for the transmembrane efflux pump.[29] Thus, it is likely that the resistance of the HL-60/ARA-C cells to CMC may be partially explained by efflux of parent drug and/or active fragments.

In summary, the current studies suggest that dual prodrug nucleotide conjugates containing mitosenes are active in sensitive cells, but do not overcome cytarabine resistance either in vitro, or in vivo. Thus, other strategies for overcoming tumor cell resistance to nucleoside-based antimetabolites are needed.

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