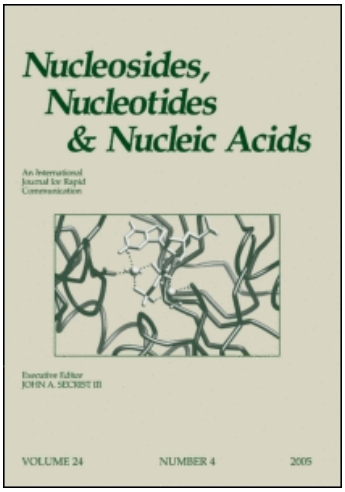

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Study of Apoptosis Induction and Deoxycytidine Kinase/Cytidine Deaminase Modulation in the Synergistic Interaction of a Novel Ceramide Analog and Gemcitabine in Pancreatic Cancer Cells

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STUDY OF APOPTOSIS INDUCTION AND DEOXYCYTIDINE KINASE/CYTIDINE DEAMINASE MODULATION IN THE SYNERGISTIC INTERACTION OF A NOVEL CERAMIDE ANALOG AND GEMCITABINE IN PANCREATIC CANCER CELLS

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□ *This study investigated the interaction between the novel ceramide analog AL6 and gemcitabine in MIA PaCa-2 and PANC-1 pancreatic cancer cell lines, harboring different polymorphic variants of the gemcitabine catabolism enzyme cytidine deaminase (CDA). AL6 dose-dependently inhibited cell growth, induced apoptosis and synergistically enhanced the cytotoxic activity of gemcitabine. Moreover, it triggered apoptosis, which was significantly enhanced by the combination, and increased the ratio between gene expression of the activating enzyme deoxycytidine kinase (dCK) and CDA, potentially favoring gemcitabine activity. In conclusion, AL6 displays synergistic cytotoxic activity, enhances apoptosis, and favorably modulates enzymes involved in gemcitabine metabolism, supporting future investigation of this combination in pancreatic cancer.*

Keywords Ceramide analogs; pancreas cancer; gemcitabine; cytidine deaminase

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-death (http://www-dep.iarc.fr/), and has the worst prognosis of any major malignancy. Because of the anatomic localization of the pancreas and the delay of clinical symptoms, in 85% of patients PDAC is detected at advanced stages, characterized by infiltration of proximal lymph nodes and vascular structures, as well as metastasis to liver or peritoneum.[1]

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The deoxynucleoside analogue gemcitabine (2′,2′-difluorodeoxy cytidine, dFdC) inhibited the in vitro and in vivo growth of human pancreatic cells resistant to several other anticancer drugs, such as platinum compounds or 5-fluorouracil,[2,3] and is now the standard therapy for advanced PDAC.[4] However, gemcitabine still yields a very limited disease control, with less than 15% of patients progression-free at 6 months from diagnosis.[5] Therefore, pancreatic cancer represents a clinical challenge and novel therapeutic approaches are warranted.

Several studies demonstrated that PDAC is characterized by dysregulation of molecular mechanisms involved in cell proliferation and apoptosis.[6] Recent studies showed that sphingomyelin enhanced chemosensitivity of the pancreatic cancer cell lines AsPc1 and PANC-1 through reducing the induction of senescence by redirecting the cell to enter the apoptotic pathway.[7] These results suggest that gemcitabine induces senescence in pancreatic cancer cells and that ceramide levels seem to be critical to this decision, with cell cycle progression being uninhibited at low ceramide levels, senescence induced at moderate levels, and apoptosis initiated at high levels.

However, several other mechanisms can be involved in PDAC cells resistance to gemcitabine, and our previous studies focussed on nucleoside transporters, such as the human equilibrative nucleoside transporter-1, whose mRNA expression correlated with clinical outcome in PDAC patients treated with gemcitabine.[8] Cellular enzymes involved in gemcitabine metabolism can also play a pivotal role in gemcitabine sensitivity/resistance. In particular, gemcitabine requires intracellular phosphorylation to its active metabolites, 2′,2′-difluoro-2′-deoxycytidine diphosphate (dFdCDP) and triphosphate (dFdCTP), which, respectively, inhibits ribonucleotide reductase and is incorporated into the DNA, leading to chain termination.[9] The rate-limiting step of drug activation is catalysed by deoxycytidine kinase (dCK), which phosphorylates gemcitabine, while cytidine deaminase (CDA) inactivates gemcitabine by deamination.[9]

In an effort to reconciliate the data on the role of cellular enzymes as determimants of gemcitabine sensitivity with the hypothesis of a sphingolipid-dependent mechanism underlying PDAC resistance to gemcitabine, the present study was undertaken to evaluate the effects of a new ceramide analog on proliferation, apoptosis induction and modulation of gemcitabine metabolism enzymes in 2 PDAC cell lines.

MATERIALS AND METHODS

Drugs and Chemicals

AL6 was prepared in the group of Prof. M. Macchia (University of Pisa, Italy, compound#1) as previously reported,[10] while gemcitabine was from Eli-Lilly (Indianapolis, IN, USA). AL6 and gemcitabine were dissolved in

DMSO and sterile distilled water and diluted in culture medium immediately before use. DMEM medium, fetal bovine serum (FBS), horse serum (HS), L-glutamine (2 mM), penicillin (50 IU/mL) and streptomycin (50 μg/mL) were from Gibco (Gaithersburg, MD, USA).

Cell Cultures

The MIA PaCa-2, and PANC-1 cell lines (ATCC, Manassas, VA, USA), were grown in DMEM with 10% FBS and 2.5% HS (MIA PaCa-2), and DMEM with 10% FBS (PANC-1) glutamine and penicillin-streptomycin. Cells were cultivated in 75 cm^2 flasks (Costar, Cambridge, MA, USA), at 37°C in 5% CO_2 and 95% air, and harvested with trypsin-EDTA when they were in logarithmic growth.

Characterization of PDAC Cell Lines for Genetic Determinants of Gemcitabine

The PDAC lines were characterized for the common non synonymous polymorphism of lysine 27 glutamine (Lys27Gln), resulting from a $A \rightarrow C$ substitution in exon 79 of the *CDA* gene, using the allelic discrimination assay with specific Taqman probes, as described previously.[11] The gene expression of *CDA* (NM_001785), and *dCK* (NM_000788) was determined with Taqman probes-based assays, as described previously.[8]

Cytotoxicity Studies

The cell growth inhibitory effect of gemcitabine and AL6 was studied using the MTT assay as described previously.[8] For this purpose, cells were plated at 10^4 cells/well in 96-well plates (Costar, Corning, NY). After 24 hours cells were treated with AL6 (0.01–100 μM) or gemcitabine (0.01–100 nM) for 72 hours. At the end of the incubation, cells were incubated for 3 hours at 37°C in 50 μl MTT and growth inhibition was expressed as the percentage of control (vehicle-treated cells) absorbance, corrected for absorbance before drug addition. The 50% inhibitory concentration of cell growth (IC_{50}) was calculated by non-linear least square curve fitting (GraphPad PRISM, Intuitive Software for Science, San Diego, CA, USA).

Drug interaction between gemcitabine and AL6 was assessed, at a concentration ratio of 1:1000, using the combination index (CI, Ref. 9), where $CI<1$, $CI = 1$, and $CI>1$ indicates synergistic, additive and antagonistic effects, respectively. Data analysis was performed by the Calcusyn software (Biosoft, Oxford, UK).

Analysis of Apoptosis

Cells were treated with gemcitabine and AL6 and their combinations at their IC_{50} levels; and, at the end of the incubation, washed twice with PBS and fixed in 4% buffered paraformaldehyde for 15 minutes. Cells were

resuspended and incubated for further 15 minutes in a solution containing 8 μg/ml bisbenzimide HCl. Then, cells were spotted on glass slides and examined by fluorescence microscopy (Leica, Germany). A total of 200 cells from randomly chosen microscopic fields were counted and the percentage of cells displaying chromatin condensation and nuclear fragmentation relative to the total number of counted cells (apoptotic index) was calculated.

PCR Analysis

Total RNA was extracted from cells treated at IC_{50} values using the TRIREAGENT-LS. RNA was dissolved in RNAse-free water and measured at 260 nm. One μg of RNA was reverse transcribed at 37°C for 1 hour and the resulting cDNA was amplified by quantitative PCR with the Applied Biosystems 7900HT sequence detection system (Applied Biosystems, Foster City, CA) as previously described.[8]

Statistics

All experiments were performed in triplicate and repeated at least three times. Data were expressed as mean values ±SE and were analysed by Student's *t* test or ANOVA followed by the Tukey's multiple comparison, setting the level of significance at $P < 0.05$.

RESULTS

Characterization of the Cell Lines

The evaluation of *CDA A79C* polymorphisms revealed that MIA PaCa-2 cells are homozygotes for the *C* allele, whereas PANC-1 have the *AA* genotype, but mRNA expression of *CDA* was comparable in the two cell lines. In contrast, PANC-1 showed a lower *dCK* expression than MIA PaCa-2 cells (Table 1).

In Vitro Cytotoxicity

AL6 caused a dose-dependent inhibition of proliferation in both PDAC cell lines, with MIA PaCa-2 being 4 times more sensitive than PANC-1. Similarly, MIA PaCa-2 were 3 times more sensitive to gemcitabine than PANC-1. The calculation of the CI for the gemcitabine-AL6 simultaneous association showed synergism at fractions affected (FA) exceeding 50% inhibition in both cell lines, as shown in Figure 1A.

Apoptosis Induction

Cells exposed to gemcitabine presented typical apoptotic morphology with cell shrinkage, nuclear condensation and fragmentation, and rupture

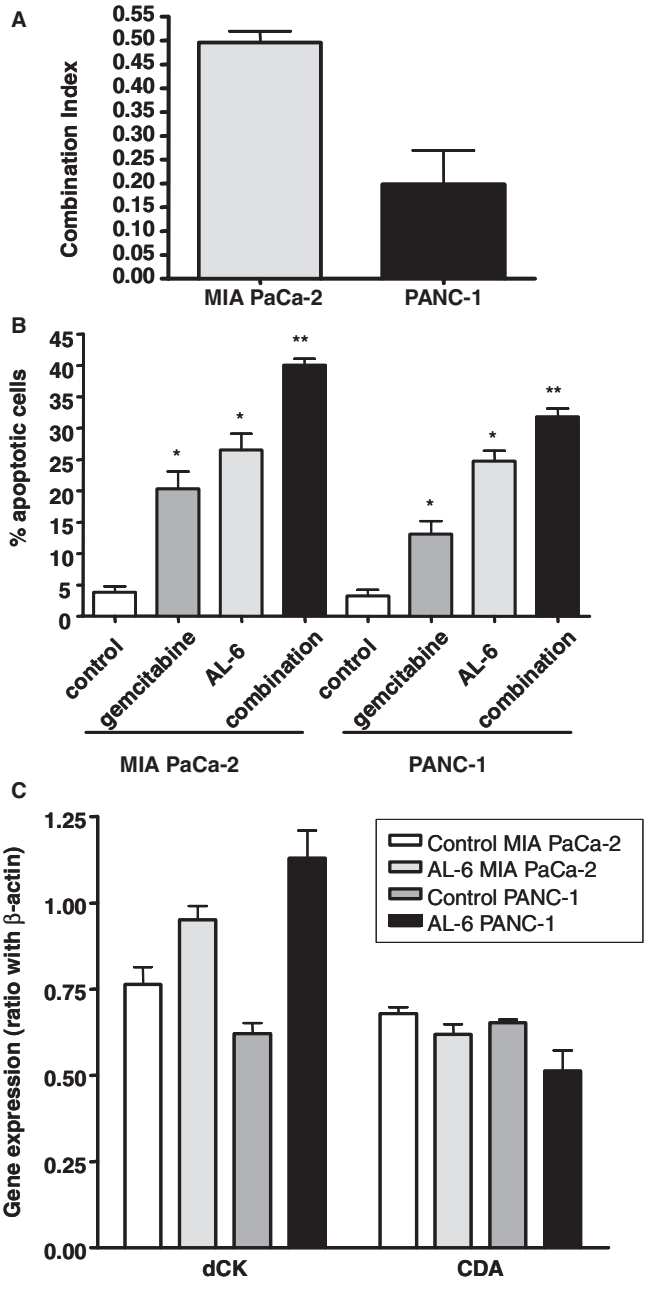

FIGURE 1 A) Mean combination index (CI) values of simultaneous AL6-gemcitabine 72-h combination in the PDAC cells. CI values at FA of 0.5, 0.75 and 0.9 were averaged for each experiment, and this value was used to calculate the mean between experiments, as described in the Matherials and Methods section. B) Induction of apoptosis by gemcitabine, AL6 and their simultaneous combination. Cancer cells were exposed to the IC_{50} value of drugs alone and in combination, at fixed 1:100 (gemcitabine:AL6) concentration ratio. C) Modulation of dCK and CDA mRNA expression as determined by PCR in PDAC cells treated for 72 hours with AL6. *Columns*, mean values obtained from three independent experiments; *bars*, SD. *Significantly different from controls ($P < 0.05$). **Significantly different from cells treated with gemcitabine ($P < 0.05$).

TABLE 1 Characterization of *CDA* gene status, and CDA and dCK mRNA gene expression, and gemcitabine and AL6 cytotoxicity in MIA PaCa-2 and PANC-1 cells

	MIA PaCa-2	PANC-1
dCK		
gene expression[a]	0.76 ± 0.05	0.62 ± 0.02
CDA		
A79C polymorphism	*CC*	*AA*
gene expression[a]	0.68 ± 0.03	0.65 ± 0.01
IC_{50}[§]		
Gemcitabine (nM)	5.9 ± 0.8	17.9 ± 1.8
AL6 (μM)	3.2 ± 0.4	12.1 ± 2.1

[a]Mean values calculated in comparison with respect to the expression values of the housekeeping gene β-actin.

[§]IC_{50} values have been calculated as described in the Materials and Methods.

of cells into debris. Gemcitabine induced apoptotic cell death in MIA PaCa-2 and PANC-1 up to 20.3 and 13.1%, respectively. Fluorescence microscopy also revealed markedly higher levels of apoptosis in the AL6-treated cells when compared with controls, with apoptotic index of 40.0 and 31.8% in MIA PaCa-2 and PANC-1 cells respectively. Furthermore, the combination of the two drugs significantly increased the apoptotic index compared with gemcitabine-treated cells (Figure 1B).

Modulation of Gene Expression

AL6 significantly enhanced *dCK* gene expression in both cell lines, while *CDA* levels were slightly reduced (Fig. 1C); as a consequence, a 1.5 and 2.2-fold increase in the ratio of dCK/CDA expression was observed in MIA PaCa-2, and PANC-1 cells, respectively ($P < 0.05$).

DISCUSSION

Standard treatments have modest impact against pancreatic cancer and new agents targeting molecular pathways involved apoptosis and enhancing gemcitabine activity are warranted. Among promising anticancer drugs, ceramide analogs have been investigated as apoptotic inducers and proliferation inhibitors of tumor cell lines. Ceramide, a lipidic second messenger involved in the sphingomyelin cycle, plays a crucial role in the processes of induction of apoptosis and inhibition of cell proliferation.[12] Its metabolism involves a hydrolysis step operated by the enzyme ceramidase to produce sphingosine, which is then phosphorylated by a specific kinase, sphingosine kinase-1, to sphingosine-1-phosphate, a metabolite which, unlike ceramide, stimulates cell growth and proliferation. The dynamic balance between levels of sphingolipid metabolites, ceramide and sphingosine-1-phosphate is an important factor that determines whether a cell survives and proliferates or

undergoes an apoptotic process. Previous studies showed that the cellular ceramide/sphingosine-1 phosphate ratio, associated with a high sphingosine kinase-1 activity, correlated with a robust intrinsic PDAC cells chemoresistance toward gemcitabine.[13]

The ceramide analog AL6 was able to trigger apoptosis in CCRF-CEM cells, and the treatment of mice bearing a WiDr colon xenograft with this compound at 50 mg/kg ip daily for 10 days resulted in an antitumor effect that was equivalent to that of cyclophosphamide, 20 mg/kg ip daily, with markedly lower systemic toxicity.[10] Therefore, we evaluated the activity of AL6 alone and in combination with gemcitabine in 2 PDAC lines, showing a strong synergistic interaction on inhibition of cell proliferation and apoptosis induction. Since sphingolipids, including ceramide, are components of complex lipid/membrane raft networks, and can also function as nodes within webs of signalling, we also evaluated the modulation of key enzymes in gemcitabine metabolism and clinical outcome.[8,9] Our data showed that AL6 increased the *dCK/CDA* gene expression ratio. These data are in agreement with previous studies showing that several anticancer drugs stimulate the *salvage* pathway initiated by dCK, accounting for the majority of nucleotide synthesis for DNA repair.[9] The upregulation of dCK without a parallel increase in the expression of the catabolism enzyme CDA, may be considered one of the most important mechanisms underlying the synergistic interaction of AL6 with gemcitabine.

In conclusion, the combination of gemcitabine with a novel ceramide analog resulted in increased apoptosis and favorable modulation of pivotal enzymes in gemcitabine metabolism and might represent a novel promising approach to improve treatment of PDAC.

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