Advances in Brief

Gemcitabine (2′,2′-Difluoro-2′-Deoxycytidine), an Antimetabolite That Poisons Topoisomerase I

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Abstract

Purpose: **Gemcitabine-containing regimens are among standard therapies for the treatment of advanced non-small cell lung, pancreatic, or bladder cancers. Gemcitabine is a nucleoside analogue and its cytotoxicity is correlated with incorporation into genomic DNA and concomitant inhibition of DNA synthesis. However, it is still unclear by which mechanism(s) gemcitabine incorporation leads to cell death.**

Experimental Design: **We used purified oligodeoxynucleotides to study the effects of gemcitabine incorporation on topoisomerase I (top1) activity and tested the role of top1 poisoning in gemcitabine-induced cytotoxicity in cancer cells.**

Results: **We found that top1-mediated DNA cleavage was enhanced when gemcitabine was incorporated immediately 3′ from a top1 cleavage site on the nonscissile strand. This position-specific enhancement was attributable to an increased DNA cleavage by top1 and was likely to have resulted from a combination of gemcitabine-induced conformational and electrostatic effects. Gemcitabine also enhanced camptothecin-induced cleavage complexes. We also detected top1 cleavage complexes in human leukemia CEM cells treated with gemcitabine and a 5-fold resistance of P388/CPT45 top1-deficient cells to gemcitabine, indicating that poisoning of top1 can contribute to the antitumor activity of gemcitabine.**

Conclusions: **The present results extend our recent finding that incorporation of 1-β-D-arabinofuranosylcytosine into DNA can induce top1 cleavage complexes [P. Pourquier *et al*. Proc. Natl. Acad. Sci. USA, *97:* 1885–1890, 2000]. The enhancement of camptothecin-induced top1 cleavage complexes may, at least in part, contribute to the synergistic or additive effects of gemcitabine in combination with topotecan and irinotecan in human breast or lung cancer cells.**

Introduction

Most of the anticancer agents presently available in clinical settings exert their cytotoxic effects by interacting with genomic DNA. One of the most common mechanisms of action is the generation of DNA strand breaks attributable to base alkylation or cross-link, to the stabilization of topoisomerase-DNA covalent complexes, or to inhibition of DNA synthesis. Nucleoside analogues are among the most potent antimetabolites. They are prodrugs that need to be phosphorylated by deoxycytidine kinase. Until now, their main cytotoxicity has been attributed to the inhibition of DNA polymerases (mainly polymerase α) after their incorporation in DNA during replication or repair. Gemcitabine is one of the most recent nucleoside analogs used in cancer chemotherapy. It is one of the most active agents in non-small cell lung cancer, and its combination with cisplatin and vinorelbine improves survival (1). Gemcitabine is also active in pancreatic cancer (2) and is used in salvage therapy for patients with tumors that are refractory to conventional therapies (3). Phase II studies suggest that the clinical activity of gemcitabine covers a large panel of solid tumors. Its association with cisplatin is now considered as a standard therapy in advanced bladder cancer (4). Interestingly, gemcitabine can be easily combined with other cytotoxic agents, frequently without dose reduction, and can also be used in the elderly (5).

Structurally, gemcitabine (2′,2′-difluoro-2′-deoxycytidine), differs from ara-C[4] by its substituents on the 2′ position of the sugar ring (Fig. 1*A*). Gemcitabine is also a more potent DNA-synthesis inhibitor (6) and is more cytotoxic than ara-C (7, 8). Its cytotoxicity is strongly correlated with the amount of its incorporation into cellular DNA (6, 9). Gemcitabine is incorporated mainly within replicating DNA, whereas ara-C is also incorporated via repair synthesis (10). The mechanism by which incorporation of nucleoside analogs into DNA leads to cell death remains unclear. Recently, we demonstrated that ara-C

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[4] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; top1: DNA topoisomerase I; CPT, camptothecin; NMR, nuclear magnetic resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Gem, gemcitabine; ICE, *in vivo* complex of enzyme.

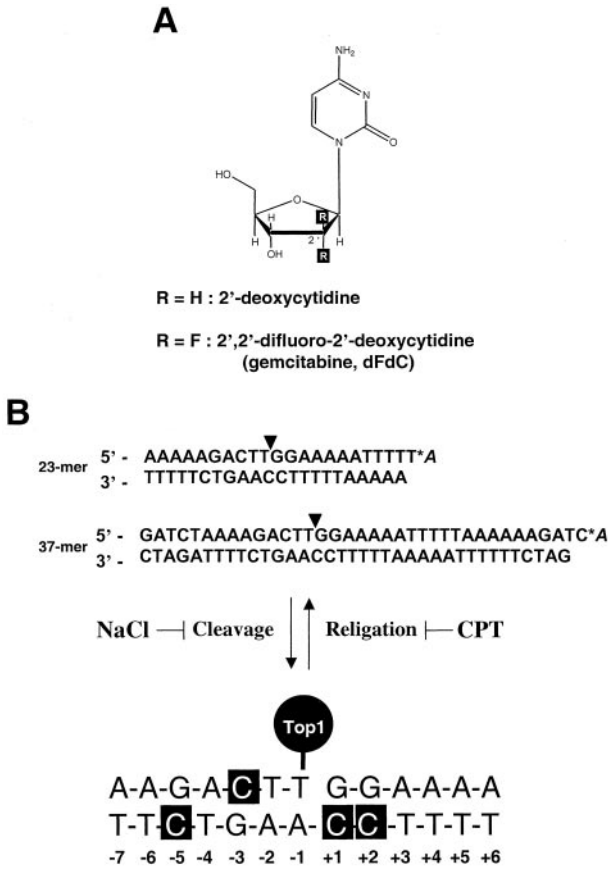

Fig. 1 *A*, chemical structures of cytosine (*R = H*) and gemcitabine (*R = F*). *B*, sequence of the 23- and 37-mer oligonucleotides derived from the tetrahymena hexadecameric ribosomal DNA sequence (38). *$_A$ corresponds to the [^{32}P]-cordycepin label at the 3′ terminus of the scissile (*upper*) strand. *Arrowhead* corresponds to the top1 cleavage site between thymine (−1 position) and guanine (+1 position). Cytosines that were replaced by gemcitabines at positions −5, +1, and +2 (nonscissile strand) and −3 (scissile strand) are *highlighted*. CPT inhibits religation, and high salt (NaCl) inhibits the formation of top1 cleavage complexes.

incorporation traps (top1)-DNA cleavage complexes and contributes to the cytotoxicity of this analogue (11).

top1 is an essential enzyme in higher eukaryotes (12, 13). It reversibly cleaves one strand of duplex DNA and allows the removal of torsional stress associated with crucial processes such as replication, transcription, chromosome condensation, and segregation during mitosis (14–16). The top1-DNA covalent intermediates are referred to as top1 cleavage complexes. Their "stabilization" can generate DNA-strand breaks by interference with advancing replication or transcription forks (17–19). These breaks are believed to be responsible for the cytotoxicity of top1 poisons such as camptothecins that inhibit the religation of the top1 cleavage complexes (15). Other DNA alterations originating from endogenous and exogenous sources such as abasic sites, uracil misincorporations, nicks, oxidized bases, alkylated bases, UV photo-lesions and carcinogenic adducts can also trap top1 by the same mechanism [see (20) for review]. Thus, top1 would act as a "sensor" of DNA damages and participate in the execution of cell death (21). Alternatively, top1 might recruit repair enzymes on the damaged site (22).

In this study, we demonstrate that gemcitabine can poison top1 both *in vitro*, when it is incorporated in purified oligonucleotides and in gemcitabine-treated cells. top1-deficient cells were also found to be resistant to gemcitabine, suggesting that the induction of top1-mediated DNA breaks can play a role in the cytotoxicity of gemcitabine.

Materials and Methods

Chemicals and Enzymes. CPT was purchased from Sigma (St. Louis, MO), [α-^{32}P]-cordycepin 5′-triphosphate was purchased from New England Nuclear (Boston, MA), and terminal deoxynucleotidyl transferase and T4 polynucleotide kinase were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Polyacrylamide was purchased from Bio-Rad, Inc. (Richmond, CA). Human recombinant top1 was purified from Sf9 cells using a baculovirus construct for the N terminus truncated human top1 cDNA as described previously (23).

Synthesis of Gemcitabine-containing Oligonucleotides. 5′-Dimethoxytrityl-*N*^{4}-benzoyl-2′,2′-difluoro-2′-deoxycytidine (1.82 mmol, 1.22 g) was dissolved in diisopropylethylamine (1.24 ml) and anhydrous chloroform (15 ml). 2-Cyanoethyl-*N*,*N*′-diisopropropyl-chlorophosphoramidite (2.4 mmol, 0.53 ml) was added, and the reaction was stirred at room temperature (2 h). Additional 2-cyanoethyl-*N*,*N*′-diisopropropyl-chlorophosphoramidite (1.4 mmol, 0.24 ml) was added, and stirring was continued (1.5 h). The reaction was stopped by the addition of ethyl acetate (0.5 ml) and dilution with chloroform (50 ml). The reaction was washed four times with aqueous NaCl and then water. Evaporation produced a yellowish foam (2.25 g) that was purified by silica gel chromatography using first 42/56/2 and then 42/53/5 (v/v/v) dichloromethane/hexane/triethylamine [yield, 1.27 g (80%); ^{31}P-NMR ($CDCl_3$): δ152.1, δ150.2]. Oligonucleotides were synthesized on Applied Biosystems 394 DNA synthesizers at the University Core DNA and Protein Services facility (University of Calgary, Alberta, Canada). Oligonucleotide synthesis was similar to that described previously (24). We used tetrazole as an activator, instead of 4,5-dicyanoimidazole, because our trial syntheses did not show any improvement with 4,5-dicyanoimidazole. Postsynthesis deprotection was performed using ammonium hydroxide (55°C for 16 h). The solutions were then evaporated to dryness and redissolved in water. Preparative gel electrophoresis was performed using 24% polyacrylamide/7 M urea gels. Product bands visualized by UV shadowing were excised and extracted with 0.1 M NaCl (16 h at 21°C), and urea was removed by desalting on Sephadex-25.

High-performance liquid chromatography-purified control and gemcitabine-containing 22-mer oligonucleotides (see Fig. 1*B* for sequence) were purchased from Macromolecular Resources (Colorado State University, Fort Collins, CO).

Oligonuceotide Labeling and Annealing Procedures. We performed 3′ labeling of single-stranded oligonucleotides with [α-^{32}P]-cordycepin using the 3′-terminal deoxynucleotidyl transferase (Invitrogen Life Technologies, Carlsbad, CA) as described previously (25). Labeling mixtures were subsequently centrifuged through a G25 Sephadex column to remove the

unincorporated nucleotide. Annealing of the unlabeled complementary strand was performed by heating at 94°C for 5 min, followed by slow cooling to room temperature.

top1 Reactions and Analysis of Cleavage Products. DNA substrates (~50 fmol/reaction) were incubated with 5 ng of top1 in the absence or presence of 10 μM CPT (final concentration) for 15 min at 25°C in reaction buffer [10 mM Tris-HCl (pH 7.5); 50 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 15 μg/ml BSA]. Reactions were stopped by the addition of SDS (0.5%, final concentration). Before loading samples to gels, we added 3.3 volume of Maxam-Gilbert loading buffer (98% formamide; 0.01 M EDTA; 10 mM NaOH; 1 mg/ml xylene cyanol; 1 mg/ml bromphenol blue) to the reaction mixtures. Twenty percent denaturing polyacrylamide gels (7 M urea) were run at 40 V/cm at 50°C for 1–2 h. After drying the gels on 3-mm Whatman paper sheets, imaging and quantitations were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell Lines and Cytotoxicity Assays. P388 and P388/CPT45 mouse leukemia cells were a kind gift from Michael R. Mattern and Randal K. Johnson (Glaxo SmithKline, King of Prussia, PA). P388/CPT45-resistant cells were obtained by exposing CPT-5 cell lines (26, 27) to stepwise increasing concentrations of CPT until they grew in the presence of 45 μM of CPT. The human T-lymphoblastoid leukemia CEM (CCRF/CEM) cell line was purchased from the American Type Culture Collection (Rockville, MD). CEM and P388 cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Inc.) containing 10% heat-inactivated FCS and 3 mM glutamine in a 5% CO_2 incubator at 37°C. Gemcitabine cytotoxicity in P388 cell lines was measured using standard MTT assays after continuous treatment with the drug for 2.5 doubling times (3 days for P388 and 5 days for P388/CPT45 cells). The concentrations used were as follows: 0, 0.003, 0.01, 0.03, and 0.1 μM. Percentages of cell survival were the mean of two independent experiments.

Detection of Covalent top1-DNA Complexes in CEM Cells. top1-DNA adducts were isolated by using the ICE bioassay (11, 28, 29). Briefly, 10^6 treated or untreated CEM cells were pelleted and immediately lysed in 1% sarkosyl. After homogenization with a Dounce homogenizer, cells lysates were gently layered on CsCl step gradients and centrifuged at 165,000*g* for 20 h at 20°C. Half-milliliter fractions were collected, diluted with an equal volume of 25 mM sodium phosphate buffer (pH 6.5), and applied to Immobilon-P membranes (Millipore) by using a slot-blot vacuum manifold as described previously (11). top1-DNA complexes were detected using the C21 top1 monoclonal antibody (a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT; see Ref. 30) using standard Western blotting procedures.

Results

Gemcitabine Incorporation Induces Position-specific top1-mediated Cleavage Complexes. We first studied the effects of gemcitabine incorporation in DNA on purified human top1 using oligonucleotides containing a single top1 cleavage site (Fig. 1*B*, *arrowhead*). Gemcitabine was incorporated at various positions on the scissile or the nonscissile strand of 23- or 37-mer duplex DNAs (Fig. 1*B*). Incorporation of gemcitabine at the +1 position of the nonscissile strand relative to the top1 cleavage site resulted in an approximately 5- to 7-fold increase of the top1-mediated cleavage complex formation in both substrates (Fig. 2, *A* and *B*). Incorporation of gemcitabine at the −5 position of the nonscissile strand had no effect on top1 cleavage at the normal site, but induced a new top1 cleavage site that was detected only in the presence of camptothecin in the control oligonucleotide (Ref. 31; Fig. 2*A*, *white arrowhead*). This new site is located one base 5′ from the gemcitabine modification, which confirms that substitution of a cytosine by gemcitabine immediately downstream from a top1 site can induce the formation of a top1 cleavage complex. This enhancement is position-specific because gemcitabine incorporation at the −3 or +2 position of the scissile strand had no effect.

Camptothecin poisons top1 by specifically inhibiting the religation step of the top1 cleavage/religation equilibrium (Fig. 1*B*). Conversely, the addition of salt to the top1 reaction shifts the cleavage/religation equilibrium toward religation probably because it prevents binding of the enzyme to the DNA once religation has taken place (15, 32). We tested whether the incorporation of gemcitabine could affect CPT activity. As shown in Fig. 2, gemcitabine incorporation did not prevent the enhancement of top1 cleavage complexes by CPT. The reversal of top1 trapping by gemcitabine incorporation in the absence of CPT was almost complete after 1 min with high-salt addition, leading to a 30-fold reduction in the amount of top1 cleavage complexes (Fig. 2*C*, compare *Lanes 8* and *9*). By contrast, gemcitabine incorporation slowed the kinetics of reversal in the presence of CPT-induced cleavage complexes. Incorporation of gemcitabine resulted in an ~10-fold increase in the amount of top1 cleavage complexes remaining after 10 min with high-salt addition (Fig. 2*C*, compare *Lanes 6* and *15*).

Taken together, these data demonstrate that gemcitabine incorporation can enhance the formation of top1 cleavage complexes and that gemcitabine-induced top1 trapping is position-specific. It is detected only when gemcitabine is incorporated immediately downstream from a top1 cleavage site (at the position +1).

Induction of top1 Cleavage Complexes in CEM Cells after Gemcitabine Treatment. We next investigated whether top1 could be trapped by gemcitabine in cellular DNA. For this purpose, we measured top1 cleavage complexes in CEM cells using the ICE assay (28, 29). Western blots of the DNA-containing fractions (fractions 8–11) showed that top1-DNA covalent complexes could be detected as early as 3 h after treatment with 1 μM gemcitabine (Fig. 3). The kinetics of gemcitabine-mediated poisoning of top1 is consistent with the incorporation of gemcitabine in replicating DNA (Fig. 3). These results demonstrate that gemcitabine can induce top1 trapping in drug-treated cells with the same efficiency as ara-C (11).

P388/CPT45 Cells Lacking Detectable top1 Are Resistant to Gemcitabine. Next, we investigated whether the top1 trapping could play a role in the cytotoxicity of gemcitabine. We used wild-type P388 cells and the CPT-resistant P388/CPT45 cells that lack detectable top1 (11) to measure growth inhibition by MTT assay (Fig. 4). We found that P388/CPT45 cells are ~4-fold cross-resistant to gemcitabine (IC_{50} of 5 nM for P388 cells versus 19 nM for P388/CPT45 cells). We found previously a comparable cross-resistance of P388/CPT45 cells to ara-C (11).

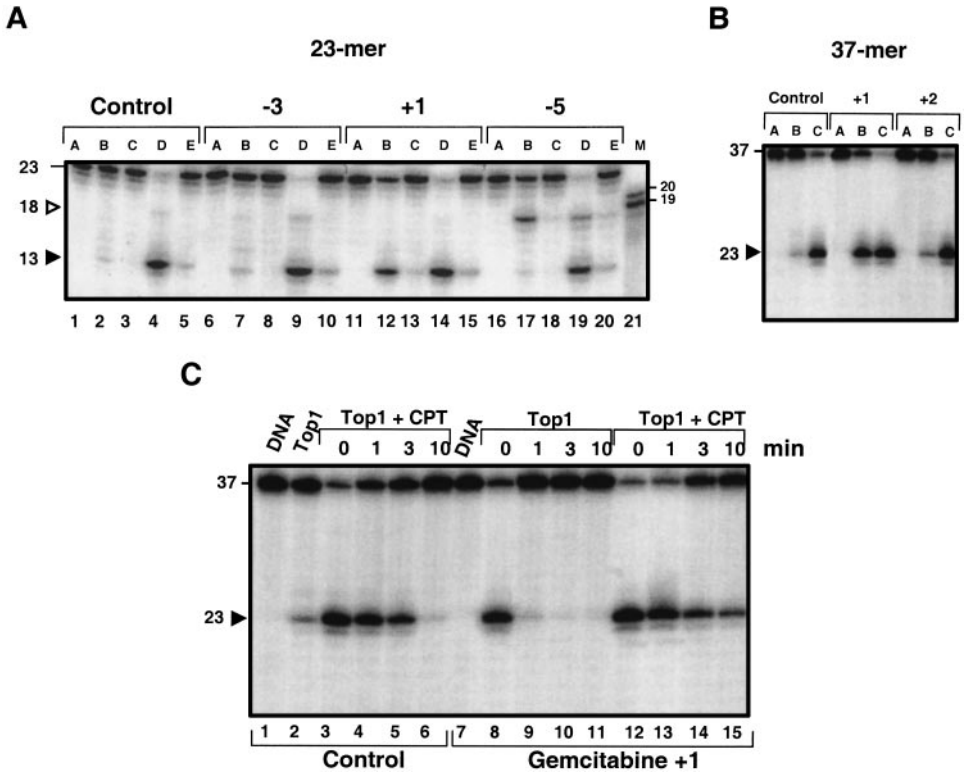

Fig. 2 Gemcitabine incorporation at the +1 position of a top1 cleavage site enhances the steady-state level of top1 cleavage complexes. *A*, effects of gemcitabine incorporation on the formation of top1 cleavage complexes using the 23-mer substrate as indicated in Fig. 1*B*. Position of the gemcitabine incorporation (Fig. 1*B*) is indicated on the top of each *panel*. *Lanes* A, DNA alone; *Lanes* B, + top1; *Lanes* D, + top1 + 10 μM CPT; *Lanes* C and E, same as *Lanes* B and D, but with further incubation in 0.5 M NaCl (final concentration) for 15 min at 25°C before the addition of 0.5% SDS. *Lane* M, 3′-labeled size markers of 20 and 19 bases. *B*, same as *A* using the 37-mer substrate. *Lanes* A, DNA alone; *Lanes* B: + top1; *Lanes* C: + top1 + 10 μM CPT. *C*, kinetics of salt-reversion. Reactions were performed for 15 min at room temperature before adding 0.35 M NaCl for the indicated times. Reversion was stopped by adding 0.5% SDS (final concentration) as described in "Materials and Methods."

Discussion

The present data demonstrate that gemcitabine incorporation, like ara-C, can trap top1 cleavage complexes. The localized effect of both deoxycytidine analogs on top1 cleavage is consistent with the NMR structures of gemcitabine- and ara-C-substituted model Okazaki fragments (33, 34), indicating that the structural perturbations resulting from analogue substitution are localized primarily to the site of substitution. Substitution of the two analogs results in distinct alterations in the mechanism responsible for enhanced top1 cleavage. In the case of ara-C, enhancement of top1 cleavage complexes is primarily attributable to the inhibition of religation (11), whereas for gemcitabine, increased cleavage results, in part, from an enhancement of the cleavage step. NMR studies of an ara-C-substituted model Okazaki fragment shows that the arabinosyl sugar adopts a C2′-endo sugar pucker. The conformational preference for this sugar pucker is based on a stereoelectronic interaction between the arabinosyl 2′-OH and O4′ of the furanose ring. In the case of the model Okazaki fragment studied by NMR, the preference for adoption of the C2′-endo sugar pucker for ara-C is greater than for deoxycytidine. Maintenance of the C2′-endo sugar pucker by ara-C inhibits maximum base overlap between adjacent purines in the complementary strand, and reduces duplex stability. The rigidity of the C_2′-endo sugar pucker for ara-C also likely contributes to religation inhibition for top1 cleavage complexes.

Gemcitabine substitution enhanced the top1 cleavage, but did not significantly inhibit the religation step of the top1 reaction. The enhancement of top1 cleavage complexes resulting from gemcitabine substitution likely is attributable to a combination of conformational and electrostatic effects. The increased density of electronegative charge resulting from the geminal-difluoro substitution may increase binding of the positively charged catalytic site of top1 to DNA near the site of substitution. The geminal-difluoro group probably also stabilizes developing negative charge in the transition state for formation of the cleavage complex. The lack of detectable religation inhibition in the gemcitabine-substituted top1 cleavage complex may result from an increased flexibility for the gemcitabine sugar relative to ara-C. Alternatively, it may indicate that adoption of a C_3′-endo sugar pucker is required for proper alignment during the religation step. The sugar pucker for gemcitabine in the NMR structure of the substituted model Okazaki fragment was C3′-endo, the conformation characteristic of ribonucleotides. The gemcitabine sugar may be more flexible than the ara-C sugar because disfavorable stereo-electronic forces can arise from interaction of either fluorine substituent relative to the furanose O4′. Most likely a combination of electrostatic and stereo-electronic forces contribute to the enhanced top1 cleavage rate for gemcitabine-substituted duplex DNA.

We found that gemcitabine can induce top1-DNA cleavage complexes in human leukemia cells. Kinetics of top1 trapping in gemcitabine-treated cells was comparable with what has been observed for ara-C (11), suggesting that incorporation of gemcitabine into cellular DNA leads to top1

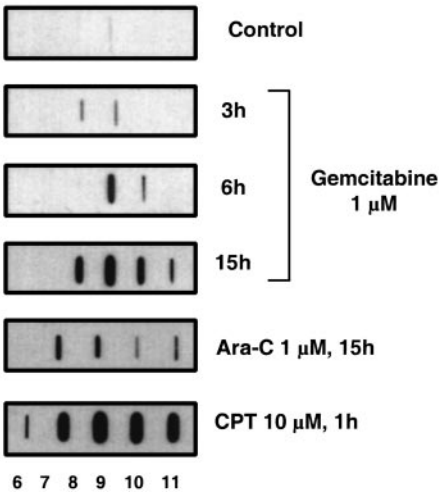

Fig. 3 Induction of top1 cleavage complexes in CEM cells treated with gemcitabine. CEM cells were treated with 1 μM gemcitabine for 0, 3, 6, and 15 h; 1 μM ara-C for 15 h; or 10 μM camptothecin for 1 h at 37°C. Approximately 10^6 cells were lysed in 1% sarkosyl and submitted to the ICE assay (see "Materials and Methods"). DNA-containing fractions (fractions 6–11) were collected from the bottom of the gradients and are indicated by *numbers*. Aliquots were blotted and top1-DNA covalent complexes were detected using the top1 C21 monoclonal antibody. *B*, comparison of gemcitabine- and ara-C-mediated induction of top1 cleavage complexes in CEM cells after treatment with 1 μM of the drug for 15 h. Treatment with 10 μM CPT for 1 h was used as a positive control.

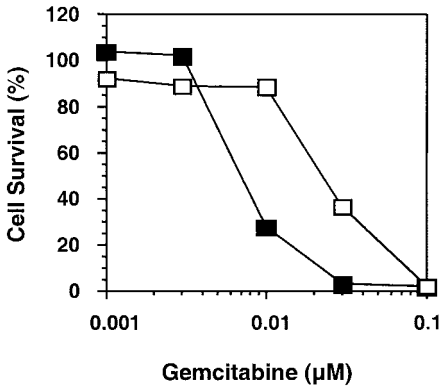

Fig. 4 Resistance of top1-deficient P388/CPT45 cells to gemcitabine. Growth inhibition in P388 (■) and P388/CPT45 cells (□) was measured by MTT assay after continuous treatment with gemcitabine for 3 and 5 days, respectively. The gemcitabine concentrations used were as follows: 0, 0.003, 0.01, 0.03, and 0.1 μM. Percentages of cell survival are the mean of two independent experiments.

poisoning. The observation that murine leukemia cells deficient for top1 and highly resistant to camptothecin are also resistant to gemcitabine also suggests that poisoning of top1 could play a role in the anticancer activity of gemcitabine. These results are consistent with a recent study showing that the human ovarian cancer cell line AG6000, highly resistant to gemcitabine, shows reduced top1 levels and a ~2-fold cross-resistance to the camptothecin derivatives CPT-11 and SN38 (35). In view of these data, we propose that nucleoside analogs could exert their antiproliferative effect not only through the inhibition of the DNA polymerases, but also by poisoning cellular top1.

We also found that gemcitabine incorporation into DNA can slow down the reversion of top1 cleavage complexes trapped by CPT *in vitro* (Fig. 2*C*). The enhanced stability of CPT-induced top1 cleavage complexes when gemcitabine is incorporated in DNA suggests that gemcitabine incorporation in cellular DNA may increase the frequency of collisions between the stabilized top1 cleavage complexes and replication and transcription complexes, leading to the accumulation of strand breaks and ultimately to enhanced cell death. This may, at least in part, contribute to the synergistic or additive effect between gemcitabine and camptothecin derivatives (36, 37). These data are of clinical importance because gemcitabine is frequently used in combination for a number of refractory tumors in association with top1 inhibitors such as topotecan or irinotecan.

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