Preclinical Evaluation of Gemcitabine Combination Regimens for Application in Acute Myeloid Leukemia

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Abstract The DNA antimetabolite gemcitabine is an anticancer agent with shown preclinical and clinical utility and a low toxicity profile. In this study, we sought to identify and optimize drug partners for binary and tertiary combinations with gemcitabine for use in the treatment of acute myelogenous leukemia (AML). Drug interaction was assessed by growth inhibition assay with metabolic end points. The combination index method was used to evaluate combinations of gemcitabine with fludarabine, paclitaxel, chlorambucil, doxorubicin, mitoxantrone, and SN-38 in U937 human AML cells. A three-dimensional method was used to determine the effect of dose ratio and schedule on drug interaction. Mechanisms underlying interactions related to cell cycle effects and apoptosis were assessed by flow cytometric and caspase-3 and -7 assays, respectively. The most synergistic binary combination was gemcitabine + fludarabine. The most synergistic tertiary combination was gemcitabine + fludarabine + paclitaxel, where the interaction was sequence dependent with paclitaxel given before gemcitabine + fludarabine, producing a 2-fold increase in synergy. Cell cycle analysis did not reveal a significant G_2-M arrest, suggesting that the synergistic effect of paclitaxel in this combination, which produced the greatest caspase activation, might be independent of microtubule stabilization. In contrast, the gemcitabine + fludarabine + mitoxantrone combination was synergistic and schedule independent. Moreover, few ratios of gemcitabine + fludarabine to mitoxantrone were antagonistic, which could be important for clinical translation. In conclusion, synergistic interactions with gemcitabine occurred with several drugs, the most promising being gemcitabine + fludarabine, gemcitabine + fludarabine + paclitaxel, and gemcitabine + fludarabine + mitoxantrone. These findings provided a rationale for clinical trials of gemcitabine + fludarabine and gemcitabine + mitoxantrone where responses were observed in heavily pretreated AML patients.

Leukemia is one of the 10 leading causes of cancer deaths in the United States, and acute myelogenous leukemia (AML) is responsible for one third of these deaths (1). Whereas several promising compounds have recently become available, single-agent therapies have been largely unsuccessful in preventing relapse (2). When optimized, combination chemotherapy regimens can offer increased efficacy, decreased toxicity, dose reductions, and decreased drug resistance.

Standard induction chemotherapy consists of cytosine arabinoside in combination with an anthracycline antibiotic or anthracenedione (usually daunorubicin, idarubicin, or mitoxantrone). Gemcitabine (2′,2′-difluorodeoxycytidine) is structurally similar to cytosine arabinoside, but creates DNA damage that is more difficult to repair. In addition, gemcitabine metabolites inhibit ribonucleotide reductase, leading to self-potentiation of cytotoxic activity (3). Gemcitabine has proven successful against a wide range of tumor types including lung, ovarian, head and neck, colon, and blood malignancies (4–7). Taken together, these characteristics, along with the novel mechanism of action of gemcitabine and relatively low toxicity profile, suggest that gemcitabine might be a good candidate for combination chemotherapy in the treatment of AML.

In this preclinical study, we evaluated the interaction of gemcitabine with fludarabine, a DNA antimetabolite; mitoxantrone, a topoisomerase II inhibitor; doxorubicin, a topoisomerase II poison; SN-38 (the active metabolite of irinotecan), a topoisomerase I poison; paclitaxel, a microtubule stabilizing agent; and chlorambucil, an alkylating agent. An AML cell line (U937) was used to assess growth inhibition. We used the combination effect analysis of Chou and Talalay (8) to identify the most synergistic combinations. Because drug interaction is often dependent on the dosage ratios of the compounds involved, we used the three-dimensional method of Kanzawa et al. (9) to evaluate these combinations across several concentration ratios. Additionally, as the documented mechanism of action of these compounds varies both in the target and the stage of the cell cycle in which they act, we also evaluated the effect of sequence of administration on the interaction of these drugs. We identified two binary combinations and two tertiary combinations that showed highly synergistic antitumor activity.

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Materials and Methods

Compounds. Gemcitabine (Eli Lilly and Company, Indianapolis, IN), fludarabine (Berlex Laboratories, Richmond, CA), mitoxantrone (Immunex, Seattle, WA), paclitaxel (Bristol Myers Squibb, Princeton, NJ), and doxorubicin (Pharmacia, Kalamazoo, MI) were obtained from the Duke University Medical Center Pharmacy and reconstituted according to the instructions of the manufacturer. Stock solutions of 10 mmol/L SN-38 (RTI International, Research Triangle Park, NC) in DMSO and 4 mmol/L chlorambucil (ICN, Aurora, OH) in 4% DMSO and sterile water were prepared and subsequently diluted.

Cell culture. U937 cells were obtained from Duke University Shared Cell Culture Resource (Durham, NC) and cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin, and 0.5% amphotericin B (Bristol Meyers Squibb) at 37°C in a humidified atmosphere containing 5% CO_2. Cell density was maintained at 10^5 to 10^6 cells/mL with viability exceeding 90%.

Isolation and evaluation of clinical samples. Clinical blood samples were collected from patients diagnosed with AML or chronic myelogenous leukemia after obtaining informed consent under a protocol approved by the Duke University Medical Center Institutional Review Board. Percent blasts ranged from 30% to >80%. The peripheral blood mononuclear cells were isolated by centrifugation in Vacutainer CPT tubes (Becton Dickinson and Company, Franklin Lakes, NJ) at 1,625 × *g* for 15 minutes. All centrifugation steps were done at room temperature. Peripheral blood mononuclear cells were harvested and resuspended in growth medium: Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 10% equine serum (Hyclone), 10 ng/mL pixy 321 (Immunex), 0.1 units/mL erythropoietin (Amgen, Thousand Oaks, CA), 25 ng/mL Flt-3 ligand (Immunex), 5 μg/mL gentamicin (Fujisawa USA, Deerfield, IL), 20 μg/mL vancomycin (Eli Lilly and Company), and 5 μmol/L hydrocortisone (Sigma, St. Louis, MO). Peripheral blood mononuclear cells were washed thrice with growth medium and collected by centrifugation at 600 × *g* for 7 minutes. Initial cell viability was assessed by trypan blue dye exclusion (Trypan Blue, Sigma). Macrophages were depleted by adherence to plastic culture flasks at 37°C for 16 to 21 hours at a concentration of 1×10^7 to 2×10^7 cells/mL. Peripheral blood mononuclear cells were further purified by density gradient sedimentation at 210 × *g* for 30 minutes over Ficoll-Paque PLUS (Pharmacia Biotech, Uppsala, Sweden). The mononuclear cell layer was harvested, washed thrice with growth medium, and stained with trypan blue to determine cell viability. Cells were seeded in 96-well microtiter plates at 250,000 cells/well (with the exception of two patient specimens plated at 50,000 cells/well) in a final volume of 200 μL with viability exceeding 90%. Cells in triplicate wells were exposed to drugs for 48 hours before 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. In samples where cells were limited, the ATPLite cytotoxicity assay (Packard Instruments, Downers Grove, IL) was used according to the protocol of the manufacturer. This assay measures cellular ATP as a marker for viable cells with a sensitivity of the chemiluminescent end point of 10 cells per well. Drug response was evaluated as described below.

Growth inhibition assay. Cells were seeded in 96-well microplates (Corning, Corning, NY) at a density of 50,000 cells/well and a final

Table 1. Antiproliferative activity of single chemotherapeutic agents against U937 cells *in vitro*

Drug	IC_{50} (nmol/L)
Mitoxantrone	3.0 ± 0.2
Paclitaxel	3.9 ± 0.9
SN-38	6.1 ± 0.3
Gemcitabine	10.4 ± 0.8
Doxorubicin	74 ± 11
Fludarabine	3,200 ± 560
Chlorambucil	13,000 ± 2,900

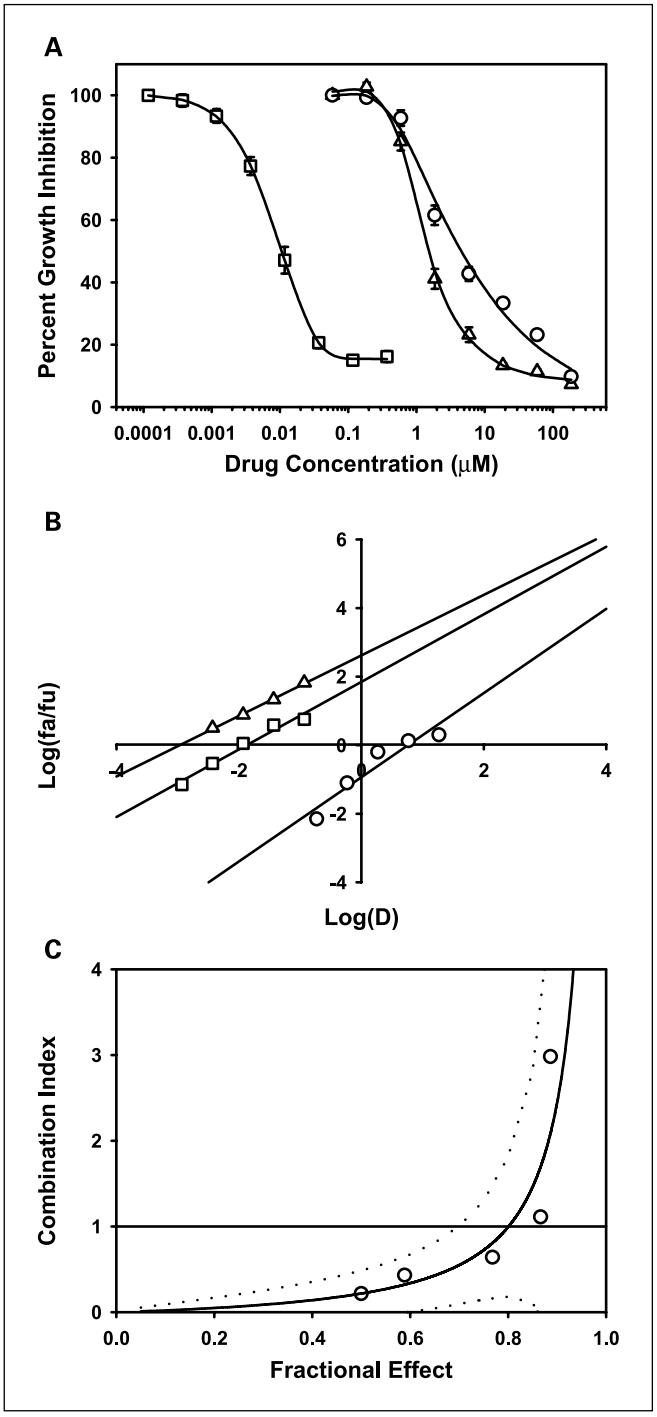

Fig. 1. Interaction of gemcitabine and fludarabine in U937 cells. *A*, composite dose-response curves for antiproliferative activity of gemcitabine (□), fludarabine (○), or the combination (△, at a ratio of gemcitabine/fludarabine = 1:500) were evaluated *in vitro* in U937 cells as described in Materials and Methods (n = 29). Error bars are the inter-assay SE. *B*, median effect plot for the interaction of gemcitabine + fludarabine in A. *C*, combination index for the interaction as a function of level of effect (fractional effect = 0.5 is the IC_{50}); dotted lines, 95% confidence intervals.

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volume of 200 μL. Cells were then exposed to varying concentrations of gemcitabine, fludarabine, mitoxantrone, paclitaxel, doxorubicin, SN-38, and chlorambucil alone, or in binary and tertiary combinations. When used in combination, these drugs were added either simultaneously or sequentially with an interval of 24 hours. All incubations were carried out for 48 hours or approximately two cell doublings. Cytotoxicity was assessed using the CellTiter 96 AQ proliferation assay (Promega, Madison, WI), a colorimetric method for quantification of viable cells that is based on the bioreduction of MTS by metabolically active cells. At 46 hours, 20 μL of MTS solution were added. Following 2-hour incubation period, absorbance at 490 nm was measured on an EL340 microplate reader (Bio-Tek, Winoski, VT). We initially determined the sensitivity of U937 cells to single administration of all compounds. Dose-response curves were constructed using Table Curve 2D software (SPSS, Inc., Chicago, IL) and were used to determine the range of concentrations to be used in combination.

Interaction analysis. We employed two methods of interaction analysis. First, the median effect method of Chou and Talalay (8) was used to identify the most promising combinations. We chose this method because it takes into account both the potency of each drug or combination of drugs and the shape of the dose-effect curve. Additionally, this is one of the few methods of interaction analysis in which three drug combinations can be evaluated. CalcuSyn software (Biosoft, Cambridge, United Kingdom), which is based on this method, was used to calculate the combination index. Synergy, additivity, and antagonism are defined as combination index < 1, combination index = 1, and combination index > 1, respectively. For this analysis, all compounds were combined at the IC_{50} level of effect. Each experiment provided quadruplicate data points for each concentration and was repeated at least four times (n = 4-29). The resulting dose-response curves were averaged, creating a single composite dose-response curve for each combination. The fraction affected [1 − (absorbance at 490 nm of treatment group − blank / absorbance at 490 nm of untreated control − blank)] was calculated for each concentration and entered into CalcuSyn software.

After identifying the two most synergistic tertiary combinations, we used the three-dimensional method of Kanzawa et al. (9) to evaluate the effect of varying concentration ratios on the interaction of these compounds. Also based on the median effect principle, this method is used to calculate a combination effect surface that characterizes the interaction across varying concentration ranges. When three drugs are combined, four variables generally exist: the concentration of the three drugs and the resulting biological effect. Two-dimensional models, such as the combination index method, require two variables to be held constant. A three-dimensional model can therefore provide a more complete analysis. As triple drug combinations contain four variables, one variable must still be held constant. Given that the purpose of this study was to identify a third drug to be used in the combination of gemcitabine and fludarabine, we combined these compounds in a fixed ratio and varied the ratio of this binary combination with a third drug (mitoxantrone or paclitaxel). In this method, synergy, additivity, and antagonism are defined as combination effect > 0, combination effect = 0, and combination effect < 0, respectively. The theoretical basis and practical application of the Kanzawa method have been previously described (4, 9). Briefly, cytotoxicity data in an 8 × 8 well matrix format from six replicate plates were entered into a Microsoft Excel (Redmond, WA) spreadsheet and the combination effect was computed. Combination effect surfaces were generated using SigmaPlot 2000 (SPSS) scientific graphics software. Optimal schedule and dosage ratios were determined.

Cell cycle analysis. Paclitaxel is known to block cells at the G_2-M boundary of the cell cycle. We did cell cycle analysis on the cells exposed to the sequential combination of paclitaxel followed 24 hours later by gemcitabine + fludarabine (paclitaxel → gemcitabine + fludarabine) to determine if blocks in the cell cycle played an important role in the synergy of this combination. Cells were exposed to concentrations of paclitaxel where synergy was observed (3, 10, and 30 nmol/L) for 24 hours, pooled together, and fixed with an ice-cold solution of 70% ethanol and distilled water. RNA was destroyed with 5 mg/mL of RNase A (Sigma) and cells were stained with 5 mg/mL of propidium iodide (Molecular Probes, Eugene, OR). Propidium iodide is

Table 2. Dose reduction index achieved with combination regimens at IC_{50}

Drug combination					Dose reduction index				
Drug A	Drug B	Drug C	Combination index	SD	Drug A	Drug B	Drug C	Molar ratios (drug A/B/C)	*n*
G	F		0.22	0.14	10.0	8.6		1:500	29
G	M		0.64	0.18	3.9	2.6		1:0.426	6
G	D		0.70	0.15	2.1	4.2		1:2.4	6
F	M		0.77	0.23	3.0	2.3		1:0.0009	6
G	T		0.86	0.22	1.5	4.8		1:0.085	5
F	D		0.87	0.25	1.6	3.8		1:0.005	6
G	C		1.05	0.13	1.4	2.8		1:540	4
G	S		1.12	0.09	16.9	0.9		1:8.5	3
F	T		1.18	0.52	1.1	3.9		1:0.0002	5
F	C		1.53	0.62	0.9	2.2		1:1.08	4
F	S		1.25	0.14	13.1	0.8		1:0.017	3
G	F	T	0.35	0.09	7.2	6.2	22.3	1:500:0.085	5
G	F	M	0.56	0.24	6.3	5.4	4.2	1:500:0.426	6
G	F	D	0.80	0.29	3.3	2.9	6.6	1:500:2.4	6
G	F	C	0.95	0.34	2.8	2.4	5.6	1:500:540	4
G	F	S	1.33	0.08	15.2	13.0	0.8	1:500:8.5	3

NOTE: Dose reduction = D_x / D, where D_x, dose of drug required to affect a given percentage (x) of cells when administered as a single agent and D, dose of drug required to affect the same percentage of cells when administered as part of a combination. SD was from CalcuSyn output.
Abbreviations: G, gemcitabine; F, fludarabine; M, mitoxantrone; D, doxorubicin; T, paclitaxel; C, chlorambucil; S, SN-38.

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a fluorescent dye that stains nucleic acids. Flow cytometry was done on stained samples to quantify DNA content, which was then correlated to cell cycle stage.

Caspase assay. Cells were seeded in 96-well microplates at a density of 25,000 cells/well and a final volume of 100 μL. Drug additions were done at the IC_{70} level of effect (as calculated from the composite dose-response curves mentioned above) as single agents and in binary and tertiary combinations. When used in combination, these drugs were added either simultaneously or sequentially with an interval of 24 hours. Caspase-3 and -7 enzyme activities were detected at various time points with the Apo-ONE Homogenous Caspase-3/7 Assay (Promega). This assay employs a profluorescent substrate that is selectively cleaved by caspases 3 and 7. At each time point, fluorescence was measured every 15 minutes at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm on an FL-600 plate reader (Bio-Tek). Enzyme kinetic data were entered into Excel worksheets and a time versus fluorescence curve was constructed. Linear regression was used to calculate the slope of the linear portion of this curve and was used as a measure of enzyme concentration.

Results

Activity of agents alone. Our initial experiments sought to determine the chemosensitivity of U937 cells to the individual agents. Composite dose-response curves were fit to the data using TableCurve 2D software (SPSS), and drug concentrations that produced 50% inhibition of growth versus untreated control (the IC_{50}) were computed. These cells exhibited a range of sensitivity as shown in Table 1. IC_{50} values ranged from 3 nmol/L to 15 μmol/L with rank order of potency of mitoxantrone = paclitaxel > SN-38 > doxorubicin > gemcitabine ⩾ fludarabine ⩾ chlorambucil.

Activity of agents in combination. The combination effect analysis of Chou and Talalay was used to identify highly synergistic binary combinations. In this analysis, combination index < 1, combination index = 1, and combination index > 1 characterize synergy, additivity, and antagonism, respectively. As an example, data for the gemcitabine + fludarabine combination are presented in Fig. 1, including the composite dose-response curves, median effect, and combination index plots. The results indicate that this combination is synergistic over a wide range of effects with combination index = 0.217 ± 0.136 at IC_{50} (fraction of cells affected = 0.5). The combination index at IC_{50}, dose reduction, and associated molar ratio for each compound used in the 11 binary and 5 tertiary combinations are reported in Table 2. For the gemcitabine + fludarabine case, the dose of both agents can be reduced nearly 10-fold when used in combination to obtain the same antitumor effect produced by the single agents. A graphic summary of the combination index analyses over all levels of effect are shown in Fig. 2. The most synergistic binary combination was gemcitabine + fludarabine, whereas combinations of SN-38 with gemcitabine or fludarabine proved largely additive to antagonistic. To improve on the marked synergy shown by the gemcitabine + fludarabine combination, we evaluated the interaction of tertiary combinations involving gemcitabine + fludarabine. Of the tertiary combinations, gemcitabine + fludarabine + paclitaxel and gemcitabine + fludarabine + mitoxantrone were most synergistic. We postulated that this synergy could be further improved if schedule of administration and drug-drug ratios were optimized. Accordingly, we applied Kanzawa interaction analysis to evaluate the effect of varying concentration ratios and schedules of administration on the synergy of the gemcitabine + fludarabine + mitoxantrone and gemcitabine + fludarabine + paclitaxel combinations. The interaction surfaces generated from the gemcitabine + fludarabine + mitoxantrone and gemcitabine + fludarabine + paclitaxel combinations are illustrated in Figs. 3 and 4, respectively. It should be noted that in contrast to the Chou and Talalay method, the Kanzawa end point or combination effect defines synergy, additivity, and antagonism as combination effect > 0, combination effect = 0, and combination effect < 0, respectively. These ranges have been color coded to aid

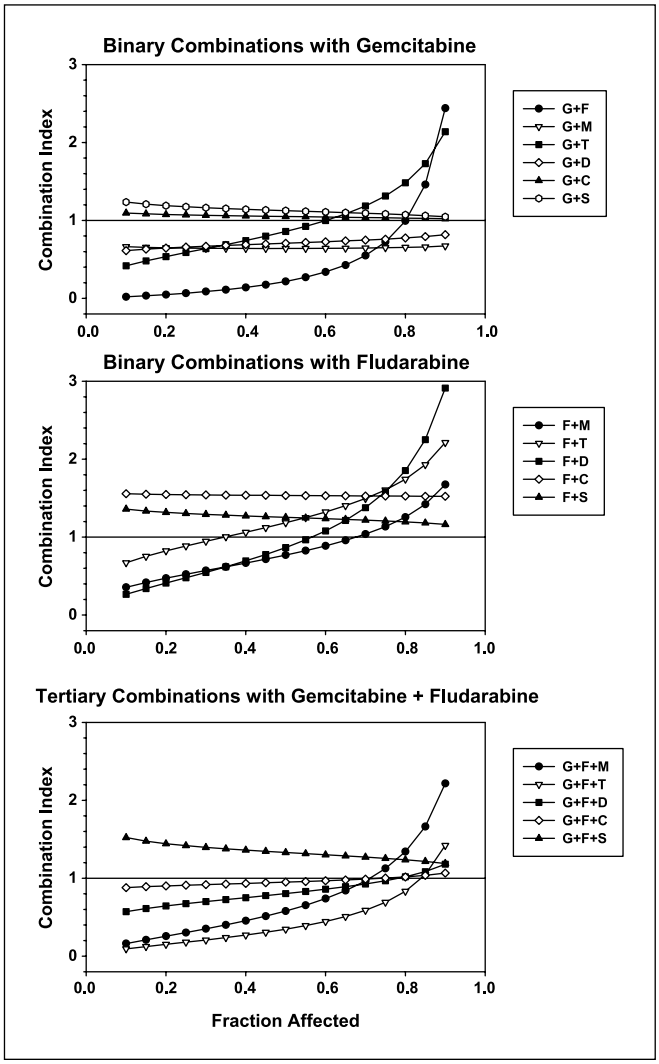

Fig. 2. Combination index values for binary and tertiary drug combinations in U937 cells. Chemotherapeutic drugs were combined simultaneously at concentrations bracketing their individual IC_{50}s and exposed to U937 cells simultaneously for 48 hours as described in Materials and Methods for growth inhibition assays. Combination index values were then calculated with Calcusyn Software as a function of the level of antiproliferative activity (fraction of cells affected = 0.5 is the IC_{50}). Combination index = 1 denotes additivity; combination index › 1 is antagonism; and combination index ‹ 1 is defined as synergy. Top, binary combinations of gemcitabine with SN-38 (*S*), chlorambucil (*C*), doxorubicin (*D*), paclitaxel (*T*), mitoxantrone (*M*), and fludarabine (*F*). Middle, binary combinations of fludarabine with SN-38, chlorambucil, doxorubicin, paclitaxel, mitoxantrone, and gemcitabine. Bottom, tertiary combinations of gemcitabine and fludarabine with SN-38, chlorambucil, doxorubicin, paclitaxel, and mitoxantrone.

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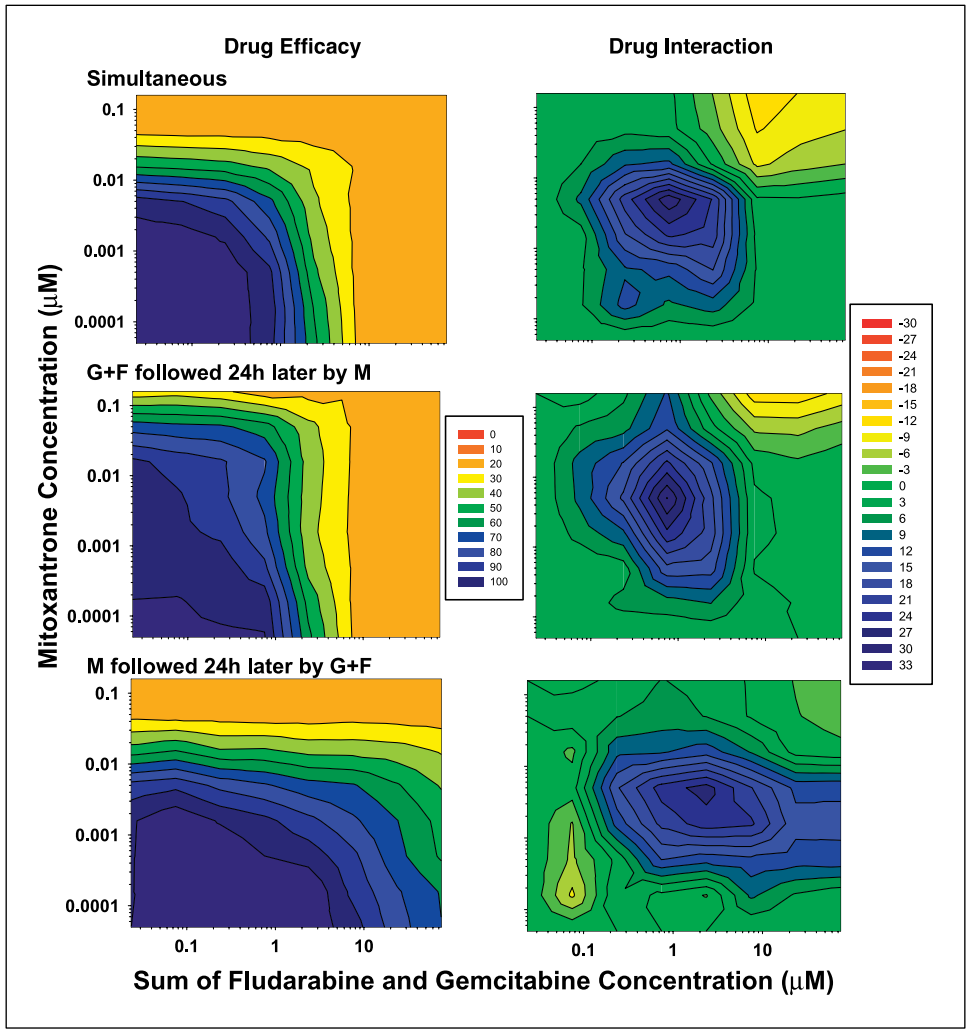

Fig. 3. Efficacy and interaction contours: gemcitabine and fludarabine with mitoxantrone in U937 cells. U937 cells were exposed to varying concentrations of gemcitabine in combination with fludarabine (at a fixed molar ratio of gemcitabine/fludarabine = 1:200) and mitoxantrone in simultaneous and sequential administrations for a total of 48 hours. The percent of control growth (*left*) and corresponding combination effect surface (*right*) are given for each combination. The graphs are color coded to indicate the range of response from minimum (*blue*) to maximum (*red*) growth inhibition and from synergistic (combination effect › 0; *blue*) to additive (combination effect = 0; *light orange*) to antagonistic (combination effect ‹ 0; *red*) drug interactions.

visualization. The combination gemcitabine + fludarabine + mitoxantrone was synergistic regardless of the sequence of administration. Important for clinical translation, very few ratios produced an antagonistic effect. In contrast, the interaction observed in the gemcitabine + fludarabine + paclitaxel combination was sequence dependent. When administered simultaneously, this combination was generally synergistic. However, when paclitaxel was administered 24 hours after gemcitabine and fludarabine, both the dose range and degree of synergy were diminished. This combination was most synergistic when paclitaxel was given first, followed 24 hours later by gemcitabine and fludarabine (paclitaxel → gemcitabine + fludarabine). When compared with simultaneous exposure, the number of concentration ratios that produced synergy increased 2-fold. In addition, the average degree of synergy across all ratios was increased nearly 5-fold.

Effect of drug combinations in clinical specimens. To gain an initial understanding of the clinical relevance of drug interaction studies done in U937 cells, a small number of patient samples were evaluated. Due to the small number of cells, only a limited number of combinations could be evaluated and thus focus was given on gemcitabine + mitoxantrone and gemcitabine + fludarabine. The results in Table 3 suggest that the gemcitabine + fludarabine combination was synergistic in two of the four patient specimens, whereas additive interactions were observed with gemcitabine + mitoxantrone. One patient specimen exhibited antagonism to both combinations. Interestingly, this specimen was unusually sensitive to fludarabine alone. Another specimen provided sufficient tumor cells to test the binary drug combinations by the Kanzawa method (Fig. 5). The contour maps revealed largely antagonistic interactions for the gemcitabine + SN-38 and gemcitabine + mitoxantrone combinations in this specimen. However, significant synergy was observed for the gemcitabine + fludarabine combination provided the fludarabine concentration did not exceed 0.6 μmol/L. This plot again illustrates the importance of drug ratio on the interaction.

Cell cycle analysis. The synergy shown in the paclitaxel → gemcitabine + fludarabine combination raised several mechanistic questions. Chiefly, we wanted to determine if paclitaxel induced a G_2-M cell cycle arrest before treatment with gemcitabine and fludarabine. The results given in Fig. 6 show that such a cell cycle arrest was not present before treatment with gemcitabine + fludarabine. Only 20% to 25% of cells were in the G_2-M phase of the cell cycle and a paired Student's *t* test revealed no significant difference in cell cycle distribution between untreated cells and cells treated with 3 to 30 nmol/L paclitaxel (P = 46).

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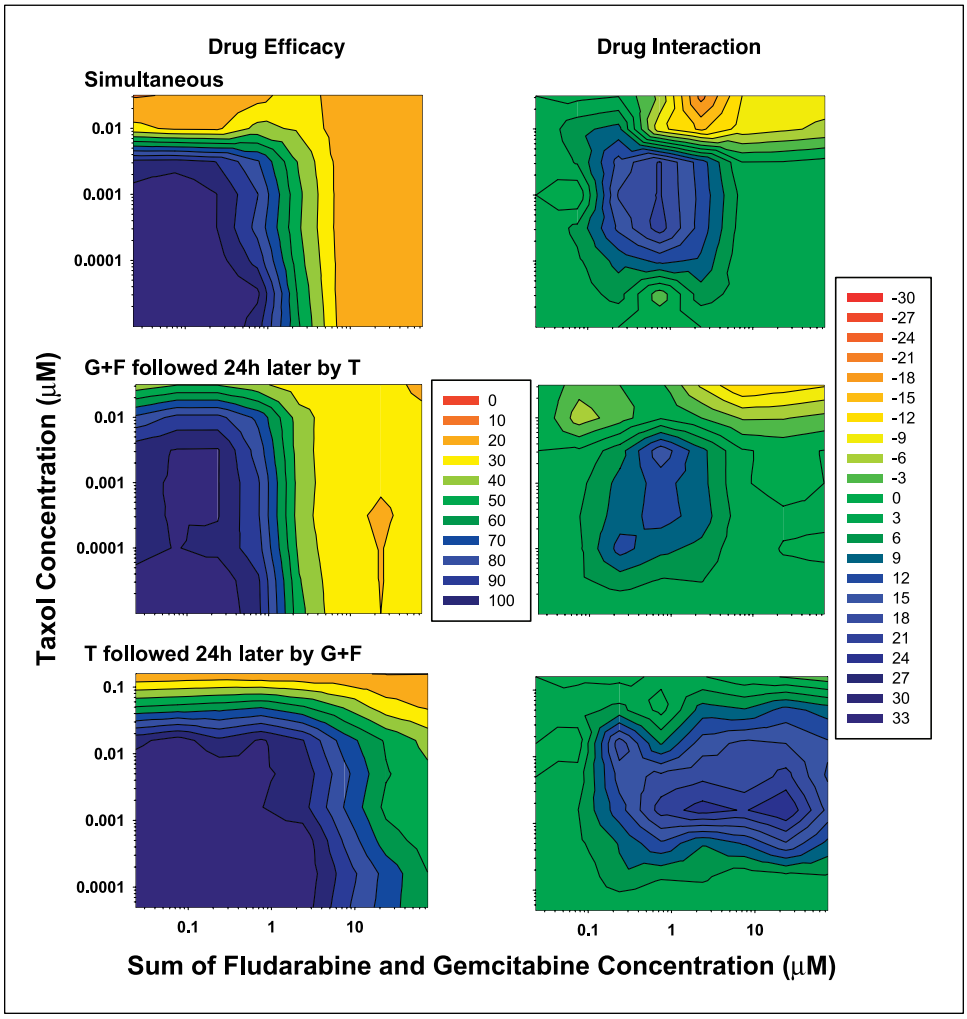

Fig. 4. Efficacy and interaction contours: gemcitabine and fludarabine with paclitaxel. U937 cells were exposed to varying concentrations of gemcitabine in combination with fludarabine (at a fixed molar ratio of gemcitabine/fludarabine = 1:200) and paclitaxel in simultaneous and sequential administrations for a total of 48 hours.

Caspase activity. To determine whether drug treatment activated the caspase-dependent apoptotic pathway, activity of caspases 3 and 7 was measured in cells exposed to gemcitabine, fludarabine, gemcitabine + fludarabine, gemcitabine + fludarabine + mitoxantrone, gemcitabine + fludarabine + paclitaxel, and paclitaxel → gemcitabine + fludarabine. The results are shown in Fig. 7. Caspase-3 and -7 activities were higher in cells treated with gemcitabine + fludarabine, gemcitabine + fludarabine + paclitaxel, and paclitaxel → gemcitabine + fludarabine than in the single and binary combinations of these drugs. The sequential combination of paclitaxel followed by gemcitabine + fludarabine showed significantly greater caspase activation compared with simultaneous exposure of gemcitabine + fludarabine + paclitaxel. These results are consistent with the drug interaction data obtained using cell viability as an end point. Interestingly,

Table 3. Activity of drugs alone and in combination in clinical specimens

		IC_{50} (μmol/L)			Combination Index at IC_{50}	
Patient	Disease	Gemcitabine	Mitoxantrone	Fludarabine	Gemcitabine + Mitoxantrone	Gemcitabine + Fludarabine
A	AML	0.131	0.114	48.80	1.0*	0.3
B	AML	0.001	0.001	3.53	1.1*	1.4
C	AML	0.009	0.002	0.05	11.3†	18.8
D	CML	10.920	0.218	3.05	1.2‡	0.6

Abbreviation: CML, chronic myelogenous leukemia.
*Simultaneous exposure at drug ratios 1:0.11 (gemcitabine/mitoxantrone) and 1:147 (gemcitabine/fludarabine).
†Simultaneous exposure at drug ratios 1:0.2 (gemcitabine/mitoxantrone) and 1:263 (gemcitabine/fludarabine).
‡Simultaneous exposure at drug ratios 1:0.22 (gemcitabine/mitoxantrone) and 1:0.9 (gemcitabine/fludarabine).

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however, the caspase activity of gemcitabine + fludarabine + mitoxantrone was less than that of gemcitabine or fludarabine alone.

Discussion

Gemcitabine is one of the most active nucleoside analogues in the chemotherapy arsenal, both in solid and increasingly also in hematologic malignancies. The basis for this activity lies in the multiple mechanisms of action and self-potentiation of gemcitabine (reviewed in refs. 5, 10). Activation of gemcitabine to its mononucleotide is primarily catalyzed by deoxycytidine kinase, although the drug is also a substrate for mitochondrial thymidine kinase 2 (11). Incorporation of the active metabolite 2′,2′-difluoro-deoxycitidine triphosphate into DNA causes "masked chain termination" wherein one additional nucleotide is added before termination of DNA synthesis. Such internucleotide linkage in DNA prevents recognition by exonuclease, making repair difficult. Other mechanisms of action include inhibition of RNA synthesis and direct induction of apoptosis (12). As important as these mechanisms of action is the pattern of self-potentiation that is unique to gemcitabine. The diphosphate metabolite, 2′,2′-difluoro-deoxycitidine diphosphate, is an inhibitor of ribonucleotide reductase, which depletes the cell of both dCTP and dATP. Depletion of dCTP in turn increases the activity of deoxycytidine kinase to enhance gemcitabine activation, whereas dATP inhibits DNA repair (5). The drug also inhibits dCMP deaminase and CTP synthetase, which decrease catabolism of gemcitabine and potentiate incorporation of 2′,2′-difluoro-deoxycitidine triphosphate into RNA and DNA, respectively (5).

Our data suggest a highly synergistic interaction between gemcitabine and fludarabine at clinically achievable plasma concentrations (13, 14). Whereas the underlying mechanism of this synergy remains unclear, there is a mechanistic rationale for combining gemcitabine with fludarabine (9-β-D-arabinofuranosyl 2-fluoroadenine 5-monophosphate). First, both drugs will act to lower the deoxynucleotide triphosphate pool, which in turn will increase incorporation of the analogue triphosphates into DNA. Second, exposure to one drug will potentiate deoxycytidine kinase activity and increase phosphorylation of the second agent.

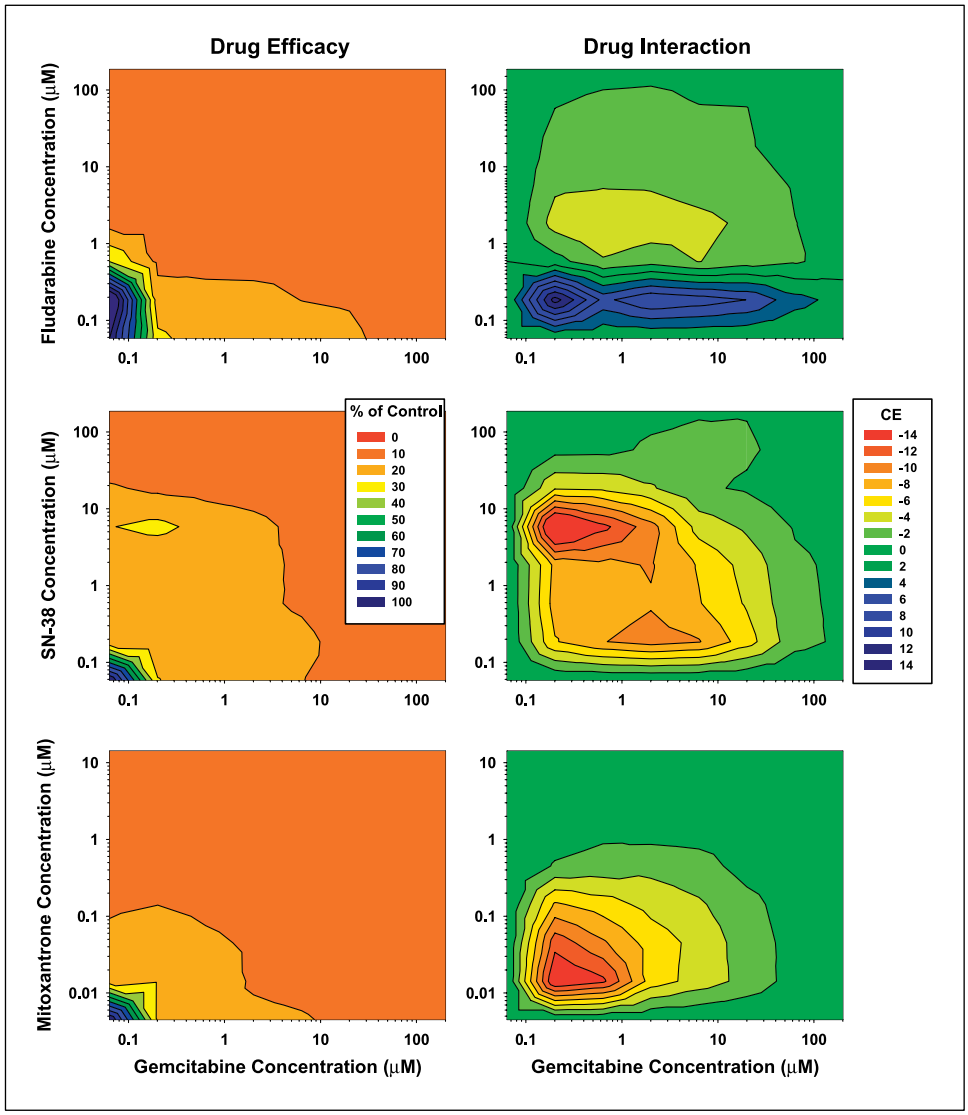

Fig. 5. Interactions of gemcitabine with fludarabine, mitoxantrone, and SN-38 in a primary AML specimen by the three-dimensional method. A patient specimen containing 89% leukemic blasts was exposed to gemcitabine alone or in combination with fludarabine, mitoxantrone, or SN-38 for 48 hours in the Kanzawa three-dimensional format. Surviving cell viability was then assessed by the ATPLite assay and the data analyzed as described in Materials and Methods to yield contour plots that compare growth inhibition (*left*) to the respective combination effect surfaces (*right*) for each combination.

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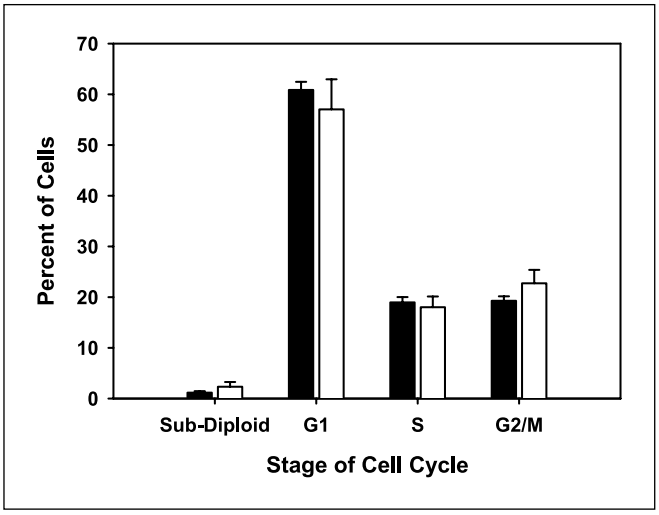

Fig. 6. Effect of paclitaxel on the cell cycle of U937 cells. U937 cells (5×10^4/well) were exposed to 3, 10, and 30 nmol/L paclitaxel for 24 hours, pooled together, and fixed with an ice-cold solution of 70% ethanol and distilled water. RNA was removed by incubation with 5 mg/mL of RNase A and DNA was stained with 5 mg/mL of propidium iodide. Flow cytometry was done on stained samples to quantify DNA content, which was then correlated to cell cycle stage. *Solid columns*, untreated control cells; *open columns*, paclitaxel-treated cells; *bars*, SE from three independent determinations.

The shown cytotoxic actions of both gemcitabine and fludarabine are dependent on phosphorylation by deoxycytidine kinase. With both compounds competing for the same enzyme, there is some question as to whether both compounds are being efficiently phosphorylated. Whereas we show no direct evidence of phosphorylation status of either compound, the doses of gemcitabine at which we observe synergy (0.3-370 nmol/L) are several orders of magnitude below the concentration (15-20 μmol/L) at which gemcitabine phosphorylation is expected to become saturated (14).

The synergistic interaction between gemcitabine and fludarabine observed here is consistent with those reported in chronic lymphocytic leukemia by Tosi et al. (15). In specimens from 10 patients, synergy was observed when relatively lower doses of gemcitabine were combined with fludarabine (gemcitabine/fludarabine = 1:10-1:100). Whereas combining chemotherapeutic agents from the same mechanistic class seems counterintuitive, a regimen including fludarabine, cytosine arabinoside, and granulocyte colony-stimulating factor (FLAG) is frequently employed in the treatment of AML with response rates as high as 70% (10). The synergy between gemcitabine and fludarabine reported here suggests that replacing cytosine arabinoside with gemcitabine may increase the efficacy of this regimen.

Cytarabine and mitoxantrone have been previously shown to interact synergistically (16). Both gemcitabine and fludarabine share similar mechanisms of action with cytarabine. These similarities, along with the documented synergy between cytarabine and mitoxantrone, would suggest that fludarabine or gemcitabine might also be synergistic with mitoxantrone. Our data provide evidence for this idea. Additionally, combination of gemcitabine with DNA damaging agents could result in synergistic antiproliferative activity based on gemcitabine diphosphate–induced inhibition of DNA repair, the effect of gemcitabine triphosphate incorporation into the DNA repair patch, or direct inhibition of repair enzymes such as DNA ligase or topoisomerase.

The synergy shown in the gemcitabine + fludarabine + mitoxantrone combination is consistent with the findings discussed above. In particular, the synergy of gemcitabine + mitoxantrone and that of gemcitabine + fludarabine suggest the triple drug combination could also be synergistic. The range of ratios in which synergistic interactions are observed is particularly important for clinical translation. Whereas achieving a precise ratio of three drugs in cell culture is relatively simple, dissimilar pharmacokinetics and physicochemical properties of each compound make *in vivo* replication of a precise ratio very difficult. The fact that synergy is observed in the gemcitabine + fludarabine + mitoxantrone combination at nearly all levels of effect is encouraging for clinical translation of this combination.

Our observation of caspase-3 and -7 activation following gemcitabine treatment is consistent with the results of Nabhan et al. (17), who found that caspases 3, 8, and 9 were activated in the MM1.S multiple myeloma cell line as early as 8 hours after 100 nmol/L gemcitabine treatment. The interaction between gemcitabine, fludarabine, and paclitaxel also produced synergistic antiproliferative activity and caspase activation, but in a sequence-dependent manner. This interaction is difficult to reconcile with the traditional mechanisms of action of these compounds. Gemcitabine and fludarabine are both thought to be active in the S phase, whereas paclitaxel has been shown to stabilize microtubules, causing a G_2-M block. Given the lack of a block at G_2-M in U937 cells at 24 hours, it seems possible that paclitaxel is working through a microtubule-independent mechanism. There is growing evidence of a microtubule-independent mechanism of paclitaxel-induced apoptosis (reviewed in ref. 18), including a recent trial from our group (19). In addition, Bahadori et al. (20) report an increase in S-phase fraction following exposure to a similar concentration

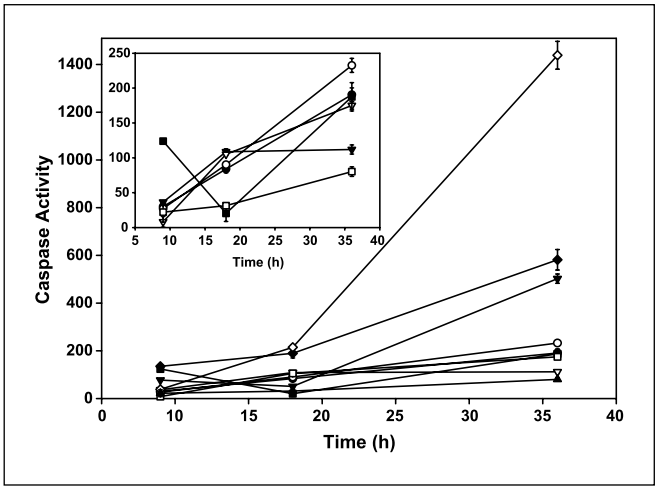

Fig. 7. Activation of caspases 3 and 7 in U937 cells. U937 cells were exposed to single agents and combinations at a concentration corresponding to the IC_{70} of each individual agent as follows: ▲, untreated control; ●, gemcitabine; ○, fludarabine; ▽, mitoxantrone; □, paclitaxel; ▼, gemcitabine + fludarabine; ■, gemcitabine + fludarabine + mitoxantrone; ♦, gemcitabine + fludarabine + paclitaxel; ◇, paclitaxel followed 24 hours later by gemcitabine + fludarabine. Caspase activity was determined by the slope of relative fluorescence units versus time (see Materials and Methods). Inset, scale expansion excluding paclitaxel, gemcitabine + fludarabine and gemcitabine + fludarabine + paclitaxel.

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(25 nmol/L) of paclitaxel, indicating that cell synchronization may be involved in the observed synergy.

Taken together, these data argue for clinical evaluation of gemcitabine combinations in hematologic malignancies. Accordingly, we have initiated phase I clinical trials to assess the activity of gemcitabine in combination with mitoxantrone and/or fludarabine in high-risk or relapsed acute leukemia and lymphoma. In one trial, 26 patients with relapsed or refractory acute leukemia received mitoxantrone daily for 3 days. Starting on the second day, they also received a 12-hour infusion of gemcitabine that generated a steady-state plasma concentration of about 25 μmol/L (21). This proved to be a tolerable induction regimen with an overall response rate of 42%. An even higher rate (63%) was achieved in a smaller number of patients who had received less than three cycles of myelotoxic chemotherapy for chronic myeloid leukemia or myelodysplasia leading to leukemia. In a second trial, 18 patients with relapsed or refractory acute myelogenous leukemia were treated with a 15-hour infusion of gemcitabine at a fixed dose rate of 10 mg/m^2/min in combination with 25 mg/m^2/d fludarabine for 5 days (22). Again, toxicity was significant but tolerable. Three complete responses and two partial responses were observed for an overall response rate of 28%. A third trial repeated this regimen in a cohort of 18 patients with very refractory acute myelogenous leukemia (23). Two complete responses were observed. In addition, Velasquez et al. (24) reported a 44% complete response rate with fludarabine and mitoxantrone in low-grade lymphoma. While preliminary, these findings suggest that such drug combinations, which would not normally be considered due to similar mechanisms and overlapping toxicities, may nevertheless provide additional therapeutic benefit in these diseases. Clearly, the ability to maintain the synergistic drug dose ratios remains a major challenge. Finally, the synergy observed for combination of gemcitabine, fludarabine, and paclitaxel raises significant mechanistic questions that warrant further investigation.

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