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ORIGINAL ARTICLE

Imexon enhances gemcitabine cytotoxicity by inhibition of ribonucleotide reductase

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Abstract
Purpose Gemcitabine (GEM) is currently the standard first line treatment for pancreatic cancer; however, the overall survival of patients with this disease remains poor. Imexon is a pro-oxidant small molecule which produced a high response rate in combination with GEM in a phase I trial in pancreatic cancer. In this study, we investigate the combination of GEM with a novel redox-active agent, imexon, in vitro and in vivo.
Methods Median effect analysis was used for in vitro combination cytotoxicity. The effect of imexon on GEM metabolism and uptake into cells and into DNA and effects on ribonucleotide reductase (RNR) were examined in vitro. The pharmacokinetics and antitumor efficacy of the imexon/GEM combination was evaluated in mouse models.
Results In three human pancreatic cancer lines, there was additivity for the imexon/GEM combination. There was significantly greater efficacy for the drug combination in Panc-1 xenograft tumors. A pharmacokinetic study in mice showed a near doubling in the AUC of imexon when GEM was co-administered, with no effect of imexon on GEM's pharmacokinetic disposition. In vitro, imexon did not alter GEM's metabolism or uptake into DNA, but significantly inhibited RNR, and this effect was greater when combined with GEM.
Conclusions These results suggest that the interaction between imexon and GEM may be due to complimentary inhibition of RNR plus an enhanced exposure to imexon when the GEM is administered in vivo. This combination is currently being tested in a randomized phase II trial in pancreatic cancer.

Keywords Imexon · Gemcitabine · Ribonucleotide reductase · Synergy · Pancreatic cancer

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Introduction

Pancreatic carcinoma remains the fourth leading cause of cancer deaths in both men and women, accounting for approximately 5% of all cancer-related deaths. In 2008, an estimated 7,680 new cases of pancreatic cancer were diagnosed in the United States and an equivalent number of patients died from the disease in the same year [1]. Chemotherapy and radiation therapy have remained largely ineffective in treating this cancer which most often presents as metastatic disease. The current standard treatment with the nucleoside analog GEM only modestly impacts the median survival of patients with pancreatic cancer [2]. Other than the combination of GEM with oral fluoropyrimidine (Capecitabine®, F. Hoffman-La Roche AG), which reported prolonged median overall survival in a subgroup of patients with good Parnofsky performance score (10.1 versus 7.4 months for GemCap versus Gem alone, respectively) [3], clinical trials combining GEM with other agents have not demonstrated clinical superiority of GEM combinations over single-agent therapy [4]. Currently, the only approved GEM combination is with the EGF receptor kinase inhibitor erlotinib, (Tarceva®, OSI Pharmaceuticals, Inc.), which produced a 15% improvement in survival over

GEM alone [5]. Hence, new treatment strategies are desperately needed for this devastating disease.

Imexon is an iminopyrrolidone aziridine small molecule currently in phase II clinical trials as an anti-cancer agent. In phase I trials, it was shown to be well tolerated and relatively non-myelosuppressive in humans [6]. Previous mechanism of action studies have demonstrated that imexon binds cellular thiols via classical aziridine ring opening to bind glutathione (GSH), or through a nucleophilic attack by the sulfur atom in cysteine at the cyclic amidine moiety of imexon [7]. In multiple myeloma cell lines, imexon decreases levels of reduced cellular thiols and induces oxidative stress. This leads to a loss of mitochondrial membrane potential (MMP) and allows cytochrome c release and caspase 3 and 9 activation [8]. We have previously shown that imexon has activity against pancreatic cancer cells in vitro as a single agent [9]. Mechanistic studies in the MiaPaCa-2 pancreatic cancer cell line demonstrated induction of oxidative stress, G_2/M arrest, and activation of caspases 3, 8, and 9 following imexon exposure. Imexon was also shown to be synergistic in vitro with alkylating agents and pyrimidine-based antimetabolites, including GEM in human myeloma and melanoma cell lines [10]. The mechanism for this synergistic growth inhibition was not known. Based upon these observations, we hypothesized that imexon might augment the cytotoxic effects of GEM in pancreatic cancer in two ways: (1) promoting increased oxidative stress in oxidatively challenged pancreatic cancer cells, and/or (2) interacting with key sulfhydryl-dependent enzymes involved with GEM metabolism, such as deoxycytidine deaminase (dCD) and ribonucleotide reductase (RNR). In this report, we demonstrate that the imexon plus GEM combination is active in pancreatic cancer cell lines in vitro and in vivo and may be due to imexon-mediated RNR inhibition and an increase in plasma imexon levels when administered in combination with GEM in vivo.

Materials and methods

Chemicals

Imexon (4-imino-1,3-diazabicyclo[3.1.0.]-hexan-one) was provided by the Pharmaceutical Resources Branch of the National Cancer Institute (Bethesda, MD) under a Rapid Access to Intervention in Development (RAID) grant to R. Dorr. The drug is supplied as a 99.6% pure lyophilized white powder (MW 111.1), with water solubility of approximately 5–10 mg/ml. A stock solution (4.4 mg/ml) was prepared in water, sterile filtered and stored at −80°C. GEM was obtained from Eli Lilly and Company (Indianapolis, IN). Radiolabeled [^{3}H]-GEM (11.0 Ci/mmol) and [^{14}C]-CDP (405 mCi/mmol) were obtained from Moravek Biochemicals (Brea, CA). [^{3}H]-cytidine was obtained from MP Biomedicals (Irvine, CA). All other chemicals were of the highest purity available and were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise noted.

Cell culture

Three human pancreatic cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cell lines included the poorly differentiated adenocarcinoma BxPC-3 (CRL-1687) [11], the largely undifferentiated epithelial carcinoma MiaPaCa-2 (CRL-1420) [12], and the ductal epithelial carcinoma Panc-1 (CRL-1469) [13]. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 media (GIBCO-BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The identity of all cell lines was validated by autosomal short tandem repeat (STR) analysis (Human Origins Genotyping Laboratory, University of Arizona).

Cytotoxicity assay

Cell viability was measured by thiazolyl blue tetrazolium (MTT) assay. This assay measures cellular dehydrogenase activity, which is considered to reflect mitochondrial activity, namely, the ability of living cells to reduce MTT to a blue formazan [14].

Median effect analysis was used to quantitate synergism, summation, or antagonism between imexon and GEM [15]. First, the IC_{50}, defined as the concentration of drug necessary to inhibit cell growth by 50%, was established for each individual agent by MTT analysis. These drugs were then combined at either a fixed molar ratio or at equipotency over concentrations required to produce a range of cell growth inhibition of 25–75% of control. According to the method of Chou and Talalay [15], a median effect combination index (CI) < 1 indicates synergism, CI $= 1.0$ indicates additivity and CI > 1 indicates antagonism.

In addition, the effect of sequential treatment with imexon and GEM was determined by 24 h pre-treatment with either imexon or GEM, and then subsequent addition of the second agent for 3 additional days. Combination indices were then obtained after 96 h of continuous exposure to both drug(s).

Combination drug activity in vivo

Female SCID mice (5–6 weeks old) were purchased from a breeding colony maintained by the University of Arizona Animal Care Facility (Tucson, AZ). Mice were housed

according to guidelines of the American Association for Laboratory Animal Care under protocols approved by the University of Arizona Institutional Animal Care and Use Committee. All mouse studies were performed to comply with the guidelines published by the National Institutes of Health (NIH Publication No 85–23, revised 1985).

For tumor growth delay analysis, SCID mice were inoculated subcutaneously with 10^7 Panc-1 cells in the right rear flank. After 24 h, mice were treated with 100 mg/kg imexon IP every day for nine consecutive days. GEM-treated mice were injected with 180 mg/kg GEM IP 1, 5, and 9 days after tumor implantation. Mice receiving combined therapy were injected with 100 mg/kg imexon every day for 9 days and 180 mg/kg GEM on days 1, 5, and 9. Tumor length and width was measured at least once per week and tumor volume was calculated as (length $\times$ width^2)/2. Tumor response was evaluated based on NCI criteria [16]. First, the tumor growth inhibition value (T/C, %) was determined at termination and was calculated as follows:

$$\mathrm{T/C}(\%) = \left(\frac{\text{mean tumor volume of treated}}{\text{mean tumor volume of control}}\right) \times 100$$

Compounds are considered active if the T/C value is $<42\%$, and highly active if $\leq 10\%$ [16]. Secondly, the tumor growth delay (T-C) value is the difference in the mean time for the tumors in the treated groups to reach 500 mm^3, when compared to control mice. Third, the tumor doubling time (Td) is determined from a log:linear growth plot in the control cells over the range of tumor cell volumes from 0 to 500 mm^3. And finally, the tumor cell kill (TCK) value is calculated as follows:

$$\mathrm{Log}_{10}\,\text{cell kill} = \frac{\mathrm{T-C(days)}}{3.32 \times \mathrm{Td}}$$

In addition to these studies evaluating tumor growth *delay* by imexon or GEM, we also tested a separate tumor *regression* model wherein drug treatment was initiated when Panc-1 xenograft tumor masses of approximately 40 mm^3 were detected.

Pharmacokinetics of imexon and GEM in mice

Adult female Balb/C mice ($n = 4$ per time point) were administered 180 mg/kg of GEM and/or 150 mg/kg of imexon intravenously and then sacrificed at six time points for the collection of blood by cardiac puncture. The blood was cooled on ice, centrifuged to separate the plasma fraction which was frozen at -80°C until analysis. The analytical method utilized reverse-phase HPLC with UV-absorbance detection based on previously reported assays for GEM in human plasma [17] and imexon in human plasma [6]. The time points chosen for blood collection were just prior to injection (0 min) and at 2, 5, 7.5, 10 and 20 min for GEM, and 0.5, 15, 30, 60 and 90 min for imexon. Pharmacokinetic parameters were analyzed using the commercial Win-NONLIN^R program (Pharsight Corporation, Cary, NC). These included the peak plasma level (C_{max}), and the time to the peak (T_{max}), the area under the plasma concentration $\times$ time curve (AUC), the clearance (Cl), measured as the Cl divided by the absorbed fraction (F), the terminal phase volume of distribution (Vb) and the plasma half-life ($t_{1/2}$). Differences between pharmacokinetic parameters for single agent and combination drug exposures were statistically compared using analysis of variation (ANOVA). Four groups were analyzed: (1) GEM alone, (2) imexon alone, (3) GEM in combination with imexon, and (4) imexon in combination with GEM, with $n = 4$ animals at each time point. For the combination, imexon was administered immediately prior to GEM.

[^{3}H]-GEM uptake assay

Panc-1 cells were simultaneously exposed to 100 nM radiolabeled GEM ([^{3}H]-GEM, 11.0 Ci/mmol) and 100 or 300 μM of unlabeled imexon for up to 4 h to determine if the presence of imexon altered GEM uptake. Control cells were treated with a tenfold excess of unlabeled GEM. After treatment, the cells were washed three times with ice-cold PBS and lysed with 500 μl of 0.1% sodium hydroxide (NaOH) and 0.1% sodium dodecyl sulfate (SDS) solution. The solution containing [^{3}H]-GEM was counted using Ecolite® scintillation cocktail (ICN Biomedicals, Irvine, CA).

[^{3}H]-GEM incorporation into DNA

Panc-1 cells were seeded at 0.5×10^6 cells/well in 60-mm^2 plates and treated with imexon, hydroxyurea, or deoxycytidine (dC) for 20 h. After 20 h, 500 nM [^{3}H]-GEM was added and incubated for an additional 4 h. The plates were rinsed two times with ice-cold PBS and two times with 5% TCA. A volume of 1 ml of 5% TCA was added to each plate and incubated at 80°C for 30 min. The remaining TCA was aspirated and each well was resuspended in 1 ml of 0.1% NaOH and 0.1% SDS, rinsed with 1 ml of PBS, and counted using Ecolite® scintillation cocktail.

Deoxycytidine deaminase assay

Deoxycytidine deaminase (dCD) activity was determined by measuring the rate of conversion of deoxycytidine monophosphate (dCMP) to deoxyuridine monophosphate (dUMP) according to the method of Camiener [18]. Panc-1 cells were lysed by freeze–thaw (3×) and protein concentration determined by BCA assay (Pierce, Rockford, IL).

The enzymatic reaction consisted of 100 mM Tris–HCl (pH 8.0), 10 mM ATP, 65 mM $MgCl_2$, 100 mM dCMP, 300 μg of Panc-1 protein lysate and increasing concentrations of imexon. The reaction mixture was incubated at 37°C for 30 min and stopped by deproteinization at 96°C for 5 min. After centrifugation, 20 μl of the supernatant was measured by HPLC analysis using an Adsorbosphere C18 nucleoside/nucleotide column, 7 μ particle size, 250 × 4.6 mm (Alltech Associates Inc., Waukegan Rd, Deerfield, IL). The gradient consisted of buffer A: 60 mM NH_4 H_2PO_4 in 5 mM tetrabutylammonium phosphate, pH 5.0 and buffer B: methanol in 5 mM tetrabutylammonium phosphate. The gradient was changed linearly from 10 to 20% B over 10 min with a flow rate of 1.5 ml/min and UV detection at 254 nm. Deoxycytidine deaminase activity was determined both as the percentage of dUMP formed from dCMP, and as the fraction of non-deaminated dCMP remaining after a fixed incubation period.

Ribonucleotide reductase activity (RNR) assay

RNR activity was determined by deoxycytidine incorporation according to Zhou et al. [19] with slight modifications and validated using ^{3}H-cytidine incorporation [20]. Briefly, Panc-1 cells were treated with drug for 2 h, rinsed twice with solution A (150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 M KPO_4, pH 7.4, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$) and permeabilized with 0.025 mg/ml lysolecithin in solution A. Plates were rinsed once with warm PBS, and then incubated with [^{14}C]-CDP (6 μl/sample) in solution B (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 8 mM DTT, 0.06 mM $FeCl_3$, 7.5 mM KPO_4, pH 7.4, 0.75 mM $CaCl_2$, 10 mM phosphoenolpyruvate, 0.2 mM dGDP, 0.1 mM dADP, and 0.2 mM dTDP) for 1 h at 37°C. Following incubation, the cells were rinsed three times with ice-cold PBS, twice with 5% TCA and incubated at 80°C for 30 min in 1 ml 5% TCA. Next, 0.1% SDS, 0.1 N NaOH was added to each well, rinsed with 1 ml PBS and counted the entire sample using Ecolite® scintillation fluid. The ^{3}H-cytidine incorporation assay for RNR inhibition was performed as described [20].

Electron paramagnetic resonance (EPR) spectroscopy of the tyrosyl radical of RNR M_2 subunit

Cell lysates from MiaPaCa-2 and Panc-1 human pancreatic cancer cells were analyzed for tyrosyl radical activity using EPR spectroscopy performed on a Bruker ESP-300E spectrometer. The microwave power was set at 2 mW with a frequency of 9.338 GHz. Scanning was performed over a magnetic field strength range of 2,900–3,500 Gauss wherein the unpaired electron from the tyrosyl radical is identified at a B_o (G) of 2.0 (magnetic field strength of approximately 3,343 Gauss or 0.3343 Tesla). The EPR amplitude modulation was set at 5G.

Statistical analysis

Data reported are the mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated. Paired sample *T*-test comparisons were used to analyze differences in the percent cell populations for flow cytometry studies. The effect of drugs on RNR activity was analyzed by using a Factorial Analysis of Variance model [21]: RNR effect $= \alpha + \beta_1$ HU $+ \beta_2$ imexon $+ \beta_3$ GEM $+ \beta_4$ imexon × GEM. The significance of coefficients β_1, β_2, β_3 and β_4 indicates the treatment effect of each drug. A dose effect was evaluated for each agent singly, and then in combination. The Tukey adjustment was applied for multiple comparisons. A logarithmic transformation was used to normalize the data.

Results

Imexon and GEM are synergistic in vivo and in vitro

To evaluate the efficacy of imexon plus GEM, we initially established drug response profiles for each agent alone in a panel of human pancreatic carcinoma cells lines. Table 1 shows the IC_{50}s and Hill coefficients for both GEM and imexon, for all three pancreatic cell lines. The three pancreatic cell lines showed a similar sensitivity to GEM with IC_{50}s of 8–11 nM in all three cell lines; however, the Panc-1 cell line required 96 h to achieve an equivalent amount of cell kill. A similar pattern of sensitivity was seen in the Panc-1 cell line against imexon.

We evaluated drug combinations of imexon (μM): GEM (nM) in 2 formats: (1) using a fixed molar ratio, and (2) using equipotent concentrations at the IC_{25}, IC_{35}, IC_{50}, IC_{65} and IC_{75}. The mean CI indices were 1.5 for MiaPaCa-2 cells and 1.58 for BxPC3 cells, with additivity seen at drug concentrations which produced less growth inhibition and

Table 1 IC_{50} values and Hill coefficients

	Gemcitabine		Imexon	
	IC_{50} nM[a]	Hill coefficient	IC_{50} μM[a]	Hill coefficient
MiaPaCa-2	11.2 ± 1.3	3.3	275.5 ± 54.2	2.2
Panc-1[b]	11.2 ± 2.4	0.9	147.4 ± 4.7	2.6
BxPC3	8.4 ± 2.5	2.1	355.7 ± 114.7	1.5

[a] Mean ± SEM ($n = 3$)

[b] IC_{50} values in Panc-1 cells were determined at 96 h

antagonism at drug concentrations which induced greater growth inhibition. In the Panc-1 cells, the mean CI index was 0.86, indicating additivity or synergy. Similar effects were seen when imexon and GEM were combined in fixed molar ratios (data not shown). We also evaluated whether the sequence of drug exposure altered the combination effects. In these studies, one agent was added 24 h prior to the addition of the second agent for an additional 72 h of exposure to both drugs. The results showed no advantage to adding one agent ahead of the second agent when compared to exposing cells to both agents at the same time (data not shown).

Because the Panc-1 cell line demonstrated the least overall response to each agent alone, but the greatest additivity in vitro, this cell line was used to investigate the combined activity of imexon and GEM in vivo. Mice receiving imexon or GEM as single agents 24 h post-tumor inoculation demonstrated no significant difference in the average number of days for the mean tumor volumes to reach 500 mm^3 (89.1, 85.3, and 84.1 for control, imexon, and GEM-treated mice, respectively (Fig. 1a). However, mice receiving the combination of imexon plus GEM showed a significant ($P < 0.05$) delay with an average of 112.4 days for the mean tumor volume to reach 500 mm^3. At termination, tumor growth inhibition values (T/C) were 104.1, 81.1, and 24.4% for imexon, GEM, and combination-treated mice, respectively (Table 2). Importantly, only the mice treated with the combination had T/C values less than 42%, the level at which a compound is considered active by NCI criteria [16]. Tumor doubling times (Td) were calculated to be 12.7, 9.9, 8.0, and 22.0 days for control, imexon, GEM, and combination treated mice, respectively. Only the GEM plus imexon combination was significantly different from the untreated control group ($P < 0.05$ by ANOVA). Finally, the $\log_{10}$ tumor cell kill (TCK) values for the combination group was 0.553, which was significantly different than either agent individually or untreated control ($P < 0.05$).

Tumor *regression* by imexon, GEM or the combination of the two was also evaluated in SCID mice with established tumors. Mice bearing Panc-1 pancreatic carcinoma xenografts were stratified into treatment groups when the average tumor volume reached approximately 40 mm^3 when treated with either imexon alone (100 mg/kg i.p. q day × 9), GEM alone (180 mg/kg i.p. days 1, 5, 9) or a combination of imexon and GEM on the same schedule. No significant tumor growth inhibition was seen in animals treated with imexon or GEM alone ($P \gg 0.05$) (Fig. 1b). However, the combination induced a mean 75% reduction in tumor growth compared to the controls ($P = 0.013$, Fig. 1b). After this initial tumor regression for the combination, no further treatment was administered and the tumors began to regrow.

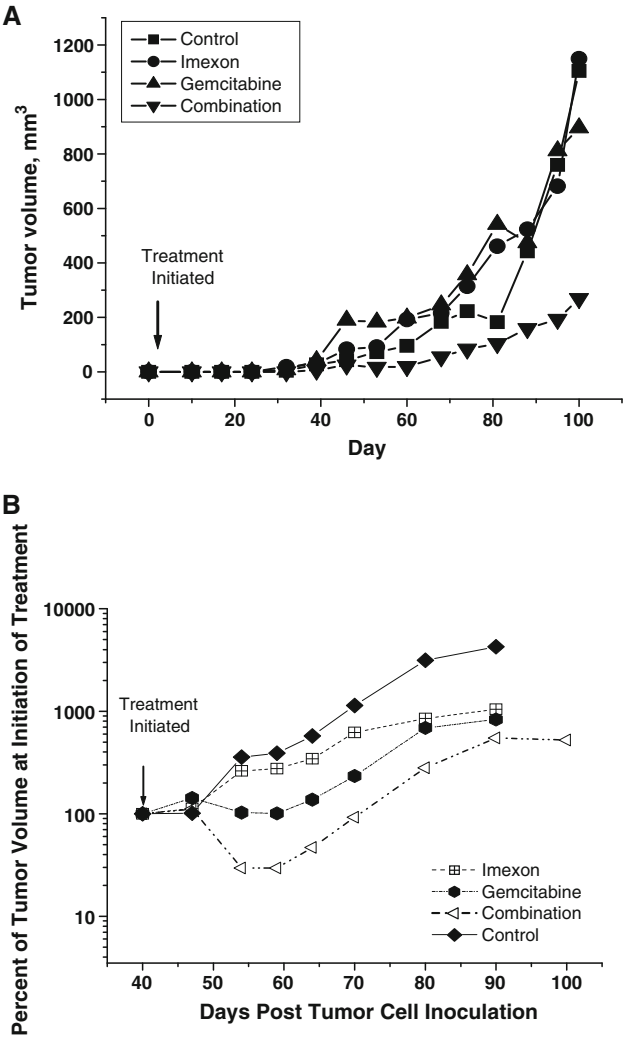

Fig. 1 Synergistic inhibition of Panc-1 tumor growth in SCID mice by the combination of GEM and imexon. **a** Tumor volume in mice treated with both 100 mg/kg imexon (daily × 9) and 180 mg/kg GEM (days 1, 5 and 9), when drug treatment was initiated 1 day after inoculation with Panc-1 cells. The data represent $n = 3 - 4$ per group. **b** Tumor regression in mice treated with both 100 mg/kg imexon (daily × 9, days 40–48) and 180 mg/kg GEM (days 40, 44 and 48), when drug treatment was initiated when tumors were palpable, approximately 40 days after Panc-1 cell inoculation. The data represent $n = 3 - 4$ for each group

Pharmacokinetics of imexon and GEM in Balb/C mice

The summary pharmacokinetic parameters for the four different groups presented in Table 3 show that the pharmacokinetics of GEM did not substantially change when imexon was added. There was an apparent delay and increase in the GEM C_{max}, but this change did not affect the overall AUC of GEM. In contrast, there were several changes in the pharmacokinetics of imexon when GEM was added, particularly a substantial increase in the C_{max} and the AUC. These distribution patterns are presented in Fig. 2a (plasma GEM levels over time) and Fig. 2b

Table 2 Panc-1 tumor response in SCID mice using NCI criteria

	(T–C) (days)	(T/C) (%)	(Td) (days)	(TCK)
Control	0.0	100.0	12.7	0.0
Imexon	<Control	104.1	9.9	<Control
GEM	<Control	81.1	8.0	<Control
Combination	23.3*	24.4*	22.0*	0.553*

The data represent $n = 3$ or 4 for each group

T–C: Tumor growth delay value is the difference in the mean time (days) for the tumors in the treated groups to reach 500 mm^3, when compared to control mice

T/C: Tumor growth inhibition value is the mean tumor volume of the treated mouse divided by the mean tumor volume of the control mouse at termination, represented as a percent

Td: Tumor doubling time, in days. The Td is calculated from a log:linear growth plot in the control cells over the range of tumor volumes from 0 to 500 mm^3

TCK: $\text{Log}_{10}\,\text{cell kill} = \frac{\text{T}-\text{C(days)}}{3.32\times\text{Td}}$

* $P \leq 0.05$ by analysis of variance

(plasma imexon levels over time). These data demonstrate that the increase in the AUC of imexon occurs early, with no change in the slope of the terminal imexon elimination curve. The addition of GEM resulted in a 73% increase in imexon's AUC, and a near-halving of imexon's clearance compared to imexon administered alone ($P < 0.05$). Of interest, there was no change in the $t_{½}$ of imexon when combined with GEM.

Biochemical interactions of imexon and GEM

To investigate potential mechanisms of the imexon-GEM interaction, we evaluated cellular uptake, activation and elimination of [^{3}H]-GEM in the presence or absence of imexon. Cellular uptake of [^{3}H]-GEM by nucleoside transporters was measured at time points ranging from 15 min to 4 h [22]. [^{3}H]-GEM transport was not significantly affected by the presence of imexon (data not shown). Control experiments demonstrated that adding a tenfold excess of unlabeled GEM competitively inhibited radiolabeled [^{3}H]-GEM uptake.

We also examined [^{3}H]-GEM incorporation into Panc-1 DNA, but did not see any difference in [^{3}H]-GEM incorporation into DNA even at concentrations of imexon up to 500 μM (data not shown). Deoxycytidine (dC) was used as a positive control, and as expected, effectively blocked [^{3}H]-GEM incorporation into DNA.

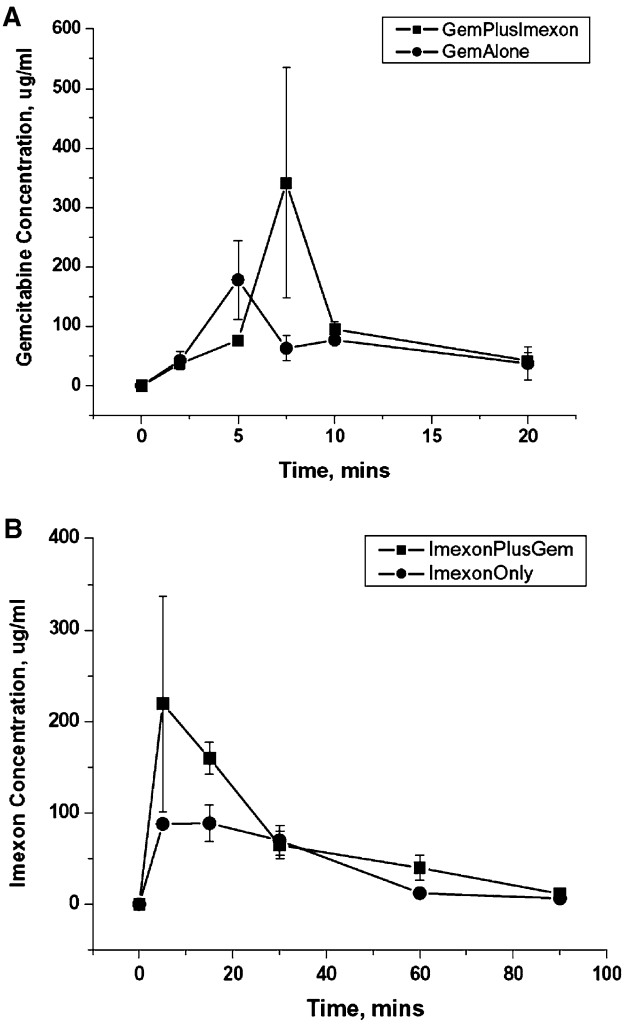

Fig. 2 The pharmacokinetics of GEM and imexon in non-tumor bearing Balb/c, mice ($n = 4$/timepoint). A single dose of 150 mg/kg imexon or 180 mg/kg GEM or a combination of agents was administered. **a** Plasma concentration × time plots for GEM as a single agent (*filled circle*) or in combination with imexon (*filled square*). **b** Plasma concentration × time plots for imexon as a single agent (*filled circle*) or in combination with GEM (*filled square*). Results are the mean ± SEM, $n = 4$

Table 3 Summary of pharmacokinetic parameters for imexon and GEM in non-tumor bearing Balb/C mice

	Mean imexon values		Mean GEM values	
Pharmacokinetic parameter	Imexon	Imexon and GEM	GEM	GEM and imexon
C_{max} (μg/mL)	89	219	237	341
T_{max} (min)	15	5*	5	7.5
AUC (μg/mL*min)	3,650.9	6,830.6*	2,197.7	2,455.9
CI/F (mL/min/Kg)	38	22*	81.9	73.3
Vb/F (L/Kg)	0.99	0.66	1.23	0.62
$T_{1/2}$ (min)	18.2	20.8	10.4	5.9

* $P < 0.05$ compared to single agent controls

Deoxycytidine deaminase (dCD) is believed to mediate the majority of GEM inactivation and this activity is reported to require reduced sulfhydryls [18]. To determine if the pro-oxidant activity of imexon could inhibit dCD activity, cell lysates from Panc-1 cells containing dCD enzyme were incubated with 100 mM dCMP in the presence of imexon or the sulfhydryl oxidant, *p*-chloromercuribenzoate (CMB) as a positive control [18]. The results show that imexon concentrations up to 1 mM had no effect on dCD activity measured either as the loss of dCMP (Fig. 3a) or the formation of the deaminated product, dUMP (Fig. 3b). In contrast, the thiol oxidant CMB significantly inhibited dCD activity in a dose-dependent manner at concentrations ≥10.0 μM (Fig. 3a, b).

Ribonucleotide reductase activity

Ribonucleotide reductase (RNR) catalyzes the rate-limiting step in DNA synthesis and repair. This enzyme is known to require reduced cysteines for proper enzymatic function [23–25]. The activity of RNR was measured by deoxycytidine diphosphate incorporation in Panc-1 cells, where we saw approximately a 50% inhibition of RNR activity by 500 μM imexon (Fig. 4a). Using the alternate method of radiolabeled cytidine incorporation, we found that both imexon and GEM significantly inhibited RNR compared to the untreated controls ($P < 0.0001$ for both agents alone). The inhibitory effects on RNR were increased for all concentrations of the imexon-GEM combinations compared to imexon alone for the MiaPaCa-2 cells, but the results did not quite reach statistical significance (Fig. 4b, $P = 0.092$). For the Panc-1 cells, the imexon/GEM combination was not more inhibitory compared to imexon alone ($P = 0.7291$) (Fig. 4c). These results were normalized for both DNA content and for a slight reduction (10.3 or 16.6%) in cellular uptake of cytidine following exposure to imexon or imexon plus GEM, respectively.

To discern whether imexon might be affecting the tyrosyl radical in the catalytic (M_2) domain of RNR, imexon-treated cells lysates from MiaPaCa-2 cells were subjected to EPR analyses. These results showed no decrease in tyrosyl radical activity with imexon. The relative EPR amplitudes were 0.7 for the control and 1.0 for imexon indicating a slight *increase* in tyrosyl radical activity with imexon. As expected, the positive (inhibitory) controls of the free radical scavenger hydroxyurea (relative EPR amplitude 0.2) and the iron chelator deferoxamine (relative EPR amplitude 0) both significantly inhibited the activity of the tyrosyl radical. These results show that imexon's effect on RNR activity does not involve inhibition of the tyrosyl radical activity at the M_2 subunit, suggesting that the inhibitory effect is at the M_1 subunit.

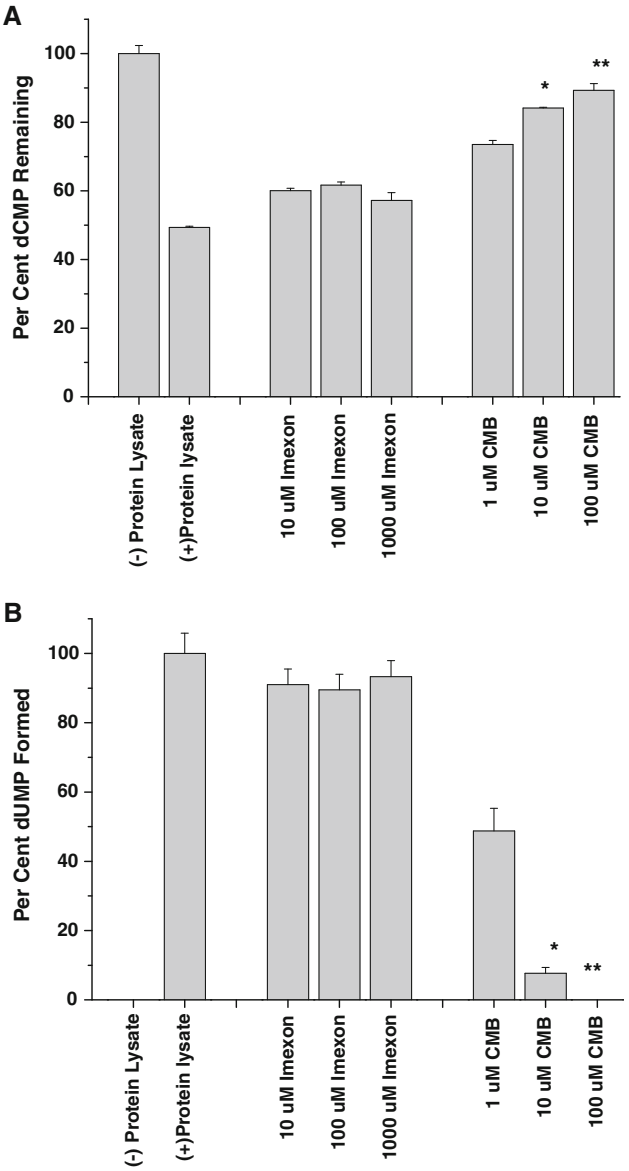

Fig. 3 Deoxycytidine deaminase (dCD) activity in Panc-1 cells. dCD activity in Panc-1 cell lysates was measured by the enzymatic conversion of dCMP to dUMP. The (−) and (+) control lanes represent activity in the absence or presence of protein lysate. **a** dCMP formation in the presence of imexon or *p*-chloromercuribenzoate (CMB), expressed as percent control, compared to dCMP remaining in the absence of protein lysate. **b** dUMP formation in the presence of imexon or CMB, expressed as percent control, compared to dUMP formed in the presence of protein lysate. The data represent the mean ± SD of three independent experiments. (* $P < 0.05$; ** $P < 0.01$)

Discussion

The present study demonstrates that both imexon and GEM have cytotoxic activity in pancreatic cancer cell lines. When imexon and GEM are simultaneously administered in vitro at fixed ratios or at equipotent concentrations required to produce 25–75% of cell growth inhibition, the result is largely additive. In contrast, mice treated with the

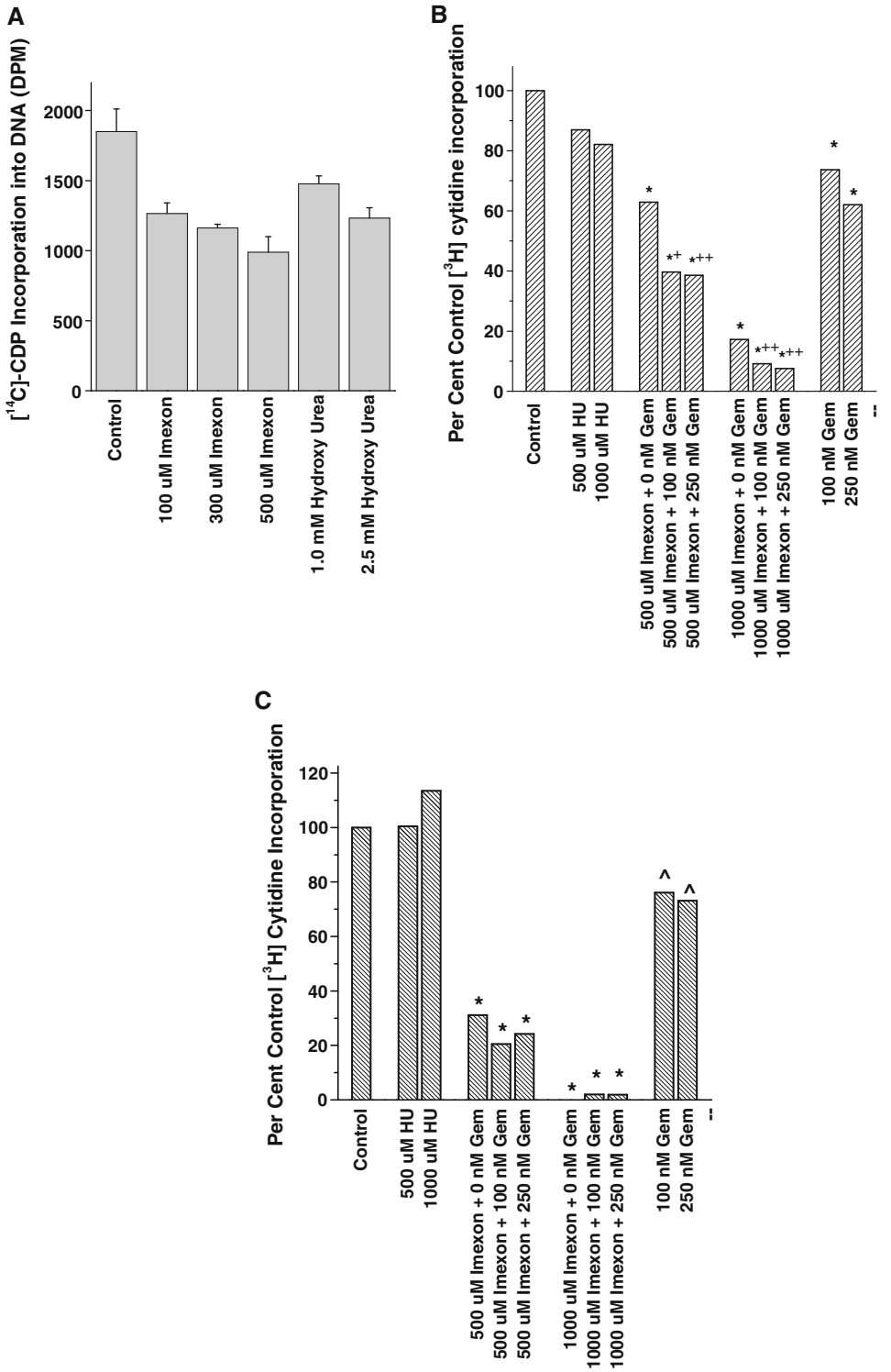

Fig. 4 Ribonucleotide reductase (RNR) activity in Panc-1 and MiaPaCa-2 cells. **a** Panc-1 cells were treated with imexon or hydroxyurea for 2 h, and then incubated with [^{14}C]-CDP for 1 h at 37°C. Cells were harvested and TCA precipitable DNA measured. Results are the mean ± SEM, $n = 3$. RNR activity was also measured in MiaPaCa-2 (**b**) or Panc-1 (**c**) cells treated with hydroxyurea, imexon, GEM, or a combination of imexon and GEM for 18 h, then pulsed with [^{3}H]-cytidine for 30 min before extracting total cellular DNA. Correction for total cellular uptake of radiolabeled cytidine and cellular protein content was made. Results shown are the mean per cent control [^{3}H]-cytidine incorporation ($n = 3$). Statistically significant differences from the control are noted by an asterisk (*, $P = 0.0001$) using ANOVA. Statistically significant differences for the combination are noted by ($^{+}$, $P = 0.012$, $^{++}$, $P = 0.001$ or ^, $P = 0.0014$) when compared to the single agent alone

combination demonstrated marked tumor growth inhibition with substantial tumor regression in a human xenograft model. The explanation for the greater degree of inhibition seen with the combination in vivo may be related to the pharmacokinetic interaction between GEM and imexon which results in increased plasma exposure to imexon when combined with GEM. The molecular basis for the pharmacokinetic interaction, which resulted in a doubled AUC and reduced clearance of imexon with no change in half-life, is not known. Alternatively, tumor growth in vivo may have greater requirements for RNR activity.

The biochemical mechanisms for an interaction between imexon and GEM were evaluated in vitro by examining the various steps involved in GEM uptake, activation and

inactivation. In short, the results showed that imexon did not affect the cellular uptake of GEM, its phosphorylation by deoxycytidine kinase (dCK) to its active metabolites [26, 27] or the incorporation of GEM triphosphate into DNA, which is believed to comprise the primary cytotoxic mechanism of GEM [28]. Conversely, we explored the inactivation of GEM which can occur by phosphorylase cleavage [29] and mainly by deoxycytidine deaminase activity [30]. Despite the fact that deoxycytidine deaminase is reported to be highly dependent on reducing equivalents for activity [18], and the fact that imexon is known to bind to thiols in tumor cells [8, 9], we found no inhibition of dCD by imexon in Panc-1 pancreatic cancer cells.

In addition to masked DNA chain termination caused by GEM triphosphate, GEM diphosphate inhibits RNR to block DNA synthesis [31, 32]. In the present study, we demonstrated significant inhibition of RNR activity using pharmacologically relevant concentrations of imexon. The degree of inhibition by 500 μM imexon exceeded the inhibition induced by 2.5 mM hydroxyurea, the only commercially available RNR inhibitor currently used as an anticancer agent. The modest inhibition of RNR by hydroxyurea in the Panc-1 cells was similar to results reported in KB epidermoid cancer cells [19]. Importantly, the inhibition of RNR was increased when imexon was combined with GEM but a statistically significant effect was limited to the MiaPaCa-2 cell line. The precise molecular site(s) of RNR inhibition by imexon have not been determined. The RNR enzyme is composed of homodimeric M_1 (regulatory) and M_2 (thiyl radical) subunits that each requires reduced cysteine residues for enzymatic activity [32]. The M_1 subunit contains essential cysteines at Cys225 and Cys462 which could be targets of imexon. During catalysis, these form an intermediate intramolecular disulfide bridge which is reduced, in turn, by two other cysteines, Cys754 and Cys759 located on the M_1 subunit. The M_2 subunit is also believed to be sensitive to thiol oxidants, since sulfhydryl groups of the active site cysteines are directly involved in the reduction of the substrate [33]. A previous thiol-oxidant anticancer agent, caracemide was similarly shown to inhibit RNR activity by oxidizing cysteine or serine groups in the M_1 subunit [34–36]. Additionally, the anticancer agent cisplatin has also been shown to inhibit RNR activity by instantaneously and irreversibly binding to thiol groups in M_1 subunit [37]. These results are compatible with the current findings with imexon wherein inhibition of RNR activity occurred without affecting the tyrosyl radical of the M_2 subunit.

In summary, the current findings show that imexon and GEM are additive in vitro but can be greater than additive in vivo. Like GEM, imexon inhibits RNR activity, possibly by oxidizing key cysteines in the M_1 regulatory subunit. Interestingly, there were no direct effects of imexon on GEM's cellular uptake, activation or degradation in vitro or its pharmacokinetic elimination in mice. The greater efficacy seen in vivo with the imexon plus GEM combination might also be due to a GEM-induced increase in plasma exposure to imexon in vivo.

The combination of imexon and GEM has been evaluated in patients with pancreatic cancer in a phase I dose-escalation trial [38]. This trial reported a 19% partial response rate and an additional 50% rate of stable disease among 76 evaluable patients. Tolerance was also good with dose-limiting gastrointestinal cramping that was reduced by lengthening the infusion time from 30 min to 1 h. Both drugs were administered at their single-agent full dose levels: 875 mg/m^2 of imexon and 1,000 mg/m^2 of GEM on days 1, 8 and 15 of a 4-week regimen. Imexon peak plasma levels achieved averaged 53 μg/mL or 470 μM, which is in the range of imexon concentrations studied herein. Furthermore, we did not see imexon clearance values for the combination that were substantially different from those reported for single-agent imexon in the initial phase I clinical trial [6]. This suggests that the reduced imexon clearance values seen in the mice given GEM may be due to the sequential rapid IV bolus dosing performed in the animal model, compared to the sequential short infusion of imexon over 60 min followed by GEM over 30 min as performed in the phase I clinical trial. The combination of imexon and GEM is currently under study in a randomized phase II trial in patients with metastatic pancreatic cancer.

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