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In vitro basis for schedule-dependent interaction between gemcitabine and topoisomerase-targeted drugs in the treatment of colorectal cancer

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Background: While combination of gemcitabine with anti-topoisomerase poisons is routinely used in oncology, little is known on the biological interactions between these drugs.

Design: To understand the cellular basis for this association, we hypothesized an interaction of the two agents at the topoisomerase level. A real-time RT-PCR method was designed to quantify topoisomerase expression after treatment with gemcitabine (GEM) in two human colon adenocarcinoma cell lines. Efficacy of drugs as single agents and in combination was analyzed on the basis of their cytotoxic effects.

Results: We showed that a) gemcitabine induces expression of all major eukaryotic topoisomerases (I, II α and β) at definite times after drug administration; b) cytotoxicity was more relevant when cells were treated with GEM and the topoisomerase poison within a short period of time. In particular synergistic effects were found when the anti-topoisomerase II agent was given 3 h after gemcitabine or when the anti-topoisomerase I drug was delivered 3 h before or after the antimetabolite.

Conclusions: These findings help explaining the effectiveness of the combined therapy GEM/topoisomerase poisons and suggest a drug administration protocol for clinical treatment.

Key words: antimetabolite, colorectal cancer, cytotoxicity, real time RT-PCR, synergism, topoisomerase poison

introduction

Colorectal cancer is one of the most frequent malignancies in humans and the second leading cause of cancer death in the U.S. [1]. The most active cytotoxic drug against this malignancy, the antimetabolite 5-fluorouracil (5-FU), was developed more than forty years ago, and as a single agent produces responses in only 10 to 15% of patients which in general last less than one year [2]. Efforts to ameliorate these poor results include the use of pyrimidine nucleoside analogues [3].

Nucleoside analogues are very potent antimetabolite prodrugs that need to be phosphorylated in their active form by deoxycytidine kinase. Among them, gemcitabine (GEM) plays an important role. As a single agent, GEM exhibits modest activity against tumors. However, response rates remarkably improve when GEM is used in combination regimens. Association of pyrimidine analogues with topoisomerase-targeted drugs has been reported to be effective in the treatment of several solid malignancies [4, 5].

Topoisomerases I and II (α and β) are essential enzymes in higher eukaryotes. They reversibly cleave one or two strands of duplex DNA, respectively, and allow the removal of torsional stress associated with crucial processes such as replication and transcription. The topoisomerase-DNA covalent intermediates are referred to as 'cleavage complexes' and their stabilization by topoisomerase poisons can generate DNA-strand breaks, which are responsible for drug cytotoxicity [6]. A large number of compounds have been shown to interfere with topoisomerase activity: the natural compound camptothecin (CPT) and its synthetic analogues topotecan and irinotecan are useful as topoisomerase I poisons, while etoposide of the epipodophyllotoxin family, is one of the several drugs listed as topoisomerase II poisons.

Most current chemotherapy regimens for cancer consist of empirically designed combinations, based on efficacy and lack of overlapping toxicity, while scheduling and possible biological interactions between drugs are often overlooked in the development of association profiles. For instance, although administration of GEM in combination with topoisomerase poisons is routinely used in tumor treatment [7], the mechanistic basis for this association is not clear.

In the present work we studied the molecular mechanisms underlying the effectiveness of the combined therapy of GEM with topotecan or etoposide on colorectal cancer *in vitro*, to help a rational optimization of administration protocols *in vivo*. The effect of GEM on topoisomerase expression was measured on two colon cancer-derived cell lines by quantification of topoisomerase I, II α and β mRNA levels through a real-time RT-PCR method. Further, the cytotoxic effects of variably

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scheduled combinations of the test drugs were measured. We were able to show that (i) GEM induces expression of all topoisomerase enzymes at definite times after drug administration; (ii) cytotoxicity was more relevant when cells were treated with GEM and the topoisomerase poison within a short period of time, and, in general, when the topoisomerase-targeted drug followed GEM.

These findings help explaining the effectiveness of the combined therapy GEM/topoisomerase poisons and suggest a drug administration protocol for clinical treatment.

materials and methods

drugs and cell lines

Gemcitabine was provided by Eli Lilly Italia, Topotecan by Smiyhkline Beecham and Etoposide by Bristol Mayer Squibb. Further dilutions were made in DMEM supplemented with heat inactivated 10% fetal bovine serum (FBS), 10 μM glutamine, penicillin (100U/ml) and streptomycin (100 μg/ml). Human colon adenocarcinoma cell lines LoVo and HT-29 (American Type Culture collection, ATCC, Rockville, Maryland) were used. Cells were routinely cultured in DMEM, 10% FBS, 10 μM glutamine, penicillin (100U/ml) and streptomycin (100 μg/ml) and kept at 37°C–5% CO_2.

cytotoxicity

1.5×10^3 LoVo or HT-29 cells/well were plated in 96 well plates in a total volume of 200 μl. The seeding cell concentration was such as to allow a logarithmic growth. After 24 h GEM was added at increasing concentrations (0.01, 0.1, 1, 10 and 100 μM). After 96 h, cell viability was evaluated by a standard MTT assay. Briefly, 10 μl/well of MTT solution (4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) were added to cells. After 3–4 h medium was removed and formazan crystals were solubilized with 200 μl of HCl 0.01 M/10% SDS. Absorbance was measured at 550–570 nm. Each drug treatment was repeated six times. Cytotoxicity was defined as the drug concentration able to induce death in 50% of the cellular population. (IC_{50}).

For drug combination cytotoxicity assay, increasing concentrations of topotecan or etoposide (0.01–10 μM) were administered to LoVo cells 3 h before, 3 h or 24 h after GEM (0.5 μM). After 96 h, cell viability was evaluated by a MTT assay, as described above. Each drug combination was repeated three times.

isolation of RNA and real-time RT-PCR analysis

Total RNA was isolated from LoVo or HT-29 cells following a single step acid guanidium phenol-chlorophorm extraction procedure employing OMNIzol (Euroclone, UK). Real-time quantitative RT-PCR analysis was performed on a Light-Cycler instrument (Roche). Random primer cDNAs were generated from total RNA using MuLV reverse trascriptase (Applied Biosystems). Oligonucleotide primers used to amplify topoisomerase I, topoisomerase II α and β were designed in order to amplify only intron sequences of interest and exclude exon portions of genomic DNA. Primers and TaqMan probes were designed using Primer Express (Applied Biosystems). For topoisomerase I gene amplification forward and reverse primers and probe sequences were, respectively 5′-TGACAGCCCCG-GATGAGA-3′, 5′-TGCCAATCGAGCTGTTGCA-3′, 5′-CATCCCAGCGA-AGATCCTTTCTTATAACCG-3′; the amplified fragment was 69 bp long. For topoisomerase II α forward and reverse primers and probe sequences were, respectively 5′-TTGAAGACGCTTCGTTATGGG-3′, 5′-CCATCA-CAACTGGCCCTCTC-3′, 5′-ACAGATCAGGACCAAGATGGTT-CCCACAT-3′; the amplified fragment was 109 bp long. For topoisomerase II β forward and reverse primers and probe sequences were, respectively 5′-GCGATTATAACCCTGGCAGGT-3′, 5′-CAGACTTCCCTACT-GAGCCACC-3′, 5′-AACATCCAAACAACAAGCAAGAAACCGAA-3′; the amplified fragment was 118 bp long. Absolute quantification was performed against a standard curve obtained by amplifying correspondent cDNAs, which were subcloned into a pGEM vector (Invitrogen). Expression of all target genes was normalized to the endogenous control GAPDH, which was quantified by real-time RT-PCR analysis using TaqMan Ribosomial Control Reagent Kit (Applied Biosystems). PCR reactions were performed in three steps: denaturation (5 min at 95°C), amplification (10 sec at 95°C, 10 sec at 60°C, 8 sec at 72°C for 40–50 cycles), cooling (10 sec at 40°C). The fluorescence signal was monitored using the Light Cycler detector (Roche). 6-carboxyfluorescein (FAM) and 6-carboxil-tetramethil-rhodamine (TAMRA) were the reporter and quencher, respectively. All PCR reagents, including primers and fluorescent dyes, were obtained from Roche Applied Sciences. PCR amplification was performed in glass capillaries (Roche), with a 20 μl final reaction mixture containing 0.25 μM forward and reverse primers, 50 nM TaqMan probe, 4 μM $MgCl_2$, ready to use 'Hot Start PCR reaction mix' (Roche Applied Sciences). Real time RT-PCR analysis was performed using six well/plates. 8×10^5 cells were plated per well.

After 24-h incubation, LoVo or HT-29 cells were treated with GEM (0.5 or 0.06 μM, respectively). Cells were harvested at 2, 3, 6 and 24 h time intervals after drug treatment and total RNA was isolated using OMNIzol; cDNA was synthesized from 3 μg of mRNA with MuLV reverse trascriptase in the presence of random hexamers.

results

GEM is differently effective on two human colon adenocarcinoma-derived cell lines

The effect of GEM as a single agent was evaluated on two different cell lines derived from human colon adenocarcinoma, LoVo and HT-29. The efficacy of GEM as antiproliferative agent was measured by its ability to induce cell death. Both cell lines were treated with increasing amounts of GEM (0.01 μM–100 μM), and after 96 h cellular viability was tested by a standard MTT assay. The results show that GEM was differently effective on the two cell lines (Figure 1). In particular, IC_{50} values corresponded to 0.5 μM and 0.06 μM for LoVo and HT-29 cells, respectively.

GEM induces expression of topoisomerases enzymes in vitro 3 h after treatment

To understand the basis of antimetabolite/topoisomerase poison drug association we measured the effects of GEM on

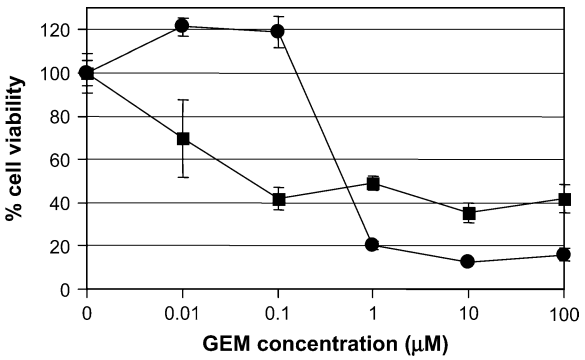

Figure 1. Cytotoxicity of GEM as a single agent. Percentages of cell viability of LoVo (●) and HT-29 (■) cells after treatment with increasing concentrations of GEM measured by MTT assay.

topoisomerases expression in human colon adenocarcinoma cell lines. Topoisomerase I, II α and II β expression was measured by means of mRNA levels, which were quantified by real-time RT-PCR (see **Materials and methods**). The GAPDH gene was used as an internal standard to normalize data. As shown in Figure 2, topological enzyme levels indeed increased after exposition to a constant amount of GEM (0.5 μM in LoVo and 0.06 μM in HT-29, which corresponded to IC_{50} values in the two cell lines, respectively). In particular, topoisomerase expression was considerably higher in LoVo cells as compared to HT-29. In LoVo cell line, enzyme expression was constantly increased in a time range of 2–3 h after treatment with GEM. At 6 h topoisomerase levels returned to almost basal intensity, hence all data were acquired within 6 h. Topoisomerase I was the most remarkably induced: after 2 h levels were 5 times as compared to basal expression (Figure 2A). Topoisomerase II α and β shared a similar degree of induction (2.5 times over basal values) (Figure 2B and C). In the HT-29 cell line, induction of expression was less relevant; nonetheless both topoisomerases I and II α were as well increased after 2–3 h of cell exposition to GEM (128% and 151%, respectively) (Figure 2A and B) and topoisomerase II β showed induction after 6 h (139%) (Figure 2C). Increased expression over basal levels was exhausted within 6 to 24 h.

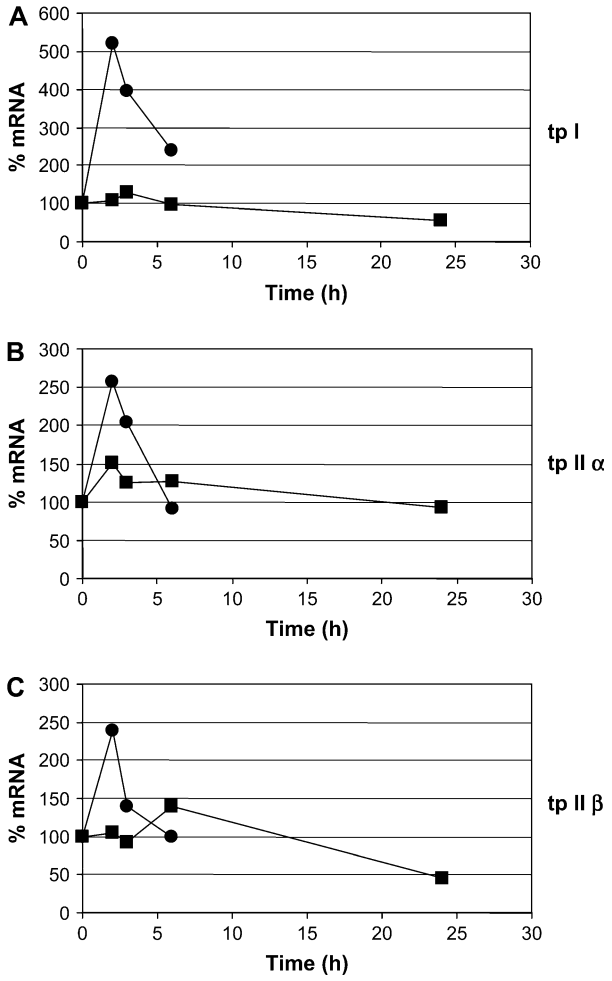

Figure 2. GEM-induced topoisomerase expression. LoVo (●) or HT-29 (■) cells were treated with IC_{50} doses of GEM. At definite time intervals (0, 2, 3, 6 and 24 h), mRNA levels of topoisomerase I (**A**), II α (**B**) and II β (**C**) were quantified by real-time RT-PCR. Values were normalized to the GADPH internal standard. An arbitrary value of 100% was assigned to topoisomerase mRNA levels at time 0 h, which all subsequent values are referred to. Tp stands for topoisomerase.

schedule dictates efficacy of antimetabolite/topoisomerase poison combination

To determine optimal administration schedule of antimetabolite/topoisomerase poison combination, cells were treated with a constant amount of GEM (0.5 μM) and topotecan or, alternatively, etoposide at increasing concentrations (0.01–10 μM), based on three different protocols: 1) topoisomerase poison given 3 h before GEM; 2) topoisomerase poison given 3 h after GEM; 3) topoisomerase poison given 24 h after GEM. Cell viability was measured 96 h after treatment by a MTT assay. Measurements were conducted on LoVo cells since they displayed the highest GEM-induced topoisomerase expression. Results of drug combination were analyzed based on the activity of the topoisomerase poison alone and on the theoretical effect of GEM associated to the anti-topoisomerase compound, calculated as the algebraic sum of the effects of each drug separately. According to the fractional effect analysis, a correspondence of the theoretical and experimental curves would reveal additive outcomes, while experimental curves below or above the theoretical curve would indicate synergistic or antagonistic effects, respectively [8] (Figure 3). Results represented an average of three different experiments for each drug combination. Differences between theoretical and experimental curves proved significant, being standard deviations less than 20% at each point. In the case of the topoisomerase I poison, additive effects were found at topotecan concentrations of 1 and 10 μM for the three different protocols (Figure 3A–C). However, at lower concentrations (0.01 and 0.1 μM), the behavior was clearly biased: synergistic when topotecan was administered 3 h before or after GEM, antagonistic when the anti-topoisomerase was administered 24 h after GEM (Figure 3A–C). In the case of the topoisomerase II agent, the effect of drug combination more evidently depended upon administration protocols: when etoposide was given 3 h before or 24 h after GEM, the outcome was antagonist, while when the anti-topoisomerase was given 3 h after GEM, the results were synergistic at lower etoposide concentration (0.01 and 0.1 μM) and additive at higher amounts (1 and 10 μM) (Figure 4D–F). To note that the synergistic effects, when the anti-topoisomerase drug was given 3 h after GEM, were slightly improved for topotecan compared to etoposide.

discussion

While combination of GEM with anti-topoisomerase poisons is routinely used in the clinical treatment of several types of cancers [7, 9], little is known on the biological interactions between these drugs. To understand the mechanistic basis for this association, we hypothesized an interaction of the two agents at the topoisomerase level. We first measured the IC_{50} values of GEM in two different human colon adenocarcinoma derived cell lines. GEM resulted more effective on LoVo than HT-29 cells, possibly due to the faster replication rate of the

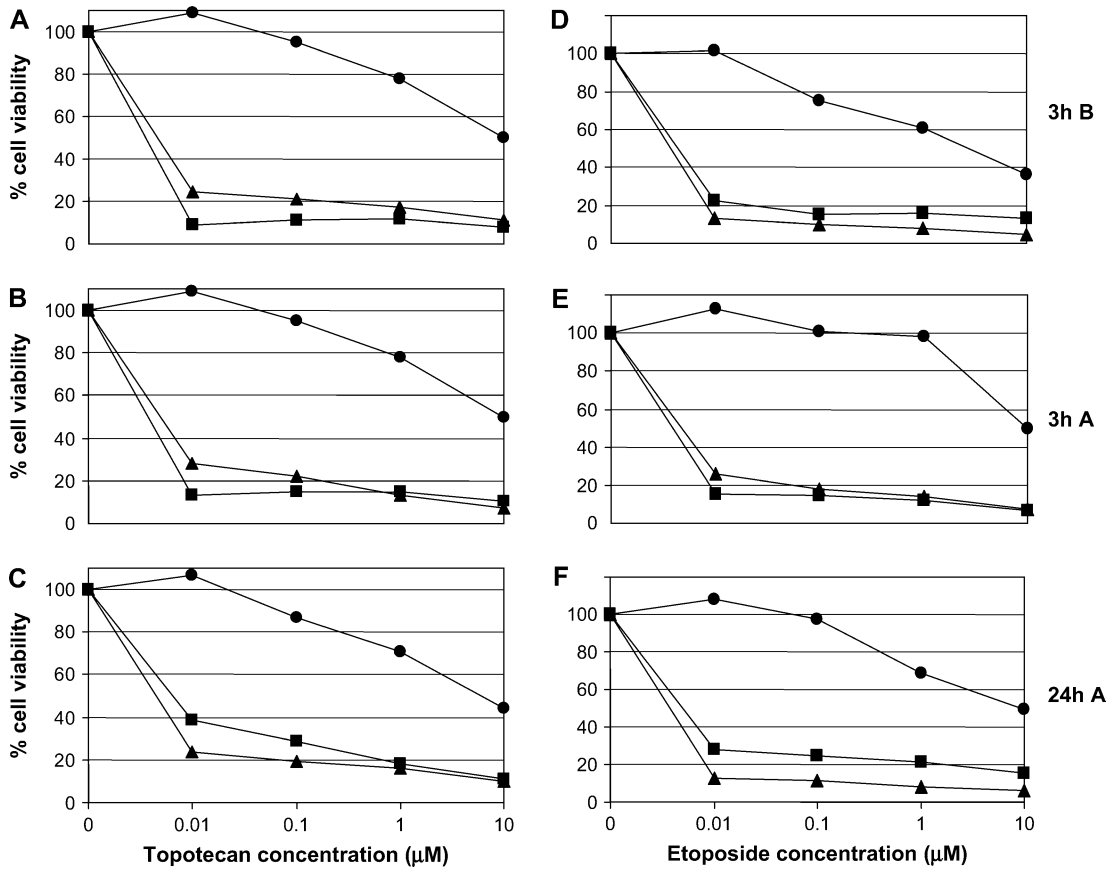

Figure 3. Cytotoxicity of the topoisomerase poisons in combination with GEM (0.5 μM). LoVo cells were treated with increasing amounts of topotecan (**A–C**) or etoposide (**D–F**) separately or in combination with GEM. When in association, the topoisomerase poison was given 3 h before (**A** and **D**), 3 h after (**B** and **E**) or 24 h after GEM (**C** and **F**), as described on the right of the graphs. After 96 h cell survival was measured by a MTT assay. Cytotoxicity curves for the topoisomerase poison alone (●), and experimental (■) and theoretical (▲) cytotoxicity curves for drug combination are reported in each graph. Theoretical curves were calculated according to the fractional effect analysis [8]. A correspondence of the theoretical and experimental curves indicates additive outcomes, while experimental curves below and above the theoretical curve imply synergistic and antagonistic effects, respectively. The results represented an average of three different experiments for each drug combination. Standard deviations were in each case less than 20%.

formers. IC_{50} values were next used as reference concentrations at which cells were treated for the subsequent measure of topoisomerase expression. All the principal eukaryotic topological enzymes were taken into consideration. In fact each has a peculiar role in the genomic DNA processing [11]. We found that treatment of both cell lines with the antimetabolite drug induced overexpression of all the topological enzymes. Interestingly, in all cases expression was maximal within 3 h from treatment with GEM and returned to basal levels within 6–24 h. Likely because of their faster growth, LoVo cells were more susceptible to GEM-mediated topoisomerase induction; in particular, topoisomerase I resulted expressed two times more than the topoisomerases II. Conversely, in HT-29 cells all three enzymes were similarly induced, with a slight preference for topoisomerase II α and β.

Based on the above results, we hypothesized that the highest cytotoxic effect could be achieved when the topoisomerase poison was given in the time range of maximal GEM-mediated induction of the topological enzymes. Hence, cells were treated with a combination of GEM and anti-topoisomerase I or II drugs, given according to three different protocols. Indeed we found that when the topoisomerase poison was given 3 h after GEM, the effect for both drug associations was synergistic at lower anti-topoisomerase doses and additive at higher concentrations. Contrary, when topotecan or etoposide were given as far as 24 h after GEM, the effect was solidly antagonistic. However, if the topological poisons were administered 3 h before GEM, the effect was biased: synergistic/additive for topotecan and antagonistic for etoposide.

We propose that in the case of the topoisomerase II poison, cytotoxicity is exerted in part by GEM-mediated inhibition of DNA polymerase α [12], with termination of nascent DNA helixes or impairing of genome repair; in part by GEM-mediated induction of topoisomerase II, which increases double strand DNA cleavage that is subsequently stabilized by etoposide, resulting in massive DNA damage and cell death. This holds true in the case of the topoisomerase I poison, which stabilizes single-stranded DNA scission. However, the fact that treatment with the anti-topoisomerase I 3 h before or after GEM gave similar results, indicates that effectiveness of GEM/topotecan association is likely mediated by additional mechanisms. It is has been reported that topoisomerase I participates in RNA polymerases I, II, and III-mediated transcription, has a kinase activity, and binds directly to at least

two helicases, nucleolin and SV40 T antigen, acting as a multifunctional protein [13]. It is then possible that impairing of the 'unconventional' topoisomerase I activities induces genome instability, which is enhanced by the following treatment with the antimetabolite agent. Hence, in the case of the topoisomerase I poison, both topotecan and GEM would augment their activity reciprocally. This is in part substantiated by the slightly improved synergistic effect of the anti-topoisomerase I drug compared to that of etoposide.

Interestingly, higher synergistic effects can be noted using low topoisomerase poison concentrations. This fact can be reasonably ascribed to saturation in the number of cleavable complex units that can be generated following stimulation with a constant concentration of GEM. While further analysis will be performed in the near future to confirm these results both including other cell lines and using various antimetabolite /anti-topoisomerase agent combinations, the present findings help explaining the rational basis of the clinical GEM/topoisomerase poisons combination, and most importantly, give a rational indication of the most appropriate drug administration schedule in the clinic.

disclosures

Dr Richter reports no financial relationships with companies whose products are mentioned in this article.

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