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ORIGINAL ARTICLE

Irinotecan–cisplatin interactions assessed in cell-based screening assays: cytotoxicity, drug accumulation and DNA adduct formation in an NSCLC cell line

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Abstract
Purpose The use of in vitro drug cytotoxicity assays for the assessment of drug–drug interactions that lead to synergy may not take into account the many cellular determinants responsible for combination effects. Administration of the anticancer drug CPT-11, for example, is associated with rapid conversion of drug from its active lactone form to the inactive carboxylate form. Thus it is difficult to model, in vitro, the behavior of this drug when used as a single agent and when used in a combination setting, this factor may contribute to the interactions measured. Therefore, the objective of this study was to examine the influence of CPT-11 lactone ratio on the cellular accumulation of CPT-11 when used as a single agent and under conditions where it is used in combination with cisplatin.

Methods A fixed ratio experimental design was used and drug ratios of CPT-11 and cisplatin were judged to be antagonistic, additive, or synergistic to the non-small cell lung cancer cell line, H460, on the basis of the median effect analysis methodology of Chou and Talalay. The influence of extracellular pH on CPT-11 accumulation was evaluated at pH 7.4 and pH 6.6 when the drug was added immediately to the cells or first pre-equilibrated at the indicated pH. These studies were completed in the presence and absence of cisplatin.

Results When CPT-11 was added as a single agent to cells in pH = 7.4 media, the drug underwent hydrolysis to the carboxylate form; however, there was a rapid accumulation of the CPT-11 lactone form which peaked at 3,800 pmol/mg protein by 30 min and drops to 570 pmol/mg protein by 24 h. In pH = 6.6 media, accumulation of CPT-11 lactone was substantially lower over a 60 min timecourse; however, the cellular uptake measured at 24 h was comparable to that observed when the drug was added into pH 7.4 media. When evaluating CPT-11 lactone accumulation in a combination setting with cisplatin no significant difference in either CPT-11 lactone accumulation or cisplatin accumulation was observed, suggesting that drug interactions that led to synergy were mechanistically based. Results are presented which suggest that when cisplatin and CPT-11 are used in combination, there was a significant prolongation of platinum association with DNA compared to results obtained when cisplatin was used alone.

Conclusion These results suggest that the CPT-11 lactone to carboxylate ratio does not influence the accumulation of the active CPT-11 lactone form in H460 cells and that CPT-11 does not influence cisplatin uptake when used in combination. It is argued, therefore,

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that the improved cytotoxicity between CPT-11 and cisplatin, as determined using cell-based assay, has the potential to be preserved in vivo assuming the optimal drug–drug ratio and concentration can be effectively delivered to the tumor.

Keywords Irinotecan · Cisplatin · Non-small cell lung carcinoma · Synergistic drug combination

Introduction

Combination chemotherapy has become the mainstay treatment regimen for the vast majority of clinical cancers. The selection of agents has been traditionally based on the effectiveness of each agent in treating the neoplasm, complementary and distinct mechanisms of activity as well as limited overlapping toxicities. Of great interest is the potential of drug combinations to act in an additive or synergistic fashion. Synergistic drug combinations should allow clinicians to achieve therapeutic effects comparable to that achieved with the single agents, but at substantially lower doses. Thus identification of synergistic drug combinations is of great clinical interest, however commonly used cell-based screening assays that define synergistic interactions are often not predictive of synergy in vivo. This is a significant problem, considering it is faster and cheaper to define interactions in vitro and these assays typically provide the initial data guiding the selection and further development of potentially effective combinations. In an effort to enhance the predictive potential of cell-based screening assays used to determine drug–drug interactions, our laboratory is pursuing pharmacodynamic-based experimental approaches which attempt to relate drug-combination delivery to therapeutic activity and drug–drug synergy [29, 34].

A drug combination that is of significant interest clinically comprises topoisomerase I (Topo I) inhibitors (e.g., CPT-11, topotecan or SN-38) in combination with platinum-based chemotherapeutic drugs (e.g., cisplatin, carboplatin and derivatives). Such combinations have been reported to be synergistically active against many tumor cell lines, including colorectal, leukemia, ovarian, squamous-carcinoma, small cell lung cancer and non-small cell lung cancer (NSCLC) [12, 16, 17, 25, 27, 30, 36]. The combination of CPT-11 and cisplatin has also demonstrated encouraging results in phase I/II/III clinical trials in patients with NSCLC [15, 26, 31]. Mechanistically, Topo I inhibitors stabilize the DNA-Topo I covalent complex leading to replication arrest and the formation of double-strand DNA breaks [24, 28], and platinum compounds form intrastrand and interstrand adducts with DNA [11]. Unrepaired DNA-Pt crosslinks can disrupt DNA replication and transcription, leading to cell death. In combination, the inclusion of topoisomerase I inhibitors with platinum compounds, such as cisplatin, have mechanistically demonstrated synergistic activity. Masumoto et al. [27] demonstrated an increased degree of intrastrand cross-linking in squamous carcinoma cells treated with cisplatin and SN-38 compared to cisplatin alone. Recently, DNA-PT adducts have been shown to exacerbate the cytotoxicity of topotecan by increasing the number of Topo I-DNA complexes [38].

Similar to all camptothecins, CPT-11 activity is dependent on the stability of the lactone ring [6, 10]. CPT-11 can undergo pH dependent lactone ring opening to an inactive carboxylate form, which is unable to interact and stabilize the Topo 1-DNA covalent complex [20, 22]. Burke et al. [5] determined at physiological pH (7.4) that only 13% of CPT-11 is in the active lactone ring form with an equilibrium half-life of 25 min. Additionally, the active metabolite of CPT-11, SN-38, is also rapidly converted to the open carboxylate ring form with an equilibrium lactone fraction of 15% at pH = 7.4 [5]. Differences in the cellular permeability of the lactone and carboxylate forms of CPT-11 have also been described, suggesting that the closed lactone ring can cross cell membranes at a significantly faster rate than the charged carboxylate form of CPT-11 [14, 19]. The higher membrane permeability of the CPT-11 lactone form has been associated with a greater degree of cytotoxicity observed in vitro [14, 19].

When tumor cells are exposed to CPT-11 in a defined drug ratio with cisplatin that demonstrates additive or synergistic cytotoxicity, it is unknown if alterations in the lactone-carboxylate ratio of CPT-11 can influence the cellular accumulation of either drug. Although several groups have characterized the cellular bioavailability of camptothecin and derivatives such as topotecan [1, 8], little information has been reported on the cellular accumulation of CPT-11. Therefore, the objective of this work was to determine the influence of the CPT-11 lactone ratio in drug combinations with cisplatin on the cellular accumulation of these two drugs in the NSCLC cell line, H460.

Materials and methods

Materials

Irinotecan hydrochloride trihydrate (Camptosar, Pharmacia) and cisplatin (Faulding, Montreal, QC) were purchased from the British Columbia Cancer Agency

pharmacy. SN-38 was obtained from LKT Laboratories (St. Paul, MN). EDTA, sodium dodecyl sulfate (SDS), triton X-100, tetrabutylammonium bromide (TBAB), HEPES, Bis–Tris, ammonium acetate, NP40, sodium borate, and sodium acetate were supplied by Sigma-Aldrich (Oakville, ON). All tissue culture flasks and plates were BD-Falcon from BD sciences (Bedford, MA). Phosphate buffer saline, RPMI 1640, and penicillin/streptomycin were purchased from StemCell Technologies (Vancouver, BC). L-glutamine and trypsin/EDTA was obtained from Invitrogen (Burlington, ON) and fetal bovine serum (FBS) was from Cansera (Rexdale, ON). HPLC grade methanol (MeOH) and acetonitrile (ACN) was from Fisher Scientific (Nepean, ON).

Cell culture

The NSCLC cell line, H460, was obtained from ATCC (Rockville, MD). H460 cells were grown in a humidified atmosphere of 5% CO_2 at 37°C and maintained in RPMI 1640 media supplemented with 10%v/v FBS, 1%v/v L-glutamine, 100 μg/ml streptomycin, and 100 UI/ml penicillin.

Assays of cellular reductive capacity

CPT-11 and cisplatin induced H460 cell toxicity was measured using the CellTiter 96 Aqueous assay (Promega, Madison, WI), which measures bioreduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in actively metabolic cells. H460 cells were seeded into flat bottom 96 well plates in a volume of 200 μL and a density of 1,500 cells/well for 72 h cytotoxicity experiments or 5,000 cells/well for 24 h cytotoxicity experiments. Cells were allowed to re-adhere for 24 h at 37°C before addition of drug(s). Media composition for pH = 7.4 was bicarbonate free RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FBS, 1% L-glutamine, penicillin/streptomycin, and 25 mM hepes. Composition for pH = 6.6 media was similar but buffer component was 10 mM Bis–Tris.

For experiments which assessed cytotoxic effects immediately after CPT-11 addition, CPT-11 concentrations were prepared approximately 5 min before addition to cells in pH = 7.4 and pH = 6.6 media. For pre-equilibrated experiments, CPT-11 solutions were incubated at 37°C in pH = 7.4 and pH = 6.6 media for 12–16 h prior to the addition to cells. This time was sufficient to obtain an equilibrium lactone–carboxylate ratio at the indicated pH. Cisplatin concentrations for all experiments were prepared just prior to the addition to cells in either media described above.

Before addition of drug(s) to the cells, the media was aspirated from each well and 200 μl of the treatment group was added and allowed to incubate for either 24 or 72 h. After the specified time, 40 μl of CellTiter solution was added to each group and allowed to incubate for 2 h at 37°C. Cell proliferation was determined spectrophotometrically at 490 nm (MRX microplate reader, Dynex Technologies, Chantilly, VA) by comparing the optical density (OD) of treatment group to OD of control (no treatment, media alone).

Determination of combination index for fixed drug ratios of cisplatin and CPT-11

To determine if fixed drug ratios of CPT-11 and cisplatin were additive, synergistic, or antagonistic to H460 cells, cisplatin and CPT-11 were combined in fixed molar drug ratios of 1:1, 1:4, 4:1, 1:10, and 10:1. CPT-11 and cisplatin was prepared in pH = 7.4 media and added immediately to H460 cells as described above. After 72 h, cell toxicity was measured using the CellTiter 96 Aqueous assay (Promega, Madison, WI) and the results obtained from this assay were subsequently analyzed using CalcuSyn software (Biosoft, Cambridge, UK) which is based on the Median Effect Principle developed by Chou and Talalay [7]. The fraction of cells affected (Fa) following addition of the indicated fixed ratios of cisplatin and CPT-11 were compared against the Fa for each drug when administered alone. Combination index (CI) values were calculated and a CI > 1 was considered to be indicative of antagonism, <1 was considered to be indicative of synergism and CI ≈ 1 was considered to be indicate additive interactions.

HPLC detection of CPT-11 and SN-38 lactone and carboxylate forms

HPLC separation of CPT-11 and SN-38 lactone and carboxylate forms was performed using a 250 × 4.6 mm C18 Symmetryshield column and C18 Symmetryshield guard column (Waters, Mississauga, ON). Gradient elution was used with mobile phase A composed of 75 mM ammonium acetate and 7.5 mM tetrabutylammonium bromide adjusted to pH = 6.4 with glacial acetic acid (Fisher Scientific, Nepean, ON) and mobile phase B was acetonitrile. Gradient profile was as follows: Time = 0 min: 78% A:22% B, Time = 10 min: 64% A:36% B, Time = 12 min: 78% A:22% B, Time = 20 min: 78% A:22% B. A 10 μl sample was injected onto the column (column temp. = 35°C) and eluted at a flow rate of 1 ml/min. CPT-11 and SN-38

forms were detected using a Waters 2475 multi-wavelength fluorescence detector (Waters, Mississauga, ON) set with time program events of λex = 370 nm; λem = 425 nm between times 0–12.5 min for CPT-11 forms, and λex = 370 nm; λem = 535 nm between times 12.5–20 min for SN-38 forms. Prior to injection all samples were maintained at 4°C to reduce conversion between lactone and carboxylate forms. Standard curves of CPT-11 and SN-38 lactone form were prepared by serial dilutions in a 2:1:1 sodium acetate (100 mM):MeOH:ACN pH = 4.0 buffer. For the carboxylate form of CPT-11 and SN-38, serial dilutions were prepared in a 2:1:1 sodium borate (100 mM):MeOH:ACN pH = 9.0 buffer. The limit of quantitation for CPT-11 and SN-38 lactone and carboxylate forms was 10 ng/ml.

Cellular accumulation of CPT-11 and cisplatin

The cellular accumulation of CPT-11 by H460 cells was evaluated over 24 h after the drug was added either with or without pre-equilibration at pH 6.6 or 7.4, as described above. H460 cells were harvested by treating with 1.0 mM EDTA in PBS for 5 min at 37°C followed by scraping. Cells were placed into 2 ml microcentrifuge tubes at a density of 5×10^6 cells/tube containing 10 μM CPT-11 in the desired pH media and a total volume of 1.5 ml. Tubes were then placed into a tumble shaker at 37°C for 15, 30, 60, 120, 240 min and 24 h. After the indicated incubation period, cells were centrifuged at 600×*g* for 3 min at 4°C and a 100 μl aliquot of the extracellular media was removed and placed on ice before the cell pellet was washed three times with ice cold PBS. The cell pellet was then lysed for 5 min with 1% Triton-X 100 at 4°C then centrifuged at 10,000×*g* for 5 min. To the extracellular media sample, 300 μl of a 1:1 ice cold MeOH:ACN solution was added to precipitate proteins and allowed to incubate on ice for 5 min after vigorous vortexing followed by centrifugation at 10,000×*g* for 5 min at 4°C. The supernatants from the extracellular media and cell lysates were injected onto the HPLC as described above for the detection of CPT-11 and SN-38 in their lactone and carboxylate forms. Cellular accumulation was normalized with respect to total protein content using the micro BCA protein assay kit (Pierce, Rockford, IL) following the method specified by the manufacturer.

The influence of cisplatin on the cellular accumulation of CPT-11 by H460 cells in fixed drug ratios was determined using a similar method as described above. All CPT-11 concentrations with or without cisplatin were allowed to pre-equilibrate in pH = 7.4 or 6.6 media to the equilibrium lactone-carboxylate ratio at 37°C for 12–16 h. Cisplatin was added to pre-equilibrated CPT-11 samples just prior to addition to the cells. H460 cells were placed into 2 ml microcentrifuge tubes at a density of 5×10^6 cells/tube containing a fixed concentration of 10 μM CPT-11 in the desired pH media with or without cisplatin at specified drug ratios to a final volume of 1.5 ml. Tubes were then placed into a tumble shaker at 37°C for 2 h. After the incubation time, cells were centrifuged at 600×*g* for 3 min at 4°C and washed three times with ice cold PBS. The cell pellet was lysed for 5 min with 1% Triton X 100 at 4°C then centrifuged at 10,000×*g* for 5 min. The supernatant was then prepared for HPLC analysis as described above for the detection of CPT-11 and SN-38 in their respective lactone and carboxylate forms. Cellular accumulation was normalized with respect to total protein content using the micro BCA protein assay kit (Pierce, Rockford, IL) following the method specified by the manufacturer.

The total cellular platinum content was utilized as a measure of cisplatin accumulation in the presence of CPT-11 in either pH = 7.4 or pH = 6.6 media. The cellular accumulation of cisplatin was evaluated using the same methodology as described above for accumulation of CPT-11. A fixed drug ratio design was used and cisplatin added alone or in combination with CPT-11 was held constant at 50 μM. After an incubation time of 2 h, the cells were washed and the pelleted cells were then lysed by addition of 1% SDS and 1% NP40 and sonication for 30 s (Sonifier W-350, Branson Sonic Power Co, Markham, ON). The resulting solution was analyzed for platinum using an AAnalyst 600 atomic absorption spectrophotometer (Perkin-Elmer Life Sciences, Woodbridge, ON). Total cellular platinum was quantitated from serial dilutions of a platinum standard (Sigma-Aldrich, Oakville, ON) and was normalized to total protein content.

Extent of platinum association with DNA

The amount of platinum (Pt) bound to DNA was determined after exposure of H460 cells to either cisplatin alone or in combination with CPT-11. H460 cells were grown on 150 mm tissue culture plates to approximately 75% confluency. Cells were then incubated with 0.1 μCi/ml of ^{3}H-thymidine for 24 h at 37°C. Subsequently, cells were washed with PBS and exposed to 50 μM cisplatin or a combination consisting of cisplatin and CPT-11 (molar drug ratio of 1:4 and 4:1 where the combination of drugs was prepared within 5 min prior to addition to cells cultured in pH = 7.4 media). After 2 h incubation at 37°C the cells were then washed again

with PBS and incubated with cell culture media at 37°C for an addition time period of 12 or 24 h. Cells were then harvested using 0.25% trypsin and DNA isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) as per manufacturer protocol. The reconstituted DNA pellet was quantified using UV spectroscopy at 260 nm (Hewlett-Packard 8453 UV–Vis spectrophotometer, Sunnyvale, CA) and specific activity determined by liquid scintillation counting (Tri-Carb 1900 TR, PerkinElmer Life Sciences, Woodbridge, ON). Platinum content was quantified using an AAnalyst 600 atomic absorption spectrophotometer (Perkin-Elmer Life Sciences, Woodbridge, ON) after a measured portion of the reconstituted DNA was mixed with 70% HNO_3 and incubated at 65°C for 4 h. The percent of DNA platination was calculated by comparing the amount of DNA bound Pt in cells isolated at 12 and 24 h after addition of the drug or drug combination. To correct for DNA dilution as a result of cell proliferation during the repair times, a dilution factor was applied using the ^{3}H-thymidine-DNA specific activity determined for each group.

Statistical analysis

All results are expressed as the mean ± SD of a minimum of three independent experiments. Comparisons between groups were performed using two tailed *t* test using SigmaStat v2.0 software (SPSS Inc, Chicago, IL) with a significance value of $P < 0.05$.

Results

Activity of CPT-11 and/or cisplatin against H460 cells exposed to pH 7.4 or pH 6.6 media

Cell toxicity, as measured by an assay assessing bioreduction of MTS in actively metabolic cells, of CPT-11 and cisplatin added as single agents to H460 cells in pH = 7.4 media is shown in Fig. 1a. Activity was measured 72 h after drug addition and IC_{50}s of cisplatin and CPT-11, as determined using CalcuSyn software, were 4.5 and 16 μM, respectively. As indicated in Table 1, if CPT-11 was pre-equilibrated in pH = 7.4 media prior to addition to H460 cells, to allow the drug to reach an equilibrium between the lactone and carboxylate forms, the IC_{50} was estimated to be 17 μM. This was comparable to that obtained when the drug was not pre-incubated.

To evaluate whether pH influenced the activity of CPT-11 the drug was pre-incubated in pH = 6.6 media

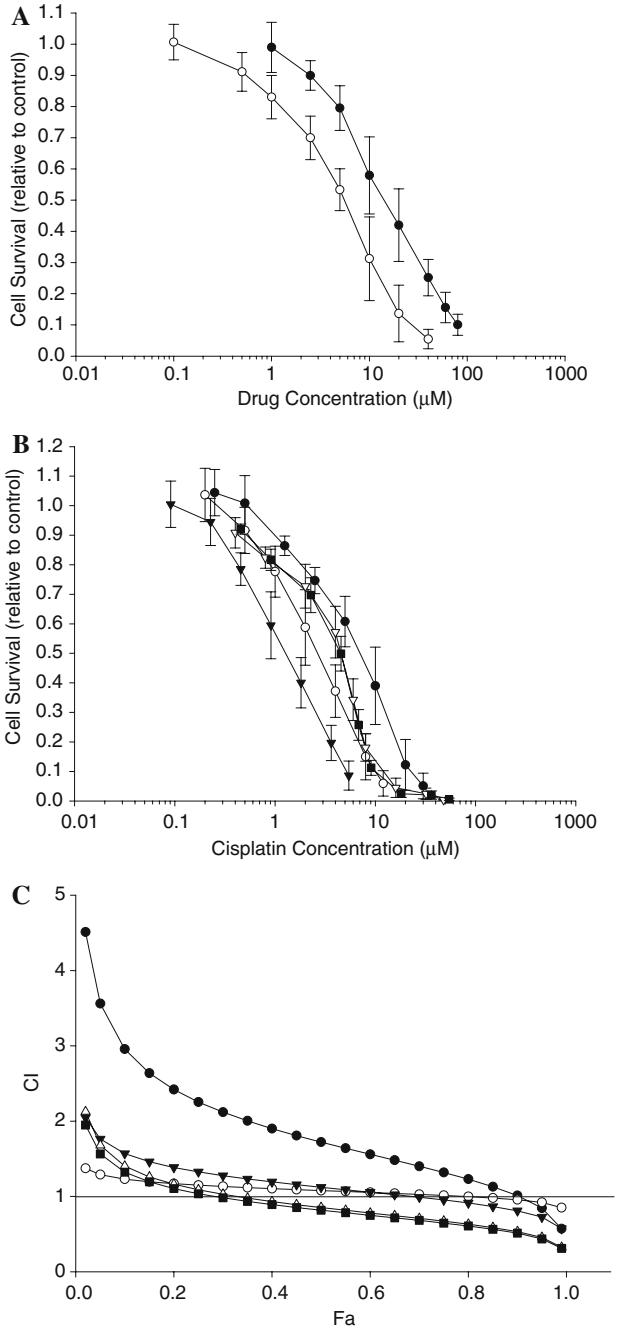


Fig. 1 **a** Individual cytotoxicity of immediately added (*filled circles*) CPT-11 and (*open circles*) cisplatin to H460 cells at 37°C in RPMI 1640 + 10% FBS with 25 mM hepes pH = 7.4 after 72 h drug exposure. Cytotoxicity was determined using the CellTiter Aqueous assay and compared to no treatment group. Results represent mean of six independent experiments ± SD. **b** Combination cytotoxicity of cisplatin:CPT-11 drug ratios of (*filled circles*) 1:1, (*open circles*) 1:4, (*filled inverted triangles*) 1:10, (*open inverted triangles*) 4:1, and (*filled squares*) 10:1 to H460 cells. **c** Fa-CI plot for cisplatin and immediately added CPT-11 combined cytotoxicity to H460 cells at 37°C in RPMI 1640 + 10% FBS with 25 mM hepes pH = 7.4 at cisplatin:CPT-11 drug ratios of (*filled circles*) 1:1, (*open circles*) 1:4, (*filled inverted triangles*) 1:10, (*open triangles*) 4:1, and (*filled squares*) 10:1

Table 1 IC_{50} of CPT-11 for 72 and 24 h cytotoxicity to H460 cells of pre-equilibrated or immediately added CPT-11 in pH = 6.6 or 7.4 media and cisplatin

Treatment group	Exposure time (h)	IC_{50} (μM)[a]
CPT-11 (pH 7.4)	72	16
Pre-incubated[b] CPT-11 (pH 7.4)	72	17
CPT-11 (pH 7.4)	24	127
Pre-incubated CPT-11 (pH 7.4)	24	154
CPT-11 (pH 6.6)	24	199
Pre-incubated CPT-11 (pH 6.6)	24	218

[a] IC_{50} values determined using CalcuSyn software

[b] CPT-11 was pre-incubated for 12 h in media with the indicated pH, a time period sufficient to achieve equilibrium between the lactone (active) and carboxylate (inactive) form of the drug

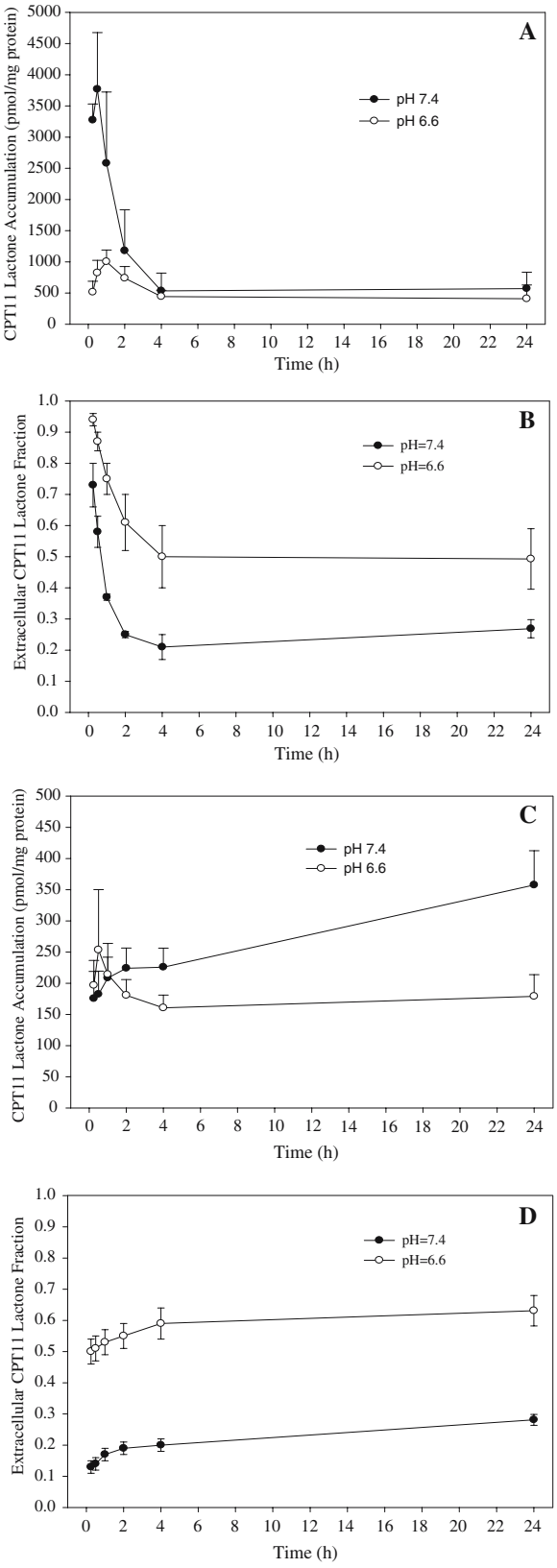
and the effects were determined following addition of the drug to cells exposed to pH 6.6 media. Drug exposure times were minimized to avoid toxicity to the cells by the acidic pH conditions. Cells exposed to pH 6.6 media for 24 h appeared healthy (cells were ~90% viable after a 24 h incubation in pH 6.6 media and this value was not different from that observed for cells culture in pH 7.4 media). Significant toxicity was observed if the cells were incubated for 72 h in the acidic media. The 24 h IC_{50} for CPT-11 at pH = 7.4 was 127 μM compared to 199 μM at pH 6.6 (Table 1). If CPT-11 was pre-incubated at pH 6.6 prior to addition to the cells the IC_{50}s were 154 and 218 μM, respectively (Table 1).

The activity of cisplatin:CPT-11 combinations was determined using a fixed ratio experimental design and the results were plotted as a function of cisplatin concentration (Fig. 1b). The CalcuSyn software was utilized to determine the nature of the drug interaction (synergistic, additive, or antagonistic) for the various fixed drug ratios. The resulting CI values were plotted as a function of fraction of affected cells (Fa) (Fig. 1c). The results suggest that regardless of ratio used, the combination exhibited additive or synergistic interactions ($CI \leq 1$) at high (>0.9) Fa values. At lower Fa values, antagonistic interactions were noted for all ratios evaluated. This is particularly true when the drug–drug ratio (molar) was 1:1, where strong antagonism was noted over a wide range of effective doses (Filled circles, Fig. 1c). When using 4:1 and 10:1 cisplatin:CPT-11 ratios, synergism was noted (as judged by calculated CI values of less than 1) when Fa values were 0.4 or higher (Fig. 1c). Please note that studies assessing drug interactions were completed over a 72 h time course using pH 7.4 media. Since the 72 h IC_{50} for CPT-11 was not significantly effected by pre-incubation (Table 1), these combination studies only considered the use of CPT-11 added without pre-incubation.

Cellular accumulation of CPT-11

Following immediate addition of CPT-11 to H460 cells cultured in pH 7.4 media there was rapid uptake of the lactone form of the drug (Fig. 2a). Maximum levels of CPT-11 (lactone form) were achieved within 30 min, where 3,800 pmol CPT-11 per mg cell protein could be measured (Fig. 2a). The level of cell associated CPT-11 in the lactone form decreased over the 24 h time course. The level of cell associated CPT-11 in the lactone form was approximately 550 pmol/mg protein following a 4 h incubation and this level was not significantly different from that measured for cells exposed to the drug for 24 h (Table 2). When CPT-11 was added immediately to H460 cells in pH 6.6 media, CPT-11 lactone accumulation was significantly lower than that measured for cells incubated in pH 7.4 media. Maximum levels of cell accumulation (1000 pmol/mg protein) were achieved after 60 min (Fig. 2a). Interestingly, following a 2 h incubation the accumulation level of CPT-11 (lactone form) in cells exposed to pH 6.6 media was not significantly different when compared to data obtained from cells cultured pH 7.4 media (Fig. 2a). This was also observed at the 4 and 24 h time points. Cell associated levels of CPT-11 in the lactone, carboxylate or total CPT-11 levels measured at the 24 h time point have been summarized in Table 2. No significant differences in the cell associated levels of CPT-11 (regardless of form) were observed at this time point for H460 cells in pH 6.6 or pH 7.4 media (Table 2). In the extracellular media, a reduction in the CPT-11 lactone fraction was observed when the drug was added immediately to pH 7.4 and pH 6.6 media. This decrease in the proportion of drug in the lactone form occurred over 4 h. Subsequently, the CPT-11 lactone fraction of approximately 0.5 for pH 6.6 and 0.2 for pH 7.4 remained constant (Fig. 2b).

When CPT-11 was pre-equilibrated in the pH 6.6 or pH 7.4 media to achieve an equilibrium lactone–carboxylate ratio prior to addition to the H460 cells, a constant CPT-11 lactone ratio of approximately 0.5 and 0.2, respectively, was maintained in the extracellular media over the 24 h exposure time to H460 cells (Fig. 2d). Interestingly, no difference in the cellular accumulation was found over a 4 h exposure period (Fig. 2b). At 24 h (see Table 2) the accumulation of CPT-11 lactone, carboxylate and total forms in cells cultured in pH = 7.4 media was significantly greater than that measured for cells cultured in pH = 6.6



media. Please note that the reproducibility of the cell accumulation data was vastly better when the drug was pre-incubated prior to addition to the H460 cells.

Fig. 2 Cellular accumulation of CPT-11 lactone form (**a** and **c**) and proportion (fraction) of CPT-11 lactone form in the extracellular media (**b** and **d**) after adding CPT-11 (10 μM final concentration) to H460 cells cultured in either RPMI 1640 + 10% FBS with 25 mM hepes pH = 7.4 (*filled circles*) or RPMI 1640 + 10% FBS with 10 mM Bis/Tris pH = 6.6 at 37°C (*open circles*). CPT-11 was either diluted into pH = 6.6 or 7.4 media immediately prior to the addition to H460 cells (**a** and **b**) or pre-equilibrated at 37°C in pH = 6.6 or 7.4 media for 12 h prior to addition to H460 cells (**c** and **d**). Cells were incubated with the indicated drug concentration for up to 24 h and CPT-11 forms were quantitated by HPLC analysis as described in the Methods section. Results represent mean of three independent experiments ± SD

Table 2 Cellular accumulation of CPT-11 lactone and carboxylate forms after 24 h incubation in either pH = 6.6 and pH = 7.4

CPT-11 form	CPT-11 accumulation (pmol/mg protein)	
	pH = 6.6	pH = 7.4
Immediate		
Lactone	408 ± 220	570 ± 264
Carboxylate	8.8 ± 10	107 ± 88
Total[a]	417 ± 230	677 ± 351
Pre-equilibrated		
Lactone	178 ± 35	358 ± 55*
Carboxylate	3.7 ± 2.3	25 ± 7.1*
Total[a]	182 ± 36	382 ± 59*

*Statistically significant differences between pH = 6.6 and pH = 7.4 using two tailed *t* test ($P < 0.05$)

[a] Total CPT-11 = lactone + carboxylate

Cellular accumulation of CPT-11 and cisplatin in fixed drug ratios

The influence of CPT-11 (lactone form) uptake into H460 cells in the presence of cisplatin is shown in Fig. 3. Because of improve reproducibility, these studies were conducted using CPT-11 that was pre-incubated in pH 7.4 or pH 6.6 media prior to addition to the cells and results were measured following 2 h incubation. The extent of CPT-11 (lactone form) accumulation by H460 cells was not significantly affected by the presence of cisplatin and consistent with the results shown in Fig. 2c, no significant differences were observed between cell uptake levels determined at 2 h for cells incubated in pH 7.4 or pH 6.6 media. Assessments of cell associated CPT-11 in the carboxylate form indicated no significant differences under conditions where cisplatin was present (Fig. 3b).

An examination of cisplatin uptake under comparable conditions indicated that the accumulation of cisplatin, as measured by total cellular platinum, was not affected by the presence of CPT-11 (Fig. 4). It is

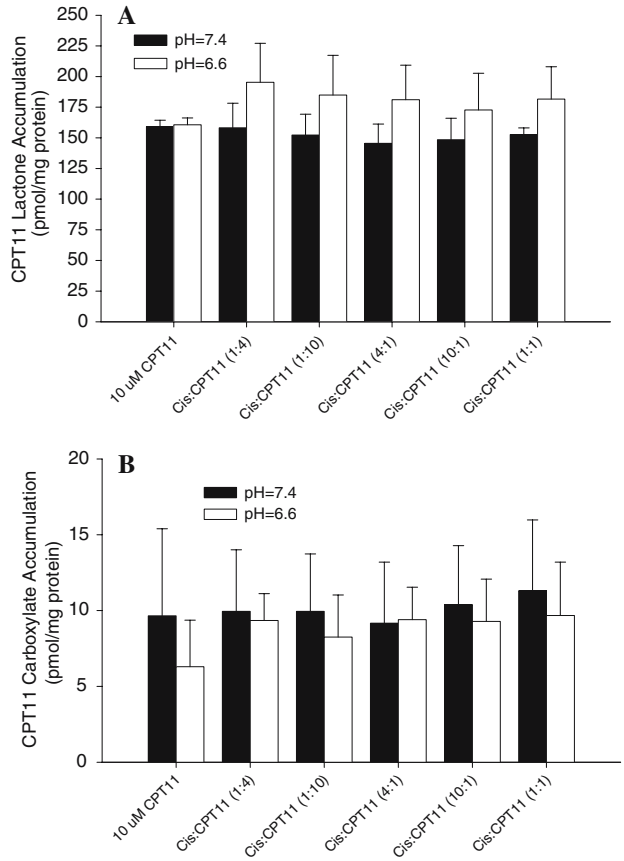


Fig. 3 Cellular accumulation of **a** CPT-11 lactone form and **b** CPT-11 carboxylate form by H460 cells after exposure to cisplatin ± pre-equilibrated 10 μM CPT-11 at defined drug ratios in either RPMI 1640 + 10% FBS with 25 mM hepes pH = 7.4 or RPMI 1640 + 10% FBS with 10 mM Bis–Tris pH = 6.6 at 37°C. CPT-11 was pre-equilibrated at 37°C in pH = 6.6 or 7.4 media for 12 h prior to addition of cisplatin. Cells were incubated with drug concentrations for 120 min and CPT-11 forms were quantitated by HPLC. Results represent mean of three independent experiments ± SD

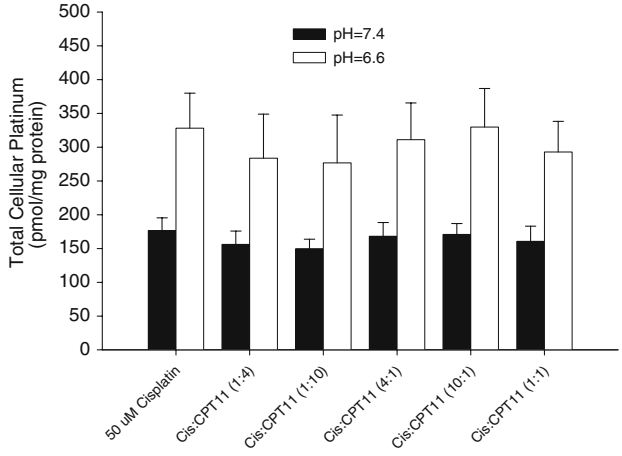


Fig. 4 Total cellular platinum accumulation by H460 cells after exposure to 50 μM cisplatin ± pre-equilibrated CPT-11 at defined drug ratios in either RPMI 1640 + 10% FBS with 25 mM Hepes pH = 7.4 or RPMI 1640 + 10% FBS with 10 mM Bis–Tris pH = 6.6 at 37°C. CPT-11 was pre-equilibrated at 37°C in pH = 6.6 or 7.4 media for 12 h prior to the addition of cisplatin. Cells were incubated with drug concentrations for 120 min and total platinum determined using atomic absorption spectroscopy. Results represent mean of three independent experiments ± SD

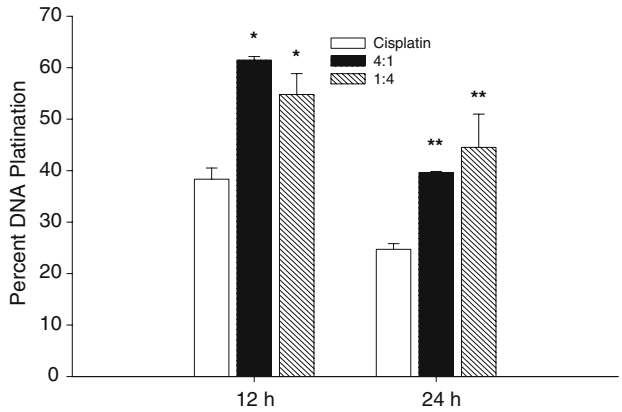


Fig. 5 Platinum association with DNA in H460 cells after exposure of either 50 μM cisplatin alone or in cisplatin:CPT-11 drug ratios of 4:1 and 1:4. Cells were exposed to drug treatments for 2 h followed by a repair time of 12 or 24 h. Cellular DNA was isolated and platinum content was quantitated using atomic absorption spectroscopy. The percent DNA platination is relative to the amount of Pt bound to DNA after 2 h exposure with no repair time. Results represent mean of three plates for each treatment group ± SD. *Asterisk* and *double asterisks* statistically significant differences ($P < 0.05$) to cisplatin alone using two tailed *t* test

interesting to note that cisplatin accumulation in H460 cells was increased almost twofold when cisplatin uptake was measured in cells exposed to pH 6.6 media when compared to cells exposed to pH 7.4 media (Fig. 4).

Association of platinum with DNA in fixed drug ratios of cisplatin and CPT-11

Figure 5 demonstrates the extent of platinum associated with DNA after exposure to either cisplatin alone or in combination with CPT-11 (in a 1:4 and 4:1 molar ratio with cisplatin). In these studies drug or drug combinations were added to cells (pH 7.4 media) and incubated for 2 h. The cells were than washed and the extent of DNA platination was measured at 12 and 24 h, time frames that should be sufficient for DNA repair. When used in combination, regardless of fixed ratio used, there was a significant prolongation in the degree of platinum association with DNA at the 12 and 24 h time points when compared to results obtained using cisplatin alone (Fig. 5).

Discussion

The in vitro assessment of drug combinations using drug cytotoxicity assays, as a guide for the in vivo administration of synergistic drug combinations, may not always account for many of the cellular determinants for drug activity. For instance, CPT-11 can undergo rapid hydrolysis to an inactive carboxylate form at physiological pH [2, 5, 10]. The anionic carboxylate form of CPT-11 and its active metabolite SN-38 (also in an anionic carboxylate form), have been shown to have lower cellular uptake rates into intestinal cells compared to the non-ionic lactone forms [19]. These results suggest that the exposure of tumor cells to high lactone fractions of CPT-11 may be of therapeutic advantage. Since NIH guidelines recommend physiological pH (7.4) for cytotoxicity assessment of chemotherapeutics, in vivo tumor exposure to the active CPT-11 lactone form may differ to that assessed in vitro due to the more acidic microenvironment that can exist in regions of poorly perfused tumor tissue [9]. Therefore, conditions used to establish synergistic cytotoxic interactions between CPT-11 and other chemotherapeutic drugs may not be accurate when considering parameters that affect in vivo bioavailability of CPT-11. This, of course is a complex topic, but considering the ease in which cell-based screening assays can be preformed it is reasonable to address some key biologically relevant variables in culture. The studies reported here focused on the role of pH in governing the activity and delivery of the ring closed and active lactone form of the camptothecin CPT-11, add alone and in combination with cisplatin.

The results shown in Fig. 1b, c indicate that combinations of CPT-11 and cisplatin interact in a manner that results in, depending on the fraction of affected cells (fa), either antagonistic, additive or synergistic cytotoxicity to H460 cells. Further, the results suggest that drug interactions are also dependent on the drug ratio used. Similar drug ratio dependence between cisplatin and CPT-11 was also seen for freshly isolated human colorectal cancer cells [36]. Although for these studies cisplatin and CPT-11 were added to H460 cells simultaneously, it may also be important to assess sequence depend interactions. For example, maximal synergistic cytotoxicity was found between SN-38 and oxaliplatin when the platinum derivative was administered 24 h prior to SN-38 in the human HT29 colon cancer cell line [41]. However, synergistic cytotoxicity was optimal when the small cell lung cancer cell line, SBC-3, were simultaneously exposed to CPT-11 and the platinum derivative, nedaplatin [17].

To model the influence that pH may have on the cellular bioavailability of CPT-11 lactone form and the cytotoxic effects of CPT-11, in vitro cytotoxicity and uptake assay conditions employed in these studies used CPT-11 that was either immediately diluted into either pH 7.4 or pH 6.6 media or pre-equilibrated for 12–16 h before addition to H460 cells. When CPT-11 was immediately diluted in pH 7.4 media, an extensive and rapid accumulation of CPT-11 (lactone form) was found that peaked after 30 min followed by a decline in cell associated drug levels by 4 h (Fig. 2a). A similar rapid and significant accumulation of CPT-11 (lactone form) at early exposure times followed by a decrease in accumulation was also described for the human colorectal carcinoma cell line, HT-29 [4]. This decline in cell associated CPT-11 levels over time was also observed for camptothecin and topotecan in L1210 mouse leukemia cells [8]. Figure 2b demonstrated that CPT-11 is rapidly hydrolyzed to its inactive carboxylate form at pH = 7.4 and an apparent equilibrium between the lactone form and carboxylate form is reached over a time frame that coincides with the decreases in cell associated CPT-11 (lactone form) levels (Fig. 2a).

Under situations where CPT-11 was pre-incubated prior to addition to the cells, significant decreases in the cellular CPT-11 lactone levels were observed and this is likely due to the significantly lower levels of CPT-11 in the lactone form when first added to the cells. In the absence of pre-incubation, CPT-11 (lactone form) accumulation in H460 cells reached maximum levels of ~3,500 pmol/mg protein at 30 min. At this time point, cells exposed to CPT-11 which had been pre-incubated at pH 7.4 exhibited cell associated CPT-11 (lactone form) levels of ~250 pmol/mg protein, a 14-fold reduction. Interestingly, the accumulation of CPT-11 to cells cultured in pH 6.6 media was approximately six-fold lower at 15 and 30 min exposure times compared to cells maintained in pH 7.4 media (see Fig. 2a). This is similar to the larger increase in accumulation of topotecan reported in a human bladder carcinoma cell line, MGH-UI, at pH = 7.4 compared to pH = 6.5 [39]. However, these results are in contrast to the greater accumulation uptake rate of CPT-11 lactone at pHs less than 6.8 compared to physiological pH range reported previously for HT-29 cells [19]. Moreover, a greater accumulation at pH = 6.2 was observed for camptothecin and topotecan in the murine leukemia cell line, L1210 [8]. The slower rate of CPT-11 lactone hydrolysis and the larger fraction of lactone form in the extracellular media at pH 6.6 compared to pH 7.4 would have suggested that we would observe increased levels of CPT-11 (lactone form) accumulation at pH 6.6 (Fig. 2a, b).

It is unclear why for H460 cells the accumulation at early times was lower at pH 6.6 compared to pH 7.4, but maybe do to offsetting differences in the rate of CPT-11 uptake in cells maintained at pH 6.6 and the fact these cells will be stressed, even though there was no change in viability as assessed using dye exclusion methods. We believe it is perhaps more important to critically evaluate CPT-11 (lactone form) accumulation at extended time points (e.g. 24 h). At this time point, the levels of CPT-11 (lactone form) accumulation (see Table 2) could be correlated to single agent cytotoxic activity (Table 1). Albiet not significant, there was a 40% reduction in CPT-11 (lactone) accumulation when comparing cells incubated in pH 6.6 media vs. pH 7.4 media. The IC_{50} at pH 7.4 was 127 μM, which was about 40% lower than 199 μM measured when the cells were incubated at pH 6.6 (see Table 1). Interestingly, the cytotoxicity of topotecan was also found to be higher when exposed to cells in pH 7.4 media, compared to pH 6.5 media [39]. Additionally, camptothecin was more cytotoxic to L1210 cells in pH 7.4 media compared to pH 6.2 [8]. Both of these studies were similar in design to ours, where cells were acutely exposed to acidic pH environments. However it may be important to consider the possibility that cells within tumors may experience acidic microenvironments for extended time periods.

CPT-11 can be metabolically converted by carboxylesterase activity in the liver to an active metabolite, SN-38, which is demonstrated to be 100- to 1,000-fold more potent than CPT-11 in vitro [18, 28]. Although our HPLC assay was able to detect SN-38 lactone and carboxylate forms as low as 10 ng/ml or 25 nM, no conversion was detectable in the cell culture media or cell lysates. In humans, conversion of CPT-11 to SN-38 in the plasma is limited due to low carboxylesterase activity [13]. Recent work has demonstrated that tumor tissue and tumor cell lines can express carboxylesterase and convert CPT-11 intracellularly to SN-38 [3, 32, 33, 35, 40]. However, no intracellular conversion was detected in H460 cells after 24 h exposure times. This may be due to the relatively low carboxylesterase activity detected in H460 cells compared to other NSCLC and SCLC tumor cell lines [37]. Undetectable levels of SN-38 intracellular was noted in two human adenocarcinoma cell lines, suggesting that CPT-11 was primarily responsible for the observed cytotoxicity [33]. Given that the IC_{50} of CPT-11 to H460 cells under our in vitro conditions was 16 μM and the quantitation limit for SN-38 was 25 nM it is reasonable to suggest that intracellular conversion of CPT-11 to SN-38 was not a major contributor to the overall cytotoxicity observed to H460 cells.

One possible explanation for the altered cytotoxicity between CPT-11 and cisplatin when given in defined drug ratios may be due to drug interactions that affect drug uptake in cells. Conflicting results have been reported suggesting a role for enhanced cellular accumulation of either CPT-11 or cisplatin in enhanced cytotoxicity. An increase in platinum accumulation was observed in ovarian cancer cell lines when treated with SN-38 [21]. However, no effect of SN-38 on cisplatin accumulation was observed in the human squamous carcinoma cell line, HST-1 [27]. Figure 3a demonstrates that the accumulation of CPT-11 (lactone form) was not influenced by the presence of cisplatin at fixed drug ratios in either pH 6.6 or pH 7.4 media. Conversely, the presence of CPT-11, regardless of media pH, did not enhance cisplatin accumulation at the various drug ratios tested (see Fig. 4). This suggests that the presence of CPT-11 did not influence the accumulation of cisplatin or CPT-11 under the culture conditions used to assess drug interactions. Interestingly, a greater cellular accumulation of cisplatin was found in cells culture in pH 6.6 media compared to pH 7.4 media (Fig. 4). This finding is similar to the larger increase in cisplatin accumulation found in EMT6 mouse mammary tumor cells cultured at pH 6.0 compared to cells cultured at pH 7.2 [23].

The lack of alterations in CPT-11 or cisplatin accumulation when combined together, suggests that the additive and synergistic activity found for cisplatin:CPT-11 drug ratios of 1:4 and 4:1 may be primarily associated with a concerted mechanistic interaction. Several groups have demonstrated that SN-38 and 9-aminocamptothecin can increase the lifetime of Pt-DNA complexes as a possible mechanism for synergistic cytotoxicity [12, 27, 36, 41]. To determine if a similar interaction of CPT-11 and cisplatin occurred in our model system, we evaluated the association of Pt with DNA at two drug ratios (1:4 and 4:1, mole:mole) which were found to be additive or synergistic over a broad range of effective doses (see Fig. 1c). A significant increase in the association of Pt with DNA was found over 12 and 24 h for both cisplatin:CPT-11 drug ratios compared to cisplatin alone (Fig. 5), suggesting a possible explanation for the increased cytotoxicity to H460 cells when these drugs are used in combination. Although only two drug ratios were assessed in these studies, it is interesting to note that when using an endpoint of Pt association with DNA no drug ratio effect was apparent, yet the combination studies relying on a metabolic endpoint determined 72 h after drug addition clearly suggest ratio-dependent interactions.

In conclusion, altering the CPT-11 lactone ratio as a function of extracellular pH environment did not appear

to influence the overall cellular accumulation of the active CPT-11 lactone form and the resulting cytotoxicity to H460 cells. Additionally, cellular accumulation of cisplatin was not influenced by the presence of CPT-11 nor did cisplatin alter CPT-11 accumulation in the H460 cells. Therefore, it can be anticipated that improved cytotoxic responses observed in vitro between CPT-11 and cisplatin may be preserved in vivo, provided that the appropriate drug:drug ratio and concentration is maintained at the tumor site. Further work is underway to explore the synergistic cytotoxicity of CPT-11 and cisplatin in cells adapted to grow in acidic environments as a revised model of assessing the synergistic potential of these two drugs. In addition, we are exploring the use of nanoscale drug delivery systems to achieve improved coordinated tumor delivery of drug combinations [29, 34].

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