Topotecan and Doxorubicin Combination to Treat Recurrent Ovarian Cancer: The Influence of Drug Exposure Time and Delivery Systems to Achieve Optimum Therapeutic Activity

Nilesh A. Patankar1,3,5, Julia Pritchard3, Mariska van Grinsven3, Maryam Ossooly5, and Marcel B. Bally1,2,3,4

Abstract

Purpose: To provide proof-of-concept data to support use of Doxil–liposomal topotecan (Topophore C) combinations to treat ovarian cancer.

Experimental Design: ES-2, OVCAR-3, and SKOV-3 ovarian cancer cell lines were treated with doxorubicin–topotecan combinations by exposing the cells to drugs from 1 to 72 hours. Pharmacokinetic analysis was conducted following administration of liposomal formulations of these drugs alone and in combination. Efficacy assessments were completed in ES-2 and SKOV-3 ovarian cancer models.

Results: On the basis of drug doses capable of achieving 50% reduction in cell viability over 72 hours, doxorubicin–topotecan combinations were additive in SKOV-3 but highly synergistic in ES-2 and OVCAR-3 cells. Favorable drug–drug interactions increased with increased drug exposure time. Topophore C pharmacokinetic remained unaffected when co-administered with Doxil. In the ES-2 model, Doxil at maximum tolerated dose (MTD 7.5 mg/kg) in combination with free topotecan (MTD 15 mg/kg) did not enhance median survival time (MST) over that achieved with topotecan alone. In contrast, MST was increased to 52 days with combination of Topophore C (MTD 2.5 mg/kg) and Doxil (7.5 mg/kg) compared with untreated animals (MST 18 days) or those treated with Topophore C alone (MTD 5 mg/kg, MST 40 days). In the SKOV-3 model, combination treatments showed better therapeutic efficacy than the individual drugs.

Conclusions: Topotecan–doxorubicin combinations produced additive or synergistic effects which were best achieved when the tumor cells were exposed to drugs over extended time. Doxil–Topophore C combinations are therapeutically superior as judged in two ovarian cancer models. Clin Cancer Res; 1–13. ©2012 AACR.

Introduction

Ovarian cancer is among the most common gynecologic cancers and is the leading cause of cancer-related deaths among female patients with cancer (1, 2). Platinum-refractory ovarian cancer is considered an incurable disease and the treatment options available at present are primarily palliative in nature (3). There is a need to develop improved treatment options for these patients, and a great deal of hope has been placed on the identification and development of molecularly targeted drugs; drugs that can affect signaling pathways uniquely expressed in the patients with ovarian cancer (4). While these more specific therapeutic agents are offering benefits in many cancers, they are not replacing the use of existing cytotoxic drugs. In fact, the targeted drugs often exhibit poor therapeutic effects when used alone and their therapeutic value is achieved primarily in the combination setting. Thus, there remains a strong rationale for exploring the use of strategies that can enhance the effects of existing cytotoxic drugs when given alone and in combination (5–7). Combination chemotherapy is an effective strategy that has shown promise in the treatment of ovarian cancer. The concept of developing drug combinations selected on the basis of synergistic drug–drug interactions has been promoted (5, 6). Synergy can be defined as an interaction that results in therapeutic effects that are greater than that which could be expected from single-agent activities (7–9). An alternative perspective is that a synergistic drug combination can achieve therapeutic effects equal to that achievable with single agents, but at significantly lower, better tolerated, drug doses (5). It is not well understood what factors influence synergy; however, existing evidence suggests that drug dose, drug–drug ratio, and drug sequencing all influence combination interactions.
Translational Relevance

Combination therapy in the treatment of recurrent ovarian cancer can be promising if the combination is designed to achieve therapeutic synergy between selected drugs with proven therapeutic value. As the therapeutic effects of anticancer drugs are highly dependent on drug concentration and exposure time, these variables are critically important in achieving optimal therapeutic outcomes for the combinations. Drug carriers are important tools to achieve increased drug concentrations at sites of tumor growth over extended time periods.

Our results showed that an approved liposomal formulation of doxorubicin (Doxil), when used in combination with a liposomal topotecan formulation, achieved significantly better treatment outcomes against models of ovarian cancer. This combination also achieved improved therapeutic effects at lower drug doses that are better tolerated, and hence will be ideally suited for use in the context of emerging targeted therapies that will ultimately lead to a time when refractory ovarian cancer is treated with curative intent and not merely as a palliative care.

In this study, the role of drug exposure time on drug–drug interaction is being explored with the goal of defining an improved drug combination for use in patients with relapsed ovarian cancer. Currently, the U.S. Food and Drug Administration (FDA) has approved topotecan (12–17) and a liposomal formulation of doxorubicin (Doxil) as single agents for use in second-line therapy for ovarian cancer (18, 19). Topotecan is a camptothecin analogue that specifically targets DNA–topoisomerase I complex and induces DNA damage by stabilizing this complex and thereby acts as a topoisomerase I (topo I) inhibitor (20). Doxorubicin, an anthracycline analogue, acts by stabilizing the DNA–topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (21). Combination of topo I and II inhibitors have been evaluated and results suggest synergy between them when administered sequentially (18, 19, 22, 23). Interestingly, the combinations of topo I and topo II inhibitors currently being pursued for treatment of patients with relapsed ovarian cancer actually involve the use of a free topo I inhibitor (topotecan) and a liposomal nanoparticle (LNP) formulation of a topo II inhibitor (Doxil). However, considering pharmacokinetic differences, the LNP formulation will exhibit remarkably different plasma elimination rates and biodistribution behavior when compared with free topotecan; therefore, the drug levels and the drug–drug ratio at the sites of tumor growth will be variable over time.

It has been shown that the pharmacokinetic and biodistribution behavior of drug combinations can be controlled better when using LNP formulations of drug combinations (24–26). This concept envisioned development of novel combination products (2 drugs formulated in a single LNP composition) or the combination of 2 different LNP formulations. A number of groups, including our own, have been pursuing the development of optimized LNP formulations for topotecan (27–30), and many of these exhibit promising therapeutic potential based on preclinical studies. The formulation recently developed in our laboratory uses pH gradient encapsulation methods combined with drug complexing ability of encapsulated copper to prepare a formulation that exhibits improved drug retention in vivo and improved therapeutic activity when compared with the free drug (30). This formulation, referred to as Topophore C, is now undergoing extensive preclinical evaluations. In an effort to guide the clinical development of Topophore C, our preclinical studies have placed emphasis on its use in relapse ovarian cancer. In the studies described here, Topophore C combinations with Doxil were assessed in an effort initiated around the hypothesis that drug–drug interactions promoting synergy will be dependent on exposure time for the drugs used. As LNP formulations provide a means to enhance exposure time of drugs, combinations of Topophore C and Doxil should provide a means to achieve optimal drug combination effects in vivo. The results indicate that the synergistic effects achieved when using topotecan and doxorubicin in combination can increase when exposure time increases and the therapeutic effects of Topophore C in combination with Doxil are significantly better than those obtained when using combinations of topotecan and Doxil.

Materials and Methods

Materials and chemicals

Doxorubicin (Adriamycin), topotecan (Hycamtin), and Doxil were purchased from the British Columbia Cancer Agency Pharmacy (Vancouver, BC, Canada). ES-2, SKOV-3, and OVCAR-3 human ovarian carcinoma cells lines were obtained from the American Type Culture Collection. SKOV-Luc-D3 cell line was obtained from Caliper Life Sciences. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids and 3H-cholesteryl hexadecyl ether (3H-CHE) from PerkinElmer Life Sciences. 14C-sucrose and Pico-Fluor 40 scintillation cocktail were purchased from PerkinElmer Life Sciences. The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH), and all other chemicals (Reagent grade) were purchased from Sigma-Aldrich.

Cell culture

ES-2 and SKOV-3 cells were grown in McCoy’s 5A medium containing 1.5 mmol/L l-glutamine and 10% FBS. OVCAR-3 cells were grown in RPMI-1640 medium supplemented with 20% FBS, 0.01 mg/mL bovine insulin, and 2.5 mmol/L l-glutamine. ES-2 cells are representative of clear cell carcinoma and in animal models grow aggressively. The SKOV-3 and OVCAR-3 cell lines represent an ovarian cancer referred to as serous adenocarcinoma and in animal
models the SKOV-3 cell line grows very slowly. Cells were subcultured when 80% to 90% confluent by rinsing with PBS and detached with 0.25% trypsin. Once detached, cells were counted using a hemocytometer and diluted in medium to the appropriate concentration (see below) before addition to 96-well microtiter Falcon plates. Cells were maintained in culture for up to 20 passages. After 20 passages, new cells were expanded from frozen stock vials and stored in liquid nitrogen.

**Cell viability assay**

The MTT assay was used as a measure of cell viability. In brief, 100 μL of cell suspension containing required cell number (6 × 10⁴ for ES-2 and SKOV-3 and 1 × 10⁴ for OVCAR-3) was added to the wells of 96-well plates and incubated at 37°C in humidified air with 5% CO₂. After 24 hours, 100 μL of cell culture medium containing appropriate concentration of either topotecan hydrochloride or doxorubicin hydrochloride (0–100,000 nmol/L) was added to these cells. Following 72 hours of this treatment, 50 μL of MTT solution was added to the wells and plates were incubated at 37°C for 3.5 hours. Supernatant was aspirated before dissolving the formazan crystals in 150 μL dimethyl sulfoxide (DMSO). Plates were agitated for 10 minutes, and the optical density of each well was read at 570 nm using a microplate reader (Thermo Multiskan Spectrum). Cell viability was determined by comparing absorbance from treated wells against that from control wells (cells treated with culture medium instead of drug). Concentrations of topotecan and doxorubicin needed to cause 50% loss in viability as judged by the MTT assay (IC₅₀) were determined. To determine the effect of the 2 drugs results in synergistic (CI < 1), additive [CI = 1 (±0.2)], or antagonistic (CI > 1) effects.

**Preparation of Topophore C**

Liposomes were prepared using DSPC and cholesterol (CH; 55:45 molar ratio) by film hydration extrusion as described previously (30). Briefly, DSPC and CH were weighed, dissolved in chloroform, and then mixed such that the final mole ratio of the 2 lipids was 55:45, respectively. A nonexchangeable and nonmetabolizable lipid marker ³H-CHE (5 μCi/100 μmol total lipid) was used to label the liposomes. This solution was then dried to a thin film with a gentle stream of nitrogen gas. The residual chloroform was removed by placing the lipid film under high vacuum for at least 3 hours. Dried lipid films were hydrated at 65°C by mixing with 300 mmol/L CuSO₄ (unbuffered, pH 3.5). Following hydration, the sample was subjected to freeze (liquid nitrogen) and thaw (65°C) cycles. The multimellar vesicles (MLV) obtained were extruded 10 times through stacked polycarbonate filters of 0.1- and 0.08-μm pore size at 65°C using an Extruder (Northern Lipids). The size of the large unilamellar vesicles generated using this method was determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp.). The external buffer of large unilamellar vesicles was exchanged with sucrose (300 mmol/L), HEPES (20 mmol/L), and EDTA (15 mmol; SHE buffer) at pH 7.5 by running the sample through a Sephadex G-50 column equilibrated with the buffer. Liposomal lipid concentration was determined by measuring ³H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer). To load topotecan, liposomes with encapsulated copper sulfate (unbuffered solution, pH 3.5) were suspended in SHE buffer (pH 7.5). Subsequently, 0.23187 (0.5 μg per 1 mg lipid) was added to the liposomes which were then incubated at 30°C for 30 minutes. This mixture and a reconstituted solution of topotecan were warmed separately at 60°C for 5 minutes using a temperature bath. Immediately before addition of the drug to the liposomes a sufficient volume of 1 N NaOH was added such that the final pH of the suspension was 7.0 to 7.5 after addition of topotecan. The final drug–liposome mixture was incubated in a water bath at 60°C for 60 minutes. Following loading, the mixture was brought to the room temperature, and unencapsulated topotecan was separated from liposomes on a Sephadex G-50 column pre-equilibrated with PBS (pH 7.5). Lipid concentrations were measured by scintillation counting of the lipid marker ³H-CHE. Topotecan concentrations were determined by measuring the absorbance at 370 nm on UV–Vis spectrophotometer (Agilent/Hewlett Packard, model: 8453, Agilent Technologies) as described previously (30). Briefly, a portion of the sample collected from the spin columns was adjusted to 100 μL followed by addition of 900 μL Triton X-100 (1% v/v). This sample was heated in a 90°C water bath until the cloud point of the detergent was observed. Subsequently, the sample was cooled to room temperature and the absorbance was determined and compared against topotecan standard curve.
Pharmacokinetic analysis of drug combinations

Doxil (7.5 mg/kg), a combination of free topotecan (15 mg/kg) and Doxil (7.5 mg/kg) or a combination of Doxil (7.5 mg/kg) and Topophore C (5 mg/kg) was administered intravenously to female Ncr-Nude mice (Taconic; 20–25 g; 4 per time point). To administer a combination, the specified doses of the respective treatment agents were mixed just before injection. Following administration, at specified time intervals via cardiac puncture, the mice were terminated by CO2 asphyxiation, and blood was collected by cardiac puncture and placed into EDTA containing microtainers (Becton Dickinson). The blood was stored on ice until they were centrifuged (2,500 rpm for 15 minutes; /C24500 g) to separate plasma from blood cells. Plasma topotecan concentrations were determined by high-performance liquid chromatography (HPLC). HPLC assays were conducted using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump, a controller and a Model 2474 Multi λ Fluorescence Detector (Waters) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by diluting in a acetonitrile:methanol mixture (50:50 v/v). About 10 µL of diluted sample was injected onto a Waters Symmetry Shield RP C18 cartridge column (100 Å, particle size 3.5 µm; 75 × 4.6 mm, Waters). Mobile phase consisted of mobile phase "A" (1% triethyleneamine in water, pH 6.4 adjusted with glacial acetic acid) and mobile phase "B" (100% acetonitrile). The sample temperature was maintained at 4°C, and the column temperature was adjusted to 55°C. Each sample was run for 14 minutes at a flow rate of 1.0 mL/min using a gradient method, where the amount of organic phase was increased from 12% to 40% over 8 minutes. Plasma area under the curve (AUC) and half-life of topotecan were determined from these data using noncompartmental pharmacokinetic (PK) model with the help of PK Solutions software (Summit Research Services).

Doxorubicin concentrations in the plasma were determined by a previously established method (31). Briefly, plasma samples were mixed with 10% SDS and 10 mmol/L H2SO4 (1/1/1) and volume was adjusted to 1 mL with water. This was followed by organic extraction using isopropanol–chloroform solution (1/1; organic phase to sample ratio of 2:1). The samples were frozen at −80°C for 48 hours to facilitate the precipitation of proteins and then thawed at the room temperature. Doxorubicin-containing organic phase was separated by centrifugation at 2,500 x g for 10 minutes at room temperature. Doxorubicin equivalent fluorescence in the organic phase was determined using a luminescence spectrophotometer (Perkin Elmer LS50B) with an excitation wavelength of 470 nm (slit width = 2.5) and an emission wavelength of 550 nm (slit width = 10). The standard curve for doxorubicin was generated by extracting it into the organic phase using the procedure described above, and fluorescence readings from the samples were compared against the freshly prepared standard curve.
Assessment of antitumor efficacy

In vivo assessments of antitumor efficacy were completed in 2 ovarian cancer models. For ES-2 model development, ES-2 cells \( (1 \times 10^5/500 \mu L) \) were inoculated intraperitoneally (i.p.) into female NCr-Fox1nu mice (Taconic). Seven days after tumor cell inoculation, treatment groups were treated i.v. (every 7 days \( \times 3 \)) at the indicated drug doses. Control mice groups were injected with saline. The drug doses were escalated to levels that were close to maximum tolerated doses (MTD), and the health status of all animals inoculated with tumor cells was monitored carefully. In the event that the health status was poor as judged using a scoring method defined in a standard operating procedure, mice were terminated by CO\(_2\) asphyxiation. A balance between measurable signs (weight loss, food intake, water intake, and stool softness) and behavioral changes (activity) as well as physical appearance (coat and eye appearance) was taken into consideration for euthanasia. Survival times of the mice were recorded for all groups, and the day of death was reported as 1 day after the mice were euthanized because of poor health status.

For the SKOV-3 tumor model, \( 5 \times 10^6 \) SKOV-3-Luc-D3 cells in 500 \( \mu L \) were inoculated intraperitoneally into female mice (Ncr-nude, 20–25 g). Tumor growth was monitored one time per week noninvasively by bioluminescent imaging with an IVIS200 imager (Xenogen). The commercially available Living Image software (Xenogen) was used to obtain and analyze images. Before imaging, mice were injected i.p. with 500 \( \mu L \) luciferin solution (15 mg/mL), anesthetized with isoflurane. Animals were imaged 20 minutes (as accurately as possible) after luciferin injection. Regions of interest covering the entire peritoneal cavity were selected for the determination of total photon counts emitted per second. One week after tumor cell inoculation, mice were treated i.v. (every 7 days \( \times 3 \)) at the drug doses specified. Control mice groups were injected with saline. Following administration of the first dose, treated and control mice were imaged once a week to monitor tumor progression. All animals were observed postinoculation at least 2 times a day, more if deemed necessary, for signs of morbidity. The health status of mice was monitored as described above. All animal studies described above were conducted according to a protocol approved by the University of British Columbia’s Animal Care Committee.

Statistical analysis
The results were analyzed using ANOVA. Significant differences between groups were identified using Student–Newman–Keul multiple comparison post hoc test (GraphPad Instat software - GraphPad). Survival curves generated using Kaplan–Meier plots were compared for statistical significance using log-rank (Mantel–Cox) test (GraphPad Prism software, GraphPad). Differences between the groups were considered significant if \( P < 0.05 \).

Results
Cell viability assays following treatment with topotecan and doxorubicin alone and in combination

Results from the MTT assays have been summarized in Supplementary Table SI and Figs. 1 (topotecan) and 2.
Regardless of the cell line used, topotecan was more potent than doxorubicin. As noted, the IC₅₀ for topotecan ranged from a low of 18 nmol/L in the SKOV-3 cell line to a high of 83 nmol/L in the ES-2 cells. Doxorubicin IC₅₀ ranged from a low of 251 nmol/L in the SKOV-3 cells to a high of 539 nmol/L in the ES-2 cells. Exposure studies showed for all 3 cell lines that drug concentration required to achieve an effect level of 50% (Fa = 0.5) decreased as the exposure time increased. The activity of topotecan (Fig. 1) was much more exposure time–dependent than doxorubicin (Fig. 2). Using ES-2 cells (Fig. 1A) as an example, the IC₅₀ was about 780 nmol/L if the exposure time was 1 hour and this concentration decreased almost 10-fold to 83 nmol/L when the exposure time was increased to 72 hours. The exposure time dependency was even more dramatic for the OVCAR-3 cells (Fig. 1C), where exposure times of 1 to 8 hours was insufficient to achieve significant impacts on cell viability regardless of the drug concentration used. The effect of exposure time on the IC₅₀ of topotecan has been summarized for all 3 cell lines in Fig. 1D. There were decreases in the IC₅₀ of doxorubicin as exposure time increased but the effect of exposure time was less than that noted for topotecan, albeit a difference of 5- to 10-fold was obtained for the ES-2 cells (Fig. 2A) and the SKOV-3 cells (Fig. 2B).

The activity of topotecan and doxorubicin used alone and in combination was also assessed and these results have been summarized in Figs. 3 and 4. Fixed ratio combinations of doxorubicin and topotecan that were used were generated on the basis of the IC₅₀ values observed in the three ovarian cancer cell lines which ranged from 15:1 to 6.5:1 (doxorubicin to topotecan). These combinations were tested over a broad range of effective doses and the dose–response curves have been summarized in Fig. 3A (ES-2 cells), B (OVCAR-3 cells), and C (SKOV-3 cells). In all examples, these 72-hour studies show that drug combination was as active (SKOV-3 cells) or more active than the agents when used alone. It is difficult to interpret drug–drug interactions on the basis of the sigmoidal dose–response curves shown in Fig. 3 and for this reason the results were analyzed using the MEP developed by Chou and Talalay (8, 9, 11, 32, 33) and functionalized in the CompuSyn program described in the Methods. CompuSyn analysis of the dose–response curves (Fig. 3D) indicated that topotecan–doxorubicin combination produced CI values of 0.22 and 0.15 against ES-2 and OVCAR-3 cell lines, respectively, and therefore appeared highly synergistic. The CompuSyn analysis of the drug combination data obtained for the SKOV-3 cells suggests that the interactions are additive (CI of 1.1).

The activities of topotecan and doxorubicin were dependent on exposure time, and for this reason, it was assumed that combinations of topotecan and doxorubicin would exhibit increased activity as the exposure time increased from 1 to 72 hours. This is illustrated by the data (obtained for cells exposed to drug for 72 hours) summarized in Fig. 4A (ES-2 cells), B (OVCAR-3 cells), and C (SKOV-3 cells). To assess how exposure time influences the activity of the drugs when used in combination, the resultant dose–response curves were then analyzed through use of the CompuSyn program and the CI values measured at Fa values of 0.5.
(IC\textsubscript{50}) were plotted for different exposure times (Fig. 4D). For ES-2 and OVCAR-3 cells, increased exposure time was associated with decreases in the CI as measured by CompuSyn. For ES-2 cells, the data collected following a 4-hour exposure suggested that the CI value was 1.1, whereas a 72-hour exposure, the CI value was 0.2. The drug combination produced additive effects when tested against the SKOV-3 cell line, regardless of the exposure time.

**Pharmacokinetic analysis of the combination**

To model increases in drug exposure time for combinations of topotecan and doxorubicin, LNP formulations of the drugs were used. Doxil, an LNP formulation of doxorubicin, is already approved for use in relapsed ovarian cancer. Topophore C, an LNP formulation of topotecan that was recently described (30, 31), was selected for the in vivo studies summarized below. Before evaluating efficacy, it was important to assess whether co-administration of the various drug combinations selected affected the pharmacokinetic of the drugs when compared with their use alone. The combinations tested included free topotecan and Doxil as well as Topophore C and Doxil. For this reason, plasma elimination was assessed (see Materials and Methods) following a single i.v. dose of Doxil (7.5 mg/kg), free topotecan (5 mg/kg), or Topophore C (5 mg/kg). These data were compared with results obtained following administration of the drugs in combination (Fig. 5A–C). Results indicated that co-administration of Doxil had a small impact on the plasma elimination profile of free topotecan (Fig. 5A). The measured drug levels at 1 and 4 hours were significantly (P < 0.05) greater than those achieved when topotecan was administered alone. This is reflected in a small increase in topotecan AUC\textsubscript{0–24 h} from 15 to 70 mg h/mL. The plasma elimination of Topophore C was not changed when co-administered with Doxil (Fig. 5B). When Doxil was administered as a single agent, the associated active ingredient doxorubicin showed biphasic plasma elimination with an initial t\textsubscript{1/2}\textsubscript{distribution} of 0.36 hours corresponding rapid tissue distribution. A second t\textsubscript{1/2}\textsubscript{elimination} of 36 hours corresponded to an extended terminal elimination phase (Fig. 5C). These observations were consistent with the earlier reports (34). Co-administration of Doxil with either free topotecan or Topophore C resulted in faster first-phase elimination with 30% of initial doxorubicin concentration remaining in the plasma after 1 hour compared with that of 50% when administered as single agent (Fig. 5C). Doxil elimination rate after first hour was not affected when the drug was co-administered with the topotecan formulations. It was possible that the more rapid initial elimination of doxorubicin following administration of Doxil was a result of topotecan-mediated increased doxorubicin release from Doxil. To assess this, an in vitro assay was conducted to measure drug loss from the Doxil formulation following incubation with free topotecan or Topophore C (in PBS buffer at 37°C for 1 hour). These data (summarized in the insert to Fig. 5C) indicated that under the conditions used, there was no loss of LNP-associated doxorubicin when Doxil was mixed with free topotecan or Topophore C; therefore, the faster initial rate of doxorubicin elimination was not caused by topotecan-mediated doxorubicin release.
In vivo efficacy in two models of ovarian cancer

Antitumor activity of single-agent or combination treatments was determined in vivo using 2 different pseudo-orthotopic models of ovarian carcinoma. As indicated in the Materials and Methods, ES-2 or SKOV-3-Luc-D3 cells were inoculated i.p., and tumor progression (SKOV-3) or tumor-related morbidity (ES-2 cells) were monitored as a function of time following treatment. Treatments were given i.v. using a schedule of every 7 days. Previous studies from our laboratory and others have shown that LNP formulations can enter the peritoneal cavity following i.v. administration in non–tumor-bearing animals (35) and that the amount of LNP localization at this site increases substantially under conditions where there is a tumor burden. Dose–response curves were generated for treatment groups where overall survival (OS) was used as an indicator of efficacy in the ES-2 model. ES-2 tumor growth and associated ascites development were observed rapidly when animals were left untreated. The median survival time (MST) for control animals was 18 days from the day of tumor inoculation with more than 80% of mice terminated by day 21 due to tumor progression (Table 1). Free topotecan administered at 5 mg/kg resulted in a 57% increase in MST (to 29 days; Table 1). This increased to 76% at 15 mg/kg (MST of 32 days), and a further increase in topotecan dose to 20 mg/kg did not show any further improvement in OS and decreases in health status were noted. Although the 20 mg/kg free topotecan dose was tolerated, 15 mg/kg was used for the drug combination studies with Doxil because it was therapeutically superior. As noted previously (30), the MTD of Topophore C was 7.5 mg/kg, at least 2-fold lower than the dose that can be achieved with free drug. Treatment with Topophore C, however, provided significantly better activity than free topotecan when administered at equitoxic doses. The MST of mice treated with Topophore C at 2.5 mg/kg was 39 days, representing a 114% increase in median lifespan when compared with controls and more than a 1.5-fold improvement in activity when compared with results obtained with free topotecan administered at 15 mg/kg. Similar to free topotecan, there was no significant dose–response curve, and when Topophore C was administered at 7.5 mg/kg, (the MTD) the MST was 42 days, representing a 127% increase in median lifespan over controls. The maximum efficacious dose of Topophore C was 5 mg/kg, and this dose was considered appropriate for the drug combination studies.

It is notable that Doxil, when used as a single agent at its MTD of 7.5 mg/kg, exhibited no measurable therapeutic activity (Table 1). Insensitivity to Doxil is consistent with previous reports indicating that clear cell carcinoma (e.g., ES-2) is an aggressive and chemorefractory subtype of ovarian cancers (36–40).

For the drug combination studies, Doxil was administered at its MTD (7.5 mg/kg) and it was co-administered with increasing doses of free topotecan or Topophore C. The dose-escalation studies were conducted to establish...
whether there was an increase in toxicity when the drugs were used in combination. When Doxil was combined with free topotecan given at the 5 mg/kg dose, the MST was 31 days (Table 1), showing a small, but not significant, increase in activity when compared with use of free topotecan alone. Increases in MST were achievable when the dose of topotecan was escalated to 15 mg/kg, where the MST was 37 days when compared with 32 days achieved following administration of topotecan alone at the 15 mg/kg dose. There was some evidence of enhanced toxicity when these drugs were used in combination. For example, significant weight loss (10–15%) was noted following the third dose of the free topotecan–Doxil combination (15 and 7.5 mg/kg, respectively) but the mice recovered within 1 week. This was in dramatic contrast to studies completed with Doxil and Topophore C, where significant increases in drug-related morbidity were noted. For example, combinations of Topophore C and Doxil were not tolerated when dosed at 7.5 and 5 mg/kg, respectively, and the combination produced a greater-than-expected (synergistic) toxicity that was reflected in significant weight loss (up to 25%) and the need to terminate animals due to poor health status. The combination of Doxil and Topophore C, however, exhibited significant therapeutic benefits when the animals were treated with well-tolerated doses. When treated with 0.625 mg/kg Topophore C in combination with Doxil (7.5 mg/kg), the MST was 28 days. Escalation of the Topophore C dose to 2.5 mg/kg resulted in significant improvements in treatment outcomes (MST of 52 days). When Topophore C was used alone at the 2.5 mg/kg dose, the MST was only 39 days. Given the lack of activity of Doxil used as a single agent, this provides strong evidence that the combination of the LNPs is synergistic. The efficacy results summarized in Table 1 have also been presented in the form of Kaplan–Meier survival plot (Supplementary Fig. SI) to highlight the improvements in treatment outcomes achievable with this combination.

The tumor burden after inoculation of SKOV-3-Luc-D3 cells was estimated by assessing increases in luminescent light emitted (photos/s) by the luciferase-modified cells following luciferin injection (see Materials and Methods). Mice with established tumors were organized into treatment groups defined on the basis of data obtained using the ES-2 tumor model and these results have been summarized in Fig. 6 which includes representative images for each treatment group. The SKOV-3-Luc-D3 cells could be visualized 1 day after tumor cell inoculation. Treatment was initiated 7 days after cell inoculation, and the animals were imaged every 7 days. The results obtained 28 days after tumor cell inoculation (the day when the last treatment was provided) and 42 days after tumor inoculation are provided. Control mice exhibited a steady increase in bioluminescence over the 42-day time course, where the increase in signal intensity from day 1 to 42 was 580% (see histogram in Fig. 6). When the mice were treated with free topotecan, they exhibited, on average, a 131% increase in the bioluminescent signal, suggesting that topotecan was therapeutically active in this model. Topophore C (2.5 mg/kg)-treated mice showed only 39% increase in signal at day 42. Doxil (7.5 mg/kg) exhibited good therapeutic effects, where there was only a 92% increase in bioluminescence signal at day 42. When this effective dose of Doxil was combined with free topotecan (15 mg/kg), the increase in signal at day 42 was comparable or slightly higher than that observed when animals were treated with free topotecan or Doxil alone. Combination of Doxil and Topophore C proved to be most effective. Only a 6% increase in bioluminescence signal was noted at day 42.

Discussion

Approaches to define effective combinations in the clinic have not taken into consideration pharmacokinetic factors that could influence drug–drug interactions and engender improvements in treatment outcomes (24, 41, 42). A variety of in vitro approaches have been used to measure drug–drug interactions in cell culture (11, 32, 43); however, the use of in vitro data to predict synergy in vivo remains challenging. We believe that this is due to 2 reasons: (i) drug pharmacology and pharmacokinetics of drugs cannot be adequately mimicked in the in vitro setting and (ii) in vitro conditions cannot be mimicked accurately in vivo. In vitro assays,

### Table 1. Antitumor activity of free topotecan, Topophore C, and Doxil in nude mice bearing ES-2 clear cell carcinoma xenografts following i.v. administration of free topotecan, Topophore C, or Doxil as single agents and as combination treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Drug dose, mg/kg</th>
<th>Median survival, d</th>
<th>% ILSa</th>
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<tr>
<td>Single-agent</td>
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<tr>
<td>Saline (control)</td>
<td>5</td>
<td>39</td>
<td>N/A</td>
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<tr>
<td>Free topotecan</td>
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<td>37</td>
<td>82</td>
</tr>
<tr>
<td>Free topotecan</td>
<td>15</td>
<td>37.3</td>
<td>84</td>
</tr>
<tr>
<td>Free topotecan</td>
<td>20</td>
<td>26</td>
<td>51</td>
</tr>
<tr>
<td>Topophore C</td>
<td>2.5</td>
<td>39</td>
<td>114</td>
</tr>
<tr>
<td>Topophore C</td>
<td>7.5</td>
<td>42</td>
<td>127</td>
</tr>
<tr>
<td>Doxil</td>
<td>7.5</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Combination treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (control)</td>
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<td>20.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Free topotecan + Doxil</td>
<td>5 + 7.5</td>
<td>31.5</td>
<td>55</td>
</tr>
<tr>
<td>Free topotecan + Doxil</td>
<td>10 + 7.5</td>
<td>37</td>
<td>82</td>
</tr>
<tr>
<td>Free topotecan + Doxil</td>
<td>15 + 7.5</td>
<td>37.3</td>
<td>84</td>
</tr>
<tr>
<td>Topophore C + Doxil</td>
<td>0.625 + 7.5</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>Topophore C + Doxil</td>
<td>1.25 + 7.5</td>
<td>43</td>
<td>112</td>
</tr>
<tr>
<td>Topophore C + Doxil</td>
<td>2.5 + 7.5</td>
<td>51</td>
<td>156</td>
</tr>
<tr>
<td>Topophore C + Doxil</td>
<td>5 + 7.5</td>
<td>20</td>
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a Percentage ILS (increase in lifespan): values were determined from MSTs of treated and control (untreated) groups.
although limited, have already shown that drug:drug synergy (or antagonism) is influenced by drug dose (as represented by measured effect level) and drug:drug ratio. These findings have profound implications particularly as drugs are typically combined in vivo under conditions where drug dose and drug:drug ratio cannot be controlled. In recent studies from our group, it was observed that surprising improvements in therapeutic activity can be achieved by controlling the ratios of drugs in various combinations (irinotecan/floxuridine, daunorubicin/cytarabine, or cisplatin/daunorubicin) in vivo through use of well-designed LNPs. Efficacy of select combinations could be achieved at doses that are far lower than those required to achieve similar effects with the free drug (25). These formulations rely on use of drug delivery methods to control the drug:drug ratio within the plasma compartment and the tumor over time. However, these formulations also extend the circulation lifetime of the associated drugs and benefits may arise simply as a result of extended exposure times. This is an important point to consider, given that many in vitro assays rely on endpoints determined 3 to 5 days after drug addition, yet in vivo, the drug exposure time may be considerably shorter. The studies described in this report were designed to assess whether drug exposure time influenced treatment outcomes for a combination that appears to have some therapeutic potential for treatment of relapsed ovarian cancer. The cell-based screening studies summarized here for 3 ovarian cancer cell lines (ES-2, SKOV-3, and OVCAR-3) show that prolonged exposure to topotecan or doxorubicin (added as single agents) enhances
therapeutic effects (see Figs. 2 and 3). This can be attributed to the cell-cycle-specific nature of the drugs used. Topotecan and doxorubicin are known to produce greater effects during the S-phase of the cell cycle (44–46), thus greater cytotoxicity observed on prolong exposure would be a consequence of a greater proportion of cells entering in the S-phase.

The enhanced treatment effects noted with increasing exposure time were also observed with the drugs used in combination (see Fig. 4). These drug combination data were analyzed using the MEP developed by Chou and Talalay (8) to determine whether synergistic interactions are increased or decreased as a function of exposure time. It should be noted that the MEP methodology is built around the concept that combination effects need to be studied at fixed drug ratios and that these effects must be determined over a broad range of effective doses (6–8).

Studies summarized here used fixed molar ratio (IC50 of doxorubicin/IC50 of topotecan, determined at 72 hours), and this ratio was tested over a range of effective doses. However, it is obvious from the data plotted in Figs. 1 and 2 that this ratio would likely change depending on the exposure time used. This highlights the complexity of these studies; however, the ratio used can be justified on the basis of 2 points: (i) the accuracy of the MEP in determining drug:drug interactions is greatest when the affect level measured is 0.5 (i.e., the IC50 dose) and (ii) the MTT viability assay was always completed 72 hours after treatment was initiated.

LNP formulations can be used as an effective means to achieve extended drug exposure in vivo and this is reflected in increased levels of drug in the blood compartment over time as well as enhanced delivery to sites of tumor growth, including tumor growth within the peritoneal cavity (35, 47–49). Doxil and Topophore C formulations have previously shown these abilities and therefore were used in the studies here to increase the exposure of doxorubicin and topotecan to the tumor site. The approach used is reliant on a clinical development plan for Topophore C that would involve a phase II study comparing the effectiveness of topotecan versus Topophore C and subsequently a study evaluating combinations of Doxil, which is already approved for use in the treatment of relapsed ovarian cancer, and Topophore C.

Importantly, the drug combination effects observed when using combinations of topotecan and doxorubicin in vitro were mirror in the in vivo efficacy studies using the LNP formulations. Combination treatments involving LNPs showed superior therapeutic efficacy over single agents in both ovarian cancer models evaluated. Doxil/Topophore C combinations were therapeutically superior to the other combinations tested. Importantly, the changes in Doxil elimination observed in Fig. 5 did not appear to have an adverse impact on therapy. This is perhaps due to the lack of Doxil efficacy in the ES-2 ovarian cancer model and may suggest that the effects in the SKOV-3 model could be better if the pharmacokinetics of Doxil were unchanged when topotecan was co-administered. It is premature to speculate on why Doxil pharmacokinetics changed; however, changes in Doxil elimination were not due to topotecan-mediated release of doxorubicin from the liposomal carrier (Fig. 5C, inset). The effect was observed for both free and LNP formulations of topotecan, so it is likely due to topotecan-mediated change in liposome elimination; however, our laboratory is not able to measure this for the Doxil. It should also be noted that the enhanced therapeutic effects of the LNP combination were also associated with an increase in toxicity. This could be due to changes in Doxil pharmacokinetics and biodistribution and/or due to synergism between the 2 drugs on normal proliferating cell populations in the gastrointestinal (GI) tract and hematopoietic system. The studies presented provide preclinical evidence to support the use of combinations comprising an LNP formulations of topotecan and Doxil, but the results also suggest that combinations of free topotecan and Doxil may provide limited if any therapeutic benefit when compared with the agents used alone. This will eventually be determined in patients.

Conclusion

Cytotoxic activity of topotecan and doxorubicin against ovarian cancer cell lines was observed to be exposure time-dependent. Synergistic interactions observed between topotecan and doxorubicin in vitro were translated very well in vivo and the results suggest that this synergy may best be achieved under conditions when the tumor cells are exposed to both drugs over extended time periods. Concurrent administration of Doxil with Topophore C proved to be effective when used to treat ovarian cancer xenograft models. These results provide proof-of-concept data to support the use of this combination for treatment of recurrent ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N.A. Patankar, M.B. Bally
Development of methodology: N.A. Patankar, M.B. Bally
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.A. Patankar, J. Pritchard, M. Osooly
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.A. Patankar, M.B. Bally
Writing, review, and/or revision of the manuscript: N.A. Patankar, M.B. Bally
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.A. Patankar, M. van Grinsven, M. Osooly
Study supervision: N.A. Patankar, M.B. Bally

Grant Support

This research was supported by grants from the Canadian Institutes of Health Research. N.A. Patankar was supported by fellowships received from BC Innovation Council and University of British Columbia.

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Received August 29, 2012; revised December 7, 2012; accepted December 18, 2012; published OnlineFirst January 9, 2013.


Topotecan and Doxorubicin Combination to Treat Recurrent Ovarian Cancer: The Influence of Drug Exposure Time and Delivery Systems to Achieve Optimum Therapeutic Activity

Nilesh A. Patankar, Julia Pritchard, Mariska van Grinsven, et al.

Clin Cancer Res  Published OnlineFirst January 9, 2013.

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doi:10.1158/1078-0432.CCR-12-2459

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