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Role of Triptolide as an Adjunct Chemotherapy for Ovarian Cancer

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Key Words
Ovarian cancer · Triptolide · Carboplatin · Phosphatidylinositol 3-kinase inhibitor · LY294002

Abstract
Background: Triptolide (TPL) has been identified as the active component of the *Tripterygium wilfordii* hook F plant and demonstrated to possess antitumor properties and induce apoptosis in a variety of tumor cell lines. Since TPL actions are associated with changes in the activities of both p53 and NFκB, which are implicated in the chemoresistance of ovarian cancer, the ability of TPL to be a potential chemotherapeutic for ovarian cancer was considered. ***Methods:*** TPL actions on human ovarian cancer cells were investigated in vitro and in vivo with a nude mouse model to monitor tumor burden both in the absence or presence of other chemotherapy agents. ***Results:*** TPL was effective as a single agent in inducing apoptosis of ovarian cancer cells in vitro, but not in vivo. TPL enhanced the cytotoxicity of carboplatin in culture and enhanced carboplatin-mediated reduction of tumor burden in nude mice inoculated with human ovarian cancer cells. Previously, a phosphatidylinositol 3-kinase (PI3 kinase) inhibitor was found to enhance carboplatin actions on ovarian cancer. Interestingly, the combined treatment of TPL, PI3 kinase inhibitor LY294002 and carboplatin was found to dramatically reduce ovarian tumor progression and burden in nude mice. ***Conclusion:*** In 44% of the animals tested the combined treatment caused complete regression of ovarian cancer. Combined observations indicate TPL may be an effective adjunct chemotherapy for ovarian cancer.

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Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy [1–3]. Each year over 23,000 new cases are diagnosed and approximately 14,000 deaths occur from this disease [1, 2]. Ovarian cancer is derived from the ovarian surface epithelial layer surrounding the ovary and has a high rate of metastasis [1, 2]. The spread of this disease into the peritoneal cavity is not associated with overt signs or symptoms and is usually not diagnosed until it has reached advanced stages [3]. Diagnosed and treated early, while still confined to the ovaries, allows for a 5-year survival rate as high as 95% [3]. However, most ovarian cancers are not diagnosed until they have become more advanced and spread throughout the peritoneal cavity [3]. At this stage, ovarian cancer has a mortality rate that exceeds 80% [3].

The introduction of combination therapy using platinum-based drugs and paclitaxel has improved survival length. However, both of these compounds have significant adverse side effects and most patients who are initially responsive to this therapy eventually become resistant [4–6]. Therefore, drug resistance remains one of the most significant obstacles in the treatment of patients with recurrent disease [5, 6].

Triptolide (TPL), a diterpenoid triexpoxide, is a purified compound of the herb *Tripterygium wilfordii* hook F and has been identified as the component responsible for its actions [7]. This herb has been used as a natural medicine for hundreds of years in China [7]. Tripterygium was initially used for the treatment of autoimmune diseases, especially rheumatoid arthritis [7]. It was later discovered

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that in addition to immunosuppressive activities TPL possesses anti-inflammatory and antifertility activities [8, 9]. Moreover, it was recently discovered that TPL has potent antineoplastic and antitumor activities [10–12]. Despite the multifunctional nature of this drug the exact molecular mechanisms of its actions are largely unknown.

Antiproliferative and proapoptotic activities of TPL have been shown with several different types of cancer cells in vitro and in vivo. TPL has been shown to induce apoptosis in leukemia cells in vitro [13, 14] and to have significant antagonistic activity against mouse leukemia in vivo [15]. Clinical trials in China have demonstrated that TPL treatment can induce a high remission rate in both mononucleocytic and granulocytic leukemias [16]. TPL was also found to be effective in inhibiting proliferation of human gastric cancer cells and prostatic epithelial cells in vitro [12, 17]. TPL can inhibit the growth of several different solid tumor types including breast, bladder, stomach and melanomas [18]. TPL was also found to be more effective on a molar basis than both cisplatin and taxol in inhibiting xenograft growth of these tumor types [18]. TPL also potentiates the activities of other agents [19–21] suggesting it may be useful not only as a single therapeutic compound, but also in combination with other cytotoxic drugs for cancer treatment.

The platinum-based compounds, cisplatin and carboplatin, bind to DNA and nuclear proteins leading to the formation of DNA cross-links that inhibit DNA replication and transcription [3, 4]. In ovarian cancer the chemoresistance that develops to these DNA-damaging agents is thought to be in part mediated through p53-induced cell cycle arrest [22–24]. TPL has been shown to enhance doxorubicin-mediated apoptosis of tumor cells by blocking p53-mediated cell cycle arrest [20]. In addition to p53, intrinsically or constitutively activated NFκB is thought to confer drug resistance in several cancer types, including ovarian cancer [25]. A recent study demonstrated that inhibition of NFκB activity resulted in increased efficacy of cisplatin in ovarian cancer cells [26]. Several studies have shown that TPL is able to inhibit the transcriptional activity of NFκB [12, 19, 27]. Therefore TPL may be effective in attenuating chemoresistance in ovarian cancer cells by altering NFκB and/or p53 activities.

An important signal transduction pathway involved in ovarian cancer progression is phosphatidylinositol 3-kinase (PI3-kinase) [28]. Abnormal activation of PI3-kinase is found in a high percentage of ovarian cancers [29]. An inhibitor of PI3-kinase LY294002 has been shown to inhibit cell survival and growth of ovarian cancer cells in vitro and in vivo [29–31]. Recently, LY294002 has been shown to be a useful adjunct chemotherapy for ovarian cancer in vivo [32]. Therefore, a combined treatment of TPL, LY294002 and carboplatin was investigated.

The objective of the current study was to determine if TPL is an effective treatment for ovarian cancer, either alone or as an adjunct therapy. A nude mouse tumor model was used that has been shown to be effective in evaluating the response of ovarian tumor xenografts in nude mice [33]. This in vivo tumor model involves a human ovarian cancer cell line, OCC1, which has been stably transfected with a secreted alkaline phosphatase (SEAP) gene. The SEAP reporter gene is constitutively expressed and has been shown to be secreted at levels proportional to the number of tumor cells present [33]. TPL was evaluated for its ability to induce apoptosis of OCC1-SEAP cells in vitro and in vivo. The capacity of TPL to enhance carboplatin-induced cell death in culture and in xenografts of ovarian cancer cells was assessed. Since the signal transduction processes influenced by TPL and the PI3-kinase inhibitor LY294002 appear distinct, a combination therapy of TPL, LY294002 and carboplatin was also investigated in the current study.

Materials and Methods

Cell Culture

The human ovarian cancer cell line OCC1 was generously provided by Dr. Gordon Mills (MD Anderson Cancer Center, Houston, Tex., USA) and cultured under recommended conditions. These cells have been modified to constitutively express the marker gene: secreted human SEAP [33]. The SEAP gene encodes a heat-stable protein that is secreted in proportion to cell number. The OCC1-SEAP cells were grown in Ham's F-12 medium (Life Technologies) plus 10% bovine calf serum (BCS). Once cells reached confluence they were trypsinized and split into appropriate plates.

Cell Survival

Cell survival was assessed as the number of cells remaining in culture following exposure to treatments. The DNA content of individual culture wells was used as an indication of cell number. Cells plated in 24-well culture plates were allowed to approach confluence (80%) in Ham's F-12 medium plus 10% BCS. Cultures were then incubated in DMEM plus 0.1% BSA and 0.1% BCS in the presence of vehicle control, carboplatin (0–100 mg/ml), TPL (100 ng/ml), or a combination of these for 24–96 h. Media aliquots were taken when appropriate for SEAP analysis. The DNA was measured fluorometrically as previously described [33].

DNA Isolation and Analysis

Following 48- to 72-hour treatment incubations, cells were suspended into culture medium and pelleted in tubes. DNA was isolated from collected cells using a Puregene™ DNA isolation kit

(Gentra Systems, Minneapolis, Minn., USA). The quantity and purity of nucleic acid preparations were estimated by measuring the absorbance of each sample (A_{260}/A_{280}). DNA preparations (10 mg/well) were loaded onto 1.2% agarose gels and visualized with ethidium bromide stain.

Western Blot Analysis

The OCC1-SEAP cells were grown to 80% confluence. Cultures were incubated in DMEM plus 0.1% BSA and 0.1% BCS to which either vehicle control, carboplatin (0–50 mg/ml), TPL (100 ng/ml) or combinations of these treatments had been added. Following 24, 48 or 72 h, cells were lysed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 003% glycerol, 0.5% β-mercaptoethanol). Total cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, Mass., USA). Membranes were immunoblotted with antibodies to both cleaved and full length caspase-3 (Cell Signaling Technology, Beverly, Mass., USA).

Nude Mouse Tumor Model

Athymic nude mice (NCR Nu/Nu) were either purchased from Taconic (Germantown, N.Y., USA) or bred in-house at Washington State University. The OCC1-SEAP cells were collected in Hanks' balanced salt solution and counted prior to injection. Treatments were initiated 7–10 days following an intraperitoneal inoculation of mice with 1×10^7 OCC1-SEAP cells. Stock solutions of carboplatin (Paraplatin; Bristol-Meyers Squibb, Princeton, N.J., USA) and TPL (Calbiochem, San Diego, Calif., USA) were prepared in sterile filtered PBS and DMSO, respectively. Animals received intraperitoneal injections of either vehicle control (PBS containing 4% DMSO) or carboplatin (60 mg/kg) alone or in combination with TPL (0–1.0 mg/kg) every other day for 5 days. Alternatively, TPL (0.15 mg/kg) was injected every day for 10 days. For these experiments carboplatin was administered under the same schedule of every other day for 5 days. Some mice were also treated with 40 mg/kg of LY294002 every other day for 5 days with an intraperitoneal injection in the presence of TPL and carboplatin. Blood samples were collected into capillary tubes from saphenous vein lancings at regular intervals during and following treatments. The capillary tubes were centrifuged and the plasma samples were frozen at –20°C until the time of SEAP assay. The Washington State University Animal Care and Use Committee approved all procedures.

SEAP Assay

Blood plasma and cell culture medium samples were assayed for SEAP activity using the Great EscAPe SEAP fluorescence detection kit (Clontech Laboratories, Palo Alto, Calif., USA) as previously described [33]. Blood plasma samples were diluted 1:100 prior to the assay to bring values within the linear range of the standard curve. The intra-assay and interassay coefficients of variation were 2.5 and 18.8%, respectively [33]. SEAP levels have been previously shown to correlate with tumor burden [33]. In the case of the experiments in which mice were treated with a combination of TPL, LY294002 and carboplatin there was some variation in the extent of tumor growth in the control mice. In order to better compare response to treatment across experiments the SEAP assay data between experiments were normalized by dividing each data point by the overall mean of all SEAP measurements from that experiment and up through day 18. The normalized data then reflect the relative tumor burden of each mouse compared to others in the experimental group and can be combined across experiments.

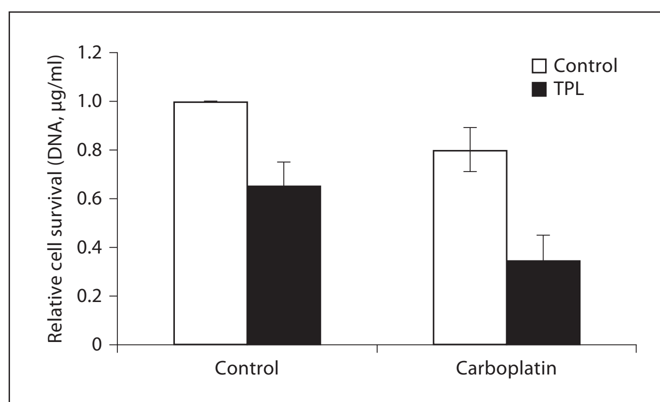


Fig. 1. Ovarian tumor cell survival following carboplatin and TPL treatment. OCC1-SEAP ovarian cancer cells were incubated for 48 h in the absence or presence of TPL (100 ng/ml) and with or without carboplatin (50 mg/ml). Cells remaining in the culture wells following the treatment period were suspended in PBS. The amount of DNA in aliquots of PBS was measured fluorometrically with ethidium bromide and considered representative of the amount of cells surviving in culture. The results are expressed as the mean ± SEM, n = 4 and are representative of three different experiments.

Statistical Analysis

Data were analyzed by a one-way or two-way analysis of variance (ANOVA). Significant differences between two treatment groups were determined using a Student t test. In some cases after analysis of variance a post hoc Bonferroni test was used to determine differences between treatment groups at particular time points. For the survival data a Mantel-Haenszel logrank test [34] was used to determine if the survival curves were significantly different. Most analyses were performed using Graphpad Prism version 4.0b for MAC (Graphpad Software, San Diego, Calif., USA). Data were expressed as means ± standard error of the mean (SEM).

Results

The ability of TPL to inhibit cancer cell survival in vitro was investigated. Ovarian cancer cell cultures were incubated in the presence of either vehicle control, TPL (100 ng/ml) or carboplatin (50 mg/ml) alone or the combination of TPL and carboplatin for 48 h. Preliminary studies determined that these were the optimal doses for the in vitro analysis. Compared to control cultures, cell survival (i.e. mg DNA/ml) was decreased by approxi-

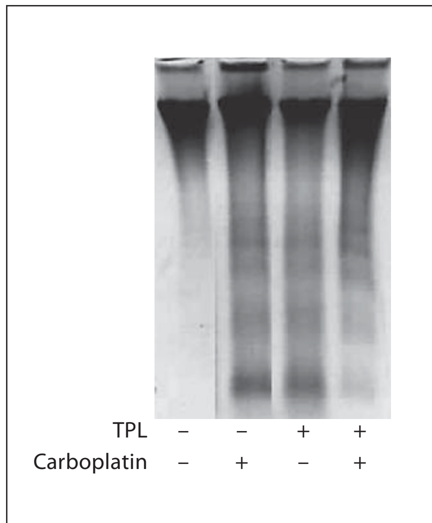


Fig. 2. DNA fragmentation in OCC1-SEAP cell cultures in response to TPL and carboplatin treatment. OCC1-SEAP cell cultures were incubated in the absence or presence of either TPL (100 ng/ml) or carboplatin (50 mg/ml) as well as combined treatments. DNA was extracted using a Puregene™ DNA isolation kit and separated by electrophoresis on a 1.2% agarose gel. Low-molecular-weight DNA fragments were visualized with ethidium bromide stain. Data is representative of three separate experiments.

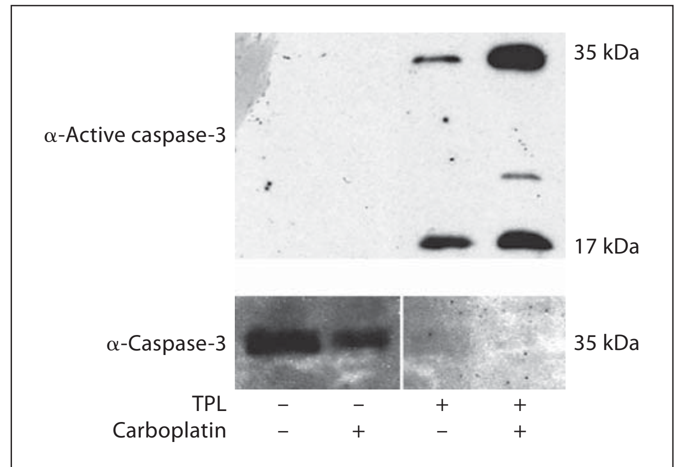


Fig. 3. Activation of the proteolytic caspase cascade in response to TPL treatment in OCC1-SEAP cell cultures. Cell cultures were incubated in the presence of either TPL (100 ng/ml) or carboplatin (50 mg/ml) or a combination of both for 24 h. Aliquots of total cell lysates from cells incubated with treatments were separated by SDS-PAGE and transferred to nylon membranes. Membranes were probed with an antibody to the cleaved (active; 35 and 17 kDa) and full-length (35 kDa) inactive forms of caspase-3 (α-active caspase-3 and α-caspase-3, respectively). Data are representative of three different experiments.

mately 40% in cultures treated with TPL alone and by 30% in cultures incubated in the presence of carboplatin (fig. 1). The reduction in cell survival (65%) was greatest in cultures exposed to the combined treatment with TPL and carboplatin. A combined treatment with TPL and the PI3-kinase inhibitor LY294002 was the same as TPL alone (data not shown).

The presence of DNA fragmentation was assessed to determine if reduction in cancer cell number correlated to an increase in cellular apoptosis. The DNA in cells undergoing apoptosis is cleaved by endonucleases resulting in DNA fragmentation that can be detected electrophoretically [35]. DNA laddering was evident in cells after a 48-hour treatment with TPL (100 ng/ml) and carboplatin (50 mg/ml), as well as with the combined treatment of TPL and carboplatin (fig. 2).

Activation of caspase-3 was used as an additional indicator of apoptosis induction. Caspase-3 is activated downstream of initiator caspase-9 or caspase-8 and is considered the major effector caspase for this proteolytic cascade [36]. Western blot analysis was performed with an antibody that is specific to the active form of caspase-3. A major band of 17 kDa and a minor band of 19 kDa are visualized with this antibody. The 35-kDa band under the activated caspase-3 label is speculated to be partially activated but not fully proteolized. Blots were also probed with an antibody to full-length (35 kDa) caspase-3. TPL exposure resulted in robust induction of active caspase-3 at 24 h and the observed stimulation of caspase-3 was visibly enhanced following combined treatment with both carboplatin and TPL (fig. 3). Cells treated with carboplatin alone do not activate caspase-3 until 48 h posttreatment (data not shown). Full length caspase-3 protein levels were visually decreased in correlation to the increase in the observed active cleaved products.

Since TPL was found to decrease ovarian cancer cell survival and enhance carboplatin activities, in vivo experiments were performed. Experiments were conducted using a previously developed nude mouse model which employs secretion of an SEAP reporter gene to monitor tumor progression and response to chemotherapeutics [33]. In initial experiments, a range of doses was used for evaluation of the toxicity of TPL. Once tumors were established in mice, as indicated by measurable SEAP protein in blood samples, doses of TPL (0.125–1.0 mg/kg) were administered by intraperitoneal injection every other day over a 5-day period (3 injections). Within 24 h, 2 of 3 mice receiving the highest dose of 1.0 mg/

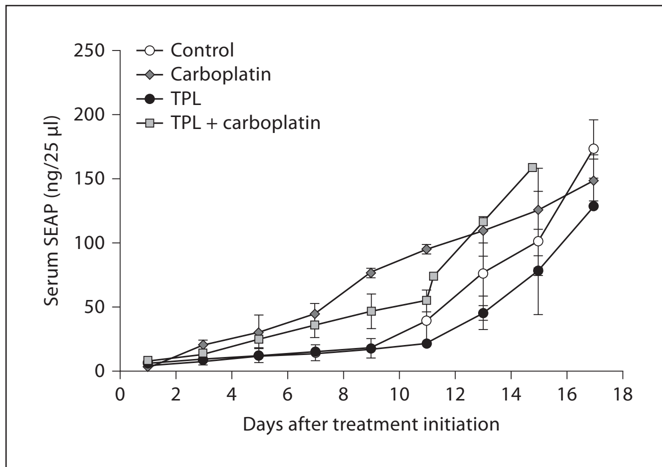


Fig. 4. Intraperitoneal tumor progression in response to a high dose and short duration of TPL treatment. Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection and treatments were initiated within 8–10 days. Mice were treated with vehicle control, carboplatin (60 mg/kg) or TPL (0.5 mg/kg) every other day for 5 days. Blood samples were taken at intervals over the treatment period and assayed for SEAP levels. Values presented are SEAP values from posttreatment tumor growth and day 0 is the first day of treatment initiation. Values are the mean ± SEM with n = 3 mice per group.

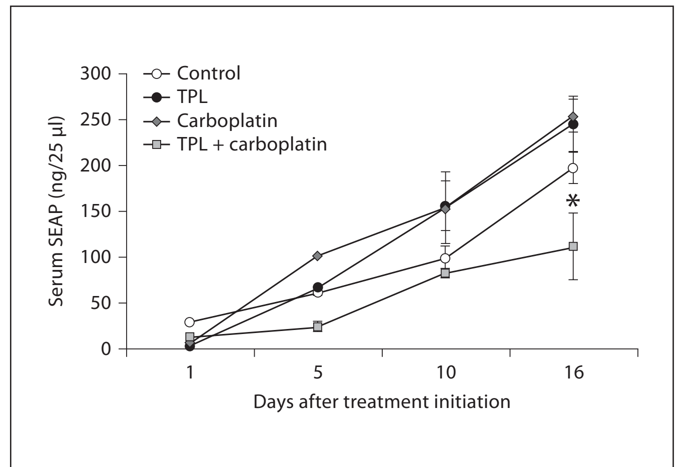


Fig. 5. Interperitoneal tumor progression in response to a low dose and long duration of TPL treatment. Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection and treatments were initiated within 8–10 days. Mice were treated with either vehicle control, carboplatin (60 mg/kg; every other day for 5 days) or TPL (0.15 mg/kg; every day for 10 days). Blood samples were taken at intervals over the treatment period and assayed for SEAP levels. Values presented are SEAP values from posttreatment tumor growth and day 0 is the first day of treatment initiation. Values are the mean ± SEM with n = 4 mice per group. * $p < 0.05$, statistical difference from control and other treatments.

kg expired. There were no significant differences in SEAP values between mice treated with either vehicle control or any of the remaining doses of TPL (0.125, 0.25 or 0.5 mg/kg).

As there was no apparent toxicity associated with the dose of 0.5 mg/kg, this dose was chosen for the following experiment. Carboplatin and TPL were administered separately or in combination using the same protocol of every other day for 5 days. One of 4 mice receiving the combined treatment of 60 mg/kg carboplatin and 0.5 mg/kg TPL expired following treatment. No apparent adverse side effects were observed in the remaining mice from this or other treatment groups. There was no significant difference in tumor burden among mice receiving vehicle control, TPL, carboplatin or the combined treatment (fig. 4).

Since no effect was seen with the dose of 0.5 mg/kg TPL administered over 5 days, the following experiments were conducted with a lower dose of TPL administered over a longer time period. The dose of 0.15 mg/kg has previously been shown to be 60% of the maximum tolerated dose in nude mice and did not appear to adversely affect the mice [17]. Therefore the dose of 0.15 mg/kg was chosen for intraperitoneal injection of mice every day for 10 days. We did not alter the dose or time frame of injection of carboplatin for these experiments. In this regard, mice received their first and last injection of carboplatin on the 4th and 8th day of TPL administration, respectively. When treatments were administered in this manner, a significant decrease in tumor burden as measured by SEAP levels was observed with the combined treatment of TPL and carboplatin (fig. 5). There was no significant difference in tumor burden between mice receiving TPL or carboplatin alone and those receiving vehicle control (fig. 5). There were no adverse side effects seen following this low dose of TPL treatment or in the combined treatment groups.

Previously the PI3-kinase inhibitor LY294002 was found to enhance carboplatin actions in vitro and in vivo [32]. The potential combined effect of TPL and LY294002 on carboplatin actions was investigated using the nude mouse ovarian tumor model [33]. LY294002 (40 mg/kg) was injected 3 times in 5 days in combination with TPL (0.15 mg/kg daily for 10 days) and carboplatin (60 mg/kg 3 times in 5 days). Alone LY294002 had negligible effects on ovarian cancer tumor progression [32], with the mod-

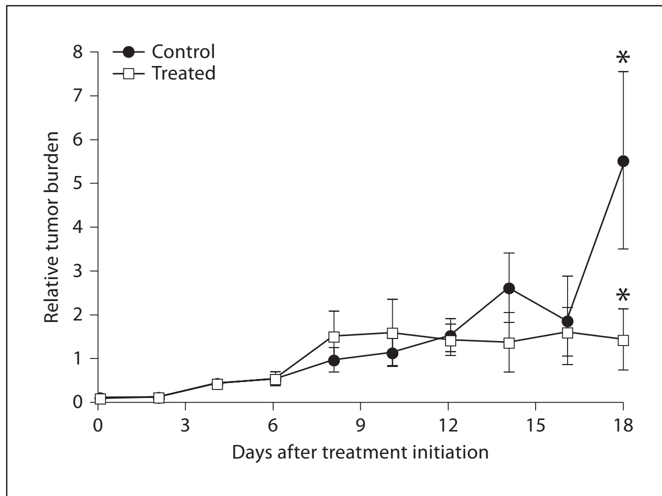
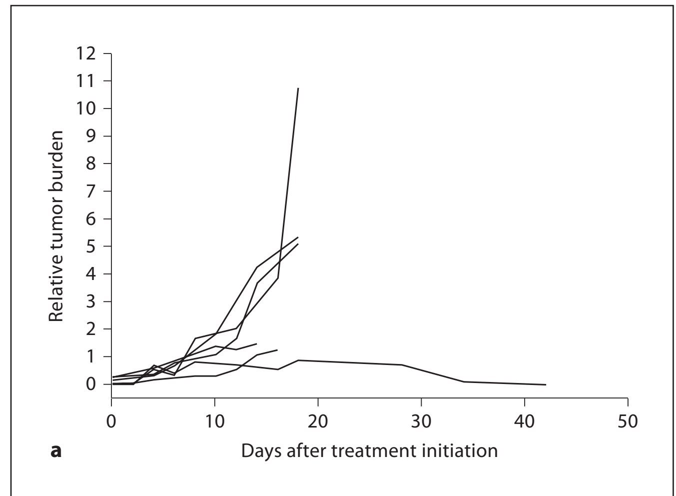


Fig. 6. Interperitoneal tumor progression in response to low dose and long duration of TPL treatment combined with LY294002 and carboplatin. Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection and treatments were initiated within 8–10 days. Mice were treated with either vehicle control or carboplatin (60 mg/kg; every other day for 5 days), TPL (0.15 mg/kg; every day for 10 days) and LY294002 (40 mg/kg; every other day for 5 days). Blood samples were taken at intervals over the treatment period and assayed for normalized SEAP levels. Values presented (relative tumor burden) are normalized SEAP values from posttreatment tumor growth and day 0 is the first day of treatment initiation. Values are the mean ± SEM from 3 experiments with n = 6 mice in the control group and n = 9 mice in the treated group. * Significant difference between control and treated groups by Bonferroni post hoc test after 2-way ANOVA.



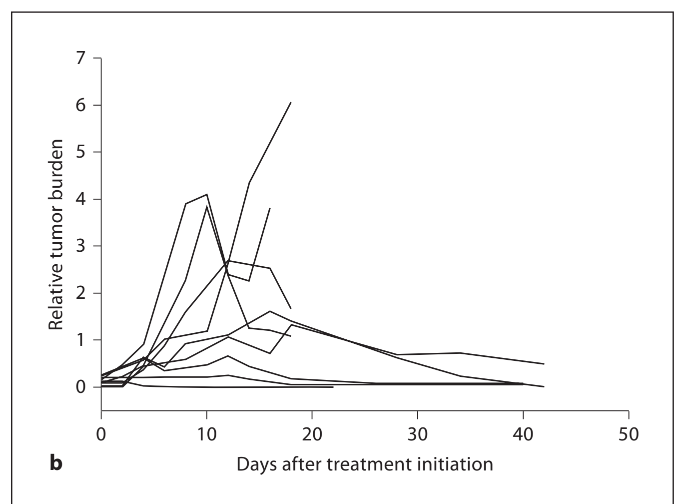
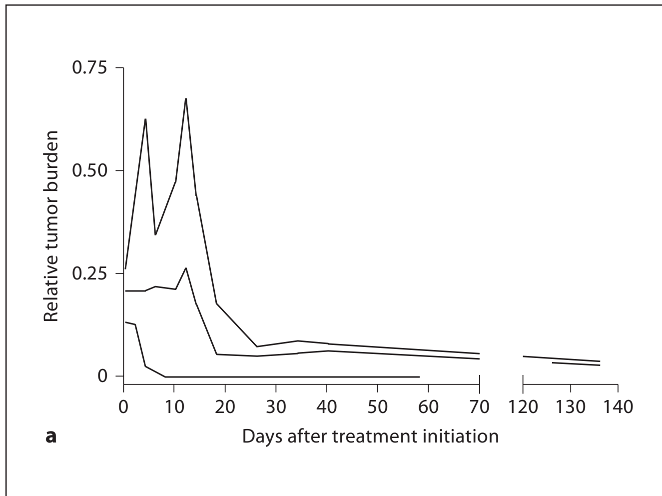


Fig. 7. Interperitoneal tumor progression in control mice (**a**) and in mice receiving combined TPL, LY294002 and carboplatin treatment (**b**). Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection and treatments were initiated within 8–10 days. Mice were treated with either vehicle control or carboplatin (60 mg/kg; every other day for 5 days), TPL (0.15 mg/kg; every day for 10 days) and LY294002 (40 mg/kg; every 2 days for 5 days). Blood samples were taken at intervals over the treatment period and assayed for SEAP levels. Values presented (relative tumor burden) are normalized SEAP values from posttreatment tumor growth and day 0 is the first day of treatment initiation. Each line follows the tumor progression of one mouse. Data are from three experiments with n = 6 mice in the control group and n = 9 mice in the treatment group.

el system used in the current study. Therefore, the in vivo control with LY294002 previously done was not repeated in the current study [32]. Since the combined in vitro treatment of TPL and LY294002 was not different than TPL alone, no in vivo experiments were initiated with only these two agents. A combination of carboplatin and LY294002 had a small decrease from control, but not complete regression [32]. The combined treatment of TPL, LY294002 and carboplatin caused a significant decrease in ovarian tumor burden and growth (fig. 6). The tumor burdens of each mouse over time are presented for both those receiving the combination treatment (fig. 7b) and controls (fig. 7a). Six of 9 treated mice (66%) responded to treatment, with response defined as a 50% or greater decrease in tumor burden from peak measured levels for that animal. Two of 9 treated mice (22%) that were euthanized for ascites and cachexia were found to have had decreasing tumor levels after having developed rather high relative tumor burdens of 3–4 using normalized SEAP units. Interestingly, 44% (4/9 animals) with the combined treatment had total regression of the ovarian tumor as determined by the absence of measurable SEAP levels after 40 days. Representative animals are shown in



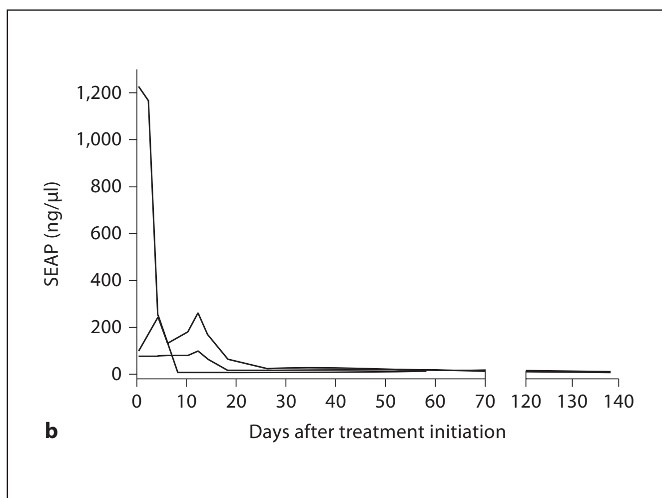


Fig. 8. Interperitoneal tumor progression in representative treated mice with tumor regression in relative tumor burden units (**a**) and in SEAP levels (**b**). Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection and treatments were initiated within 8–10 days. Mice were treated with carboplatin (60 mg/kg; every other day for 5 days), TPL (0.15 mg/kg; every day for 10 days) and LY294002 (40 mg/kg; every 2 days for 5 days). Blood samples were taken at intervals over the treatment period and assayed for SEAP levels. Values presented are SEAP values from posttreatment tumor growth and day 0 is the first day of treatment initiation. Each line follows the tumor progression of 1 mouse. Data are from 3 representative mice.

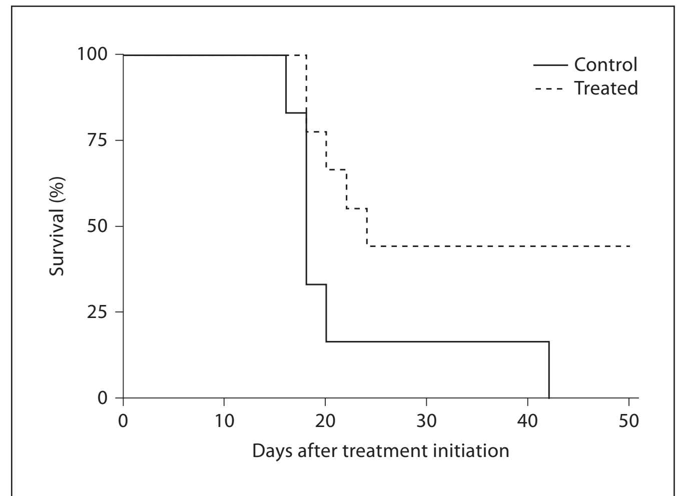


Fig. 9. Survival curves for control mice versus mice receiving combined TPL, LY294002 and carboplatin treatment. The curves reflect when animals were sacrificed or died due to tumor progression. Nude mice were inoculated with OCC1-SEAP tumor cells by interperitoneal injection. Mice were treated within 8–10 days with carboplatin (60 mg/kg; every other day for 5 days), TPL (0.15 mg/kg; every day for 10 days), and LY294002 (40 mg/kg; every 2 days for 5 days). The curves for control and treated animals are significantly ($p = 0.03$) different as determined by the Mantel-Haenszel logrank test.

figure 8 demonstrating the long-term absence of ovarian cancer. Three surviving mice from the treated group were still tumor-free 2 months after treatment (fig. 8a, b). One mouse from the control group was able to suppress tumor growth without treatment, although SEAP measurements never fell completely to baseline (fig. 7a). This mouse was euthanized for ascites production 42 days after initiation of treatment.

Mice in these studies were euthanized when abdominal distention from ascites fluid or cachexic weight loss reached certain levels as directed by the approved animal use protocol. Survival curves for the combined TPL, LY294002 and carboplatin-treated and control mice are shown in figure 9. These survival curves were found to be significantly ($p < 0.05$) different, indicating that mice receiving the combined treatment survived significantly longer. Taken together, these studies indicate that TPL did enhance the chemosensitivity of ovarian cancer to carboplatin with the appropriate treatment regimen, but the optimum effect was observed with a combined TPL, LY294002 and carboplatin treatment.

Discussion

TPL has been tested as a potential chemotherapeutic for a variety of cancer types. The present studies demonstrate that this herb extract may be a plausible adjunct chemotherapy for the treatment of ovarian cancer. TPL

was a potent stimulator of apoptosis in OCC1-SEAP cell cultures as indicated by DNA fragmentation and induction of caspase-3 activity. The observed increase in apoptosis correlated with a decrease in cell number. The combined treatment of TPL and carboplatin increased DNA laddering and caspase-3 activity, suggesting that TPL enhances the ability of carboplatin to induce OCC1-SEAP cell death in vitro. TPL also enhanced the cytotoxicity of carboplatin in vivo. There was not an observable increase in the effects of carboplatin when TPL was used at a high dose over a short time period. However, when administered at a low dose on a daily basis, TPL increased the ability of carboplatin to inhibit OCC1-SEAP tumor growth in nude mice.

As TPL was able to induce apoptosis in OCC1-SEAP cultures, it was surprising that TPL alone had a negligible effect on in vivo tumor growth. The observed results are most likely due to the narrow range of efficacy of TPL. In the current experiments a dose of 1.0 mg/kg TPL was lethal. Half this dose (0.5 mg/kg) was not effective in reducing tumor burden alone or in conjunction with carboplatin and had toxic effects when administered with carboplatin. A study by Shamon et al. [10] found the dose of 0.5 mg/kg to be lethal in athymic mice with breast cancer xenografts. Because of the toxicity observed with the dose of 0.5 mg/kg, further experiments were conducted using a lower dose of 0.15 mg/kg TPL. In addition, the period of injection was increased from every other day for 5 days to every day for 10 days. The dose of 0.15 mg/kg was demonstrated to be potent in inhibiting growth and metastasis of several solid tumor types in nude mice studies conducted by Yang et al. [18]. In the current study TPL was not effective as a single agent but did exhibit the ability to increase the efficacy of carboplatin in this system.

The mechanism by which TPL is able to induce apoptosis and render cells sensitive to DNA-damaging agents is largely unknown and was not directly addressed in the current experiments. Several studies have demonstrated the ability of TPL to alter the transcriptional actions of NFκB [12, 19, 27]. Recently, NFκB activation has been connected with multiple aspects of oncogenesis, including control of apoptosis and cell cycle progression [25]. Additionally, activation of NFκB in cancer cells by chemotherapy can blunt the ability of the cancer therapy to induce cell death [25]. In this regard, constitutive activation of NFκB appears to mediate chemoresistance of ovarian cancer cells and inhibition of NFκB activation sensitizes the ovarian cancer cells to cisplatin [26]. Several studies have shown that TPL makes cells sensitive to TNF-α and chemotherapy-induced apoptosis through inhibition of NFκB activity [19, 27]. Therefore the ability of TPL to increase cell death induced by carboplatin may be partially due to its ability to inhibit NFκB activity.

TPL appears to inhibit the growth of tumor cells regardless of their p53 activity status. Yang et al. [18] found that TPL blocks the growth of xenografts of solid tumor cells that possess both wild-type and mutated forms of p53. However, several previous studies have demonstrated the role of p53 in the ability of TPL to induce apoptosis [12, 20]. In prostatic epithelial cells protein levels of p53 were increased and predominantly accumulated in the nuclei in conjunction with a high-dose TPL treatment [17]. Chang et al. [20] showed that TPL enhancement of chemotherapy-induced apoptosis was accompanied by increased translation and accumulation of p53 in fibrosarcoma cell lines. Furthermore, Jiang et al. [12] demonstrated that p53 expression was upregulated in gastric cancer cell lines after TPL treatment, and suppression of p53 expression by antisense oligonucleotides significantly abolished TPL-induced apoptosis. Mutations in the p53 gene are common genetic alterations in ovarian cancer and have been associated with the progression and chemoresistance of this disease [22, 37]. Mutations in the p53 protein are associated with a lack of response to high-dose cisplatin therapy in ovarian cancer patients [22]. In this regard, abrogation of the actions of p53 could contribute to the ability of TPL to induce apoptosis and increase the sensitivity of ovarian cancer cells to carboplatin. Clearly, the role p53 plays in the actions of TPL needs to be further investigated.

Additional molecular targets of TPL include cell cycle regulators and apoptotic pathways. The current study confirmed that TPL induced apoptosis by activation of a proteolytic caspase cascade [12, 17]. TPL treatment was shown to result in a reduction in levels of cell cycle-promoting factors such as cyclin A/cdk2, cyclin B/cdc 2, cyclin D1 and c-myc, as well as the phosphorylated form of retinoblastoma protein (pRB) [17]. Microarray analysis of TPL-treated normal and transformed bronchial epithelia demonstrated a reduction in expression of the cell cycle regulators and survival genes such as cyclin D1, cyclin B1 and cyclin A1, cdc 25, bcl-x and c-jun [38]. Changes in the expression of these cell cycle regulators are most likely a secondary effect of TPL actions on an upstream target. However, it is difficult to ascertain what upstream target might be responsible for the actions of TPL from these findings. The reduction in cell cycle regulators could result in the initiation of apoptosis and conversely the apoptosis could reduce the synthesis of cell cycle-promoting factors.

An interesting result from the current study was that the combined treatment of TPL, LY294002 and carboplatin caused a dramatic reduction in tumor growth and burden with 44% of the animals having complete tumor regression. TPL has not been shown to influence the PI3-kinase/Akt signal transduction pathway that LY294002 inhibits. Observations suggest that the combined block of PI3-kinase by LY294002 and actions of TPL to partially inhibit p53 and NFκB are required to obtain an optimal adjunct chemotherapy. The TPL and LY294002 apparently cause a more dramatic suppression of ovarian cancer carboplatin chemoresistance than either factor alone. Observations provided suggest a combined chemotherapeutic regimen involving TPL, LY294002 and carboplatin may provide a more effective therapy for advanced ovarian cancer.

A limitation to the current study is that a single human ovarian cancer cell line (i.e. OCC1) was used. Although this cell line is considered chemoresistant and grows intraperitoneal tumors, the efficiency of the TPL and combined therapy in vivo is focused on only this cell line alone. Analysis of other human ovarian cancer cell lines and primary ovarian tumors is required before the efficiency of TPL as an adjunct chemotherapy can be conclusively determined. The current analysis does suggest further analysis of TPL and combined chemotherapies with PI3-kinase inhibitors would be useful and may lead to better treatments for advanced ovarian cancer.

The current study demonstrated that TPL was effective as a single agent in inducing apoptosis of ovarian cancer cells in vitro, but not in vivo. TPL did enhance the cytotoxicity of carboplatin in culture and enhanced carboplatin-mediated reduction of tumor burden in nude mice inoculated with human ovarian cancer cells. TPL appears to target multiple signaling molecules critical to cell survival, including those that mediate cell survival following chemotherapy exposure. Future research should be directed toward the identification of the upstream molecules that are directly influenced by TPL. Perhaps with this knowledge rational drug design could be undertaken so that the therapeutic efficacy of TPL may be enhanced and its toxicity reduced. These efforts would lead to the development of novel adjunct chemotherapy options for women with ovarian cancer.

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