Effect of Intratumoral Injection of Carboplatin Combined with Pluronic P85 or L61 on Experimental Colorectal Carcinoma in Rats

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Pluronic, a poly(ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) block copolymer, has been shown to enhance the cytotoxic activity of anticancer drugs in various cell lines. In the current study the effect of Pluronic P85 (P85) and Pluronic L61 (L61) on the intratumoral chemotherapy of an experimental adenocarcinoma in rats was examined. A total of 120 subcutaneous tumors (4 per rat) were inoculated in 30 BDIX rats and were treated weekly for 4 weeks with intratumoral injection of carboplatin (CPt) alone or with either P85 or L61. Tumors were monitored weekly and were excised at the endpoint for histologic evaluation. The effect of Pluronic on levels of intracellular ATP was explored as a possible mechanism of sensitization. Results showed that tumors treated with low-dose CPt (2.8 mg/kg) and P85 or L61 exhibited significant reductions in tumor volume after 28 days relative to Day 0 (112.7% ± 34.4%, *n* = 15; 131.3% ± 55.6%, *n* = 8) compared with tumors treated with free drug (339.4% ± 75.0%, *n* = 16). Control tumors treated with either P85 or L61 alone or with saline showed volume increases of 1079.4% ± 143.6% (*n* = 16), 729.4% ± 202.2% (*n* = 7), and 1119.2% ± 6.1% (*n* = 16), respectively. Treatment with high-dose CPt (20.7 mg/kg) led to a 79.3% ± 4.2% reduction in tumor volume, and no differences were noted with addition of P85 or L61. *In vitro* ATP measurements showed that 28.0 mg/kg of P85 significantly reduced levels of intracellular ATP to 44.7% ± 1.5% of controls, whereas L61 at this concentration depleted ATP levels completely. Results confirm that Pluronic P85 and L61 act as potent sensitizers to carboplatin chemotherapy of the experimental colorectal carcinoma, leading to a significant reduction of tumor growth compared to carboplatin alone. ATP depletion is a possible mechanism for these observed differences. Exp Biol Med 232:950–957, 2007

Key words: Pluronic; carboplatin; intratumoral injection; chemosensitizer; DHD/K12/ TRb rat colorectal carcinoma; ATP

Introduction

Platinum-based drugs are used frequently in treatment of lung, ovarian, and colorectal cancers (1, 2). Systemic administration of these potent chemotherapeutics can be effective, but the treatment is often impaired or ineffective due to dose limiting toxicity and/or insufficient drug accumulation in tumors. Although a higher drug dose can temporarily control tumor growth, most chemotherapeutic agents exhibit nonspecific toxicity and affect both tumor and normal cells. Consequently, toxic side effects are unavoidable in systemic treatments (3, 4). In addition, most anticancer agents can be less effective due to either innate or acquired resistance factors of the tumors.

Pluronic triblock copolymers, commonly used in the food and pharmaceutical industries, are a family of amphiphilic polymers consisting of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic EO_x-PO_y-EO_x structure (5). Recently, these polymers have been shown to increase the susceptibility of multidrug-resistant (MDR) cancer cells to drugs either by reducing the lethal drug concentration at the site of action (6, 7) or by increasing the drug-induced damage to the cells through modulation of apoptosis signaling pathways (8, 9). Studies by our group have shown that Pluronic P85 also can increase cytotoxic effects of carboplatin on nonresistant cancer cells *in vitro* (10) and *in vivo* when administered intratumorally and/or with radiofrequency ablation (11).

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The chemosensitizing effects of Pluronic appear to be mainly due to inhibition of the P-glycoprotein (Pgp) drug efflux transport system (12, 13). It has been further suggested that the inhibition of Pgp-mediated drug efflux is a direct result of ATP depletion by Pluronic (14). Additionally, Pluronic may decrease the activity of glutathione/glutathione *S*-transferase (GSH/GST) detoxification molecules, which reduce drug toxicity by preventing the drug's interaction with its molecular target (7, 15). It has also been suggested that Pluronic-cell interactions are cell type dependent due to differences in lipid composition of the cell membrane (16–18).

Although numerous studies have been conducted to explore the sensitization effects of Pluronic on cancer cells *in vitro,* little attention has been given to demonstrating the therapeutic value of the *in vivo* sensitization of either drug-sensitive or MDR cell lines. In the current study we hypothesize that the addition of either Pluronic P85 or L61 will significantly increase the efficacy of intratumoral drug treatment in an experimental, drug-sensitive model of colorectal carcinoma. The platinum anticancer agent, *cis*-diammine(1,1-cyclobutane dicarboxylato) platinum (carboplatin), an analogue of cisplatin, was used in this study and was selected because its toxicity (19–22), antitumor activity, and pharmacokinetics (23–25) all have been determined. This agent also was selected based on our prior work assessing its cytotoxicity *in vitro* in the DHD/K12/TRb cell line (a colorectal tumor cell) in the presence of three different polymers of the Pluronic family (10).

This study is part of a broader effort to develop a minimally invasive, injectable, intratumoral chemotherapy system for the treatment of solid tumors. Locally injected chemotherapy focuses treatment directly on the site of action, thereby reducing systemic side effects and removing dose-limiting toxicity. Including a biologically active molecule, such as P85, as a component of an intratumoral drug delivery system may make the local chemotherapy approach more robust by further decreasing the toxic drug dose and improving the chances of successful treatment.

Materials and Methods

Materials. Pluronic P85 (EO_{26}-PO_{40}-EO_{26}) and L61 (EO_2-PO_{30}-EO_2) were donated by BASF Corp. (Florham Park, NJ). Carboplatin was purchased from Sigma-Aldrich (St. Louis, MO). Trypsin-EDTA, Dulbecco's phosphate-buffered saline (without calcium or magnesium), RPMI medium 1640 (with L-glutamine and phenol red), and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). The DHD/K12/TRb rat colorectal carcinoma cell line was obtained from the laboratory of Dr. W.G. Pitt, Brigham Young University (original source was the European Collection of Cell Cultures). DispoDialyzer system (Spectra/Por) and sterile 0.22-μm syringe-driven filter units (Millex-GP) and opaque-walled 96-well cell culture plates were purchased from Fisher Scientific Inc. (Lansing, MI). The CellTiter-Glo luminescent ATP assay was purchased from Promega (Madison, WI).

Preparation of Pluronic/Carboplatin Solutions. The *in vivo* treatment solutions were prepared by first dissolving Pluronic P85 or L61 at a concentration of 10.0 mg/ml in normal saline (0.9% NaCl). The polymer was completely dissolved under refrigeration at 4°C, and carboplatin was added into the solution at either 1.0 mg/ml or 7.5 mg/ml and allowed to dissolve. Control solutions included carboplatin in saline, saline without drug or polymer, and 10.0 mg/ml P85 or L61 in saline without drug. The treatment doses of carboplatin and Pluronic were selected based on previously published work (10, 26). The test solutions were filtered with sterile 0.22-μm syringe-driven filter units (Millex-GP), aliquoted, and stored at –20°C. The solutions were returned to room temperature immediately before use.

Cell Culture. All studies were carried out using the DHD/K12/TRb rat colorectal carcinoma line (27). This metastatic cell line originated from a 1, 2-dimethylhydrazine–induced colon adenocarcinoma in BDIX rats and has well-defined properties essential for study of metastasis of colon carcinomas, such as a reproducible pattern of *in vivo* behavior and the ability to be propagated both *in vitro* and *in vivo*. The cells were maintained in completed RPMI medium containing 10% FBS and 1% (v/v) penicillin/streptomycin in a 37°C humidified incubator with 5% CO_2. The cells were passaged weekly. All cells used for the tumor inoculation were obtained from the second passage.

***In Vitro* Carboplatin Release.** The release of carboplatin from solutions containing Pluronic P85 and L61 in RPMI at 37°C was evaluated *in vitro* using a disposable dialysis membrane system (Spectra/Pro-6; 2 kDa; Spectrum Laboratories Inc., Houston, TX). Briefly, 500 μl test solution containing 1.0 mg/ml carboplatin with or without 10.0 mg/ml P85 or L61 was loaded into each dialysis bag that was placed into a 50-ml centrifuge tube containing prewarmed PBS. The release system was placed in an orbital shaker incubator (New Brunswick Scientific Cooperation C24, Edison, NJ) at 37°C. At 2, 4, 6, 24, 48, 72, and 168 hrs, 1 ml PBS solution was sampled and replaced with 1 ml fresh PBS. The release of carboplatin was determined with HPLC (Perkin-Elmer 200, Aquapore OD-300 column; Waltham, MA). The carboplatin concentration was detected using absorbance at 220 nm with a PBS mobile phase. The eluent speed was 0.5 ml/min, and the retention time of carboplatin was approximately 1.87 min.

Tumor Inoculation in BDIX Rats. All procedures involving animals were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Thirty adult male BDIX rats (6–7 wks old) were obtained from Charles River Laboratories (Wilmington, MA). On the day of tumor inoculation, cells were harvested by trypsin-EDTA, washed in RPMI, centrifuged at 115 *g* for 5 mins,

and resuspended at a final concentration of 2×10^6 cells/ml. Cells then were loaded into individual 1-ml syringes equipped with 27-gauge needles, and a separate syringe was used for each injection site. For tumor inoculation, the rats were anesthetized with 1% isoflurane with an O_2 flow rate of 1 l/m (EZ150 Isoflurane Vaporizer [Baxter, Deerfield, IL], EZ Anesthesia [Euthanex, Palmer, PA]). A total of 50 μl cell suspension was injected subcutaneously into each thigh and above each shoulder bilaterally.

***In Vivo* Efficacy Studies.** Tumors were grown for 4 wks after inoculation. To begin the treatment, the animals were anesthetized as described above, rat weights were recorded, the site was cleaned, and the tumors were measured with calipers. The rats then were randomly divided into nine groups of either 16 tumors or 8 tumors. Throughout the text, *n* represents the number of tumors, not the number of animals. All rats received weekly intratumoral injections of 200 μl treatment solution per tumor. Tumors (*n*) were treated with P85 ($n = 16$), L61 ($n = 7$), low-dose (1.0 mg/ml) CPt ($n = 16$), low-dose CPt with P85 ($n = 15$) or L61 ($n = 8$), high-dose (7.5 mg/ml) CPt in saline ($n = 16$), or high-dose CPt with P85 ($n = 15$) or L61 ($n = 8$). The control group received injections of saline ($n = 16$).

The treatments were repeated weekly for 4 wks. The total weekly carboplatin dose administered was 2.8 mg/kg for rats receiving the low-carboplatin dose and 20.7 mg/kg for rats receiving the high-drug dose. The total weekly Pluronic dose per rat administered was 28 mg/kg. Tumor size was monitored by weekly caliper measurements. Tumor volume (V) was calculated based on maximum longitudinal diameter, a, and the transverse diameter, b ($V = 0.5 \times ab^2$). Tumor volumes were standardized using the following formula: $(V_t - V_0) / V_0 \times 100$, where V_t is the tumor volume immediately before each treatment and V_0 is the initial tumor volume. The therapeutic efficacy of intratumoral chemotherapy and the enhancement of carboplatin cytotoxicity by Pluronic P85 or L61 were assessed by comparing serial measurements of tumor size. The percent changes of tumor volumes were compared between each group.

Histologic Analysis. One week after the last treatment the rats were euthanized using an approved method. Tumors were measured and excised, and portions were fixed in 10% buffered formalin solution for at least 24 hrs. The tissue was then processed by the Histology Core Facility of the Case Comprehensive Cancer Center. Briefly, the tissue was dehydrated and embedded in paraffin, and microscopic sections were cut (5 μm) and stained with hematoxylin and eosin (H&E) and Masson's trichrome (MTC) stains. Independent pathologic review of all specimens was performed by two observers.

***In Vitro* Assessment of ATP Levels.** DHD/K12/TRb cells were maintained as described above. Upon reaching 80% confluence, cells were detached with trypsin-EDTA, diluted to 10^5 cells/ml, and plated in 96-well opaque-walled flat-bottom plates. The plates were returned to the incubator to allow cell adhesion. After 24 hrs, the supernatant was aspirated, and 50 μl test solutions with 0.0 (untreated control), 0.01, 0.1, 1.0, 3.0, 5.0, 10.0, 50.0, and 70.0 mg/ml of either Pluronic P85 or L61 were transferred into each well and incubated for 6 hrs. Following treatment, the cells were washed twice, replenished with RPMI, and incubated overnight. Levels of ATP from each treatment condition were assessed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega). This assay is based on following reaction: ATP + D-luciferin + O_2 in the presence of luciferases produces oxyluciferin + AMP + PP_i + CO_2 + LIGHT. When D-luciferin and luciferase are present in a saturated level, the light produced by the reaction is directly proportional to the ATP concentration in the unknown sample. Luminescence data were acquired using the Xenogen IVIS Imaging 200 system (Xenogen Corp., Alameda, CA) with a 0.5-sec exposure time. The mean gray values from the acquired images of each well were analyzed with Image J (National Institutes of Health, Bethesda, MD).

Data Analysis and Statistical Calculations. In Vitro *Release*. Cumulative release of carboplatin in either Pluronic P85 or L61 solution was compared with Student's *t*-test, with $P < 0.05$ defined as statistical significance.

In Vivo *Treatment Efficacy*. The percent change in tumor volume from the first day of treatment was calculated from the caliper measurements for each individual tumor. Linear regression was used to estimate changes for every treatment condition as a function of time (week), which resulted in the calculation of an independent growth rate for each treatment group. The growth rate between treatment groups was compared to determine whether any difference existed with a 0.05 level of significance and the 95% confidence interval.

In vitro *ATP Assessment*. To compare the levels of intracellular ATP expression from cells treated with 0.1 mg/ml to 70.0 mg/ml of L61 or P85 relative to control, a two-tailed, unpaired Student's *t*-test was performed. For multiple comparisons, significance levels were corrected using a Bonferroni adjustment. All results are represented as mean ± SEM.

Results

***In Vitro* Drug Release.** Carboplatin release in solutions with or without Pluronic P85 or L61 was assessed by dialysis (Fig. 1). Results showed a delay in carboplatin release in P85 solutions compared with solutions containing L61 and carboplatin alone. After 2 hrs, 0.06 ± 0.002 mg carboplatin was released when P85 was present, compared to 0.2 ± 0.06 mg and 0.3 ± 0.13 mg for L61 and Pluronic-free solvents, respectively. In addition, it appears that both L61 and P85 prolonged the zero-order release phase compared with Pluronic-free solvent. At 24 hrs, significantly slower carboplatin release was seen between Pluronic-free solvent (0.49 ± 0.08 mg) compared with solvent in the presence of P85 (0.14 ± 0.006 mg). This difference continued to the terminal stage of the study.

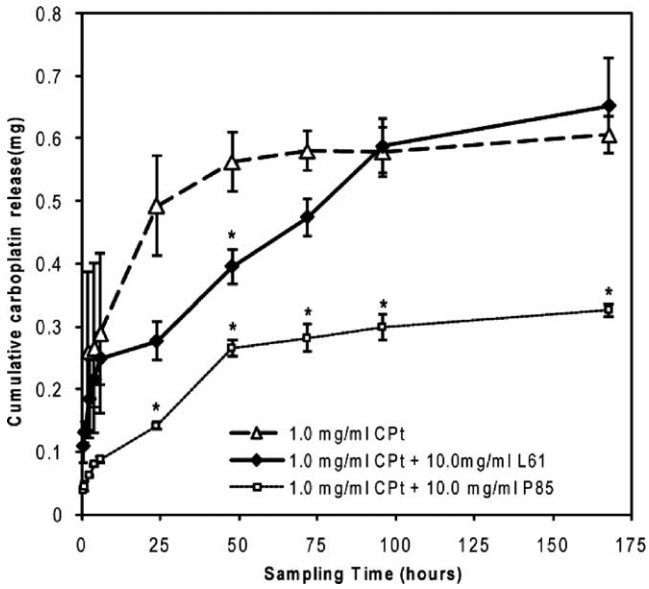

Figure 1. *In vitro* release of carboplatin (mean ± SEM). *Significant differences ($P < 0.05$) at each sampling time compared with control.

However, significant differences between control solution and solution with L61 was only seen at 48 hrs, with 0.40 ± 0.03 mg and 0.56 ± 0.05 mg carboplatin being released from L61 and Pluronic-free solutions, respectively.

Tumor Inoculation and Development. To minimize the number of animals used in the study, four tumors per rat were inoculated. To assess the tumor burden on each animal, general health and body weight were monitored after tumor inoculation and throughout the treatment. No difference in weight gain before treatment was observed between these animals and others receiving two tumors for an unrelated study. The mean tumor diameter at the initiation of treatment was 12.0 ± 0.3 mm, and the tumors were roughly spherical in shape (initial tumor volume: 221.3 ± 12.7 mm^3). Of the 30 animals inoculated with bilateral tumors on the shoulders and thighs (four tumors total), all developed four tumors, except two rats that developed only three tumors. Tumors with an initial size <5 mm in diameter were excluded from further study.

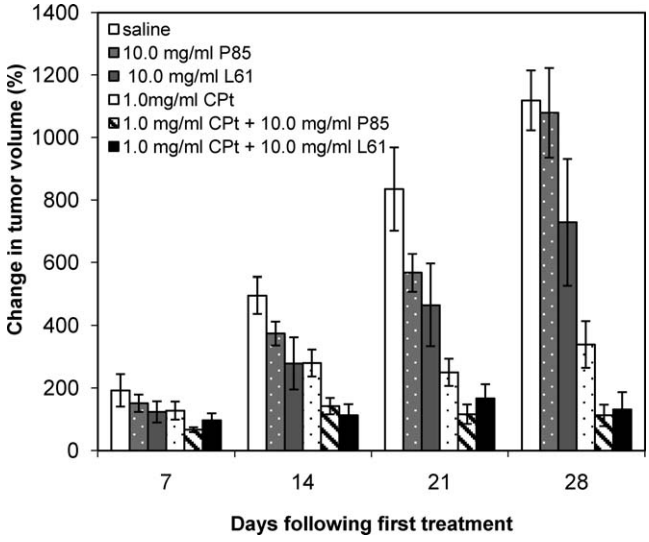

Figure 2. Change in tumor volume from the first day of treatment with 1.0 mg/ml carboplatin and corresponding controls (mean ± SEM). Corresponding growth rate data are shown in Table 1.

***In Vivo* Efficacy of Intratumoral Low-Dose Carboplatin Combined with Pluronic P85 or L61.** As shown in Figure 2, at 1.0 mg/ml carboplatin, tumors treated with CPt and P85 exhibited significantly reduced change in volume after 28 days (112.7% ± 34.4%; $n = 15$) compared with tumors treated with free drug (339.4% ± 75.0%; $n = 16$). CPt in the presence of L61 resulted in a tumor volume increase of 131.3% ± 55.6% ($n = 8$). Control tumors treated with either P85, L61 alone, or saline showed volume increases of 1079.4% ± 143.6% ($n = 16$), 729.4% ± 202.2% ($n = 7$), and 1119.2% ± 96.1% ($n = 16$), respectively. Using linear regression analysis, the independent rate of change of tumor volume as a function of week was also obtained for each treatment condition. Treatment with saline control showed a volume change of 272.2% per week throughout the treatment period. Tumors treated with low-dose CPt alone showed a volume increase of 93.2% per week. However, tumors treated with CPt in the presence of L61 showed a reduced increase in volume of 44.9% per week, whereas P85 exhibited a notably slower change of 38.4% per week during the course of the study. Statistically significant differences ($P < 0.05$) were noted between tumors treated with saline injection and all the other treatment groups except 10.0 mg/ml P85 injection alone (Table 1). In addition, a significant difference also was seen between tumors treated with 1.0 mg/ml CPt with P85 compared with CPt alone.

***In Vivo* Efficacy of Intratumoral High-Dose Carboplatin Combined with Pluronic P85 or L61.** The capability of Pluronic P85 and L61 to enhance intratumoral chemotherapy at a higher carboplatin concentration also was assessed. As shown in Figure 3, all treatment groups exhibited shrinkage of tumor volume relative to initial tumor volume. However, no significant differences were seen between tumors treated with carbo-

Table 1. Tumor Growth Rate for All Treatment Groups with Low-Dose CPt

Treatment	Growth rate, % per week (95% CI)
Saline	272.2 (248.9, 295.5)
10.0 mg/ml P85	230.7 (207.4, 254.0)
10.0 mg/ml L61	165.4 (119.4, 211.5)[a]
1.0 mg/ml CPt	93.2 (69.9, 116.6)[a]
1.0 mg/ml CPt + 10.0 mg/ml P85	38.4 (14.3, 62.4)[a,b]
1.0 mg/ml CPt + 10.0 mg/ml L61	44.9 (11.9, 77.9)[a]

[a] Statistically significant improvement over saline ($P < 0.05$).
[b] Statistically significant improvement over carboplatin alone ($P < 0.05$).

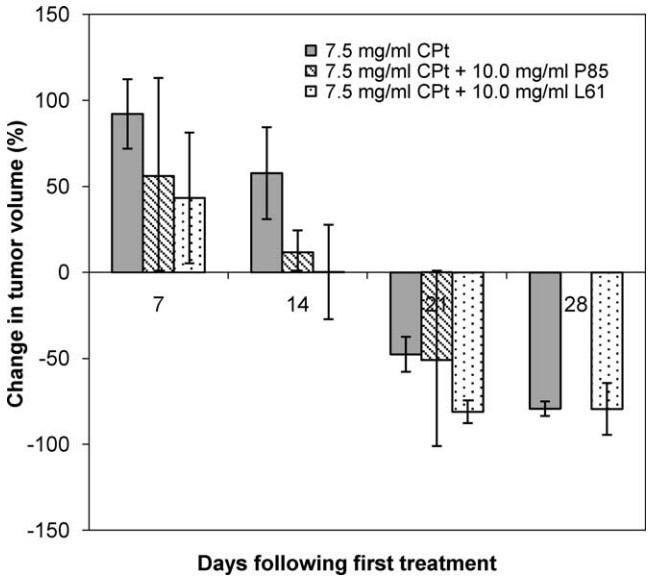

Figure 3. Change in tumor volume from the first day of treatment with 7.5 mg/ml carboplatin (mean ± SEM). Corresponding growth rate data are shown in Table 2.

platin alone and tumors treated with carboplatin combined with P85 or L61 (Table 2).

The toxicity of each treatment was evaluated on the basis of change in rat body weight and percent of survival through the entire treatment duration. Zero of four rats survived the 4 wks of treatment with 7.5 mg/ml carboplatin and P85. The symptoms included extreme weight loss and excessive urination. Also, one of two rats that died in treatment groups was from the L61 (at the second week) and the other was from the high-dose CPt with L61 (at the third week). Among these two groups, no weight loss or renal dysfunction was observed.

Histologic Analysis. To examine the tumor response to the various treatments at the cellular level, all tissue samples were stained with H&E and MTC. It was noted that the cellular morphology varied widely between treatment groups. Tumors treated with saline (Fig. 4A) and P85 or L61 (Fig. 4B) with no drug showed similar responses. Cells in these tissue sections showed pleomorphism with a high mitotic index and limited collagen deposition. Little to no fibrosis was noted in areas other than the capsule surrounding the outer tumor region. Inflammatory cells were numerous at the periphery of the tumor nodules, and some desmoplasia (connective) could be seen in the muscle and tumor interface. Relatively more basophilic nuclear debris was visible in the drug-free group in the presence of P85. In the CPt-only group, a response to the chemotherapy was readily apparent as necrosis and a fibrotic response to the treatment were visible but not extensive, and relatively more nuclear debris was observed. The groups that received CPt and P85 or L61 showed a reduced area of viable tumor and a high degree of necrosis (Fig. 4C). In general, an elevated desmoplastic response was noted in tumors treated with CPt in the presence of Pluronic. Finally, tumors treated with the high dose of carboplatin either with or without Pluronic L61 showed extensive collagen deposition and little or no viable tumor cell aggregates (Fig. 4D).

Table 2. Tumor Growth Rate for Treatments with High-Dose CPt

Treatment	Growth rate, % per week (95% CI)
7.5 mg/ml CPt	−52.5 (−65.7, −39.4)
7.5 mg/ml CPt + 10.0 mg/ml P85	−63.1 (−86.7, −39.4)
7.5 mg/ml CPt + 10.0 mg/ml L61	−62.7 (−80.4, −44.9)

Effects of Pluronic P85 and L61 on Cell ATP Levels. Adherent DHD/K12/TRb cells were treated with 0.0–70.0 mg/ml Pluronic L61 or the same dose of P85 for 6 hrs. Our results show that intracellular ATP levels were significantly reduced by P85 and L61 at all concentrations except 0.01 mg/ml P85 compared with untreated control. Figure 5 illustrates the dose-dependent reduction of intracellular ATP. The lowest P85 concentration (0.01 mg/ml) reduced ATP to 83.4% ± 2.3% of untreated control, whereas the highest concentration (70.0 mg/ml) reduced ATP to 26.2% ± 1.6% of untreated control. Levels of intracellular ATP in cells treated with L61 at 0.01 mg/ml were reduced to 76.6% ± 2.2% of control. In addition, for concentrations of 1.0 mg/ml and above, L61 caused 100% ATP depletion in these cells.

Discussion

Pluronic possesses the ability to modulate cellular function (7, 28), and several studies have demonstrated that certain members of the Pluronic family have the ability to increase the cytotoxic effects of chemotherapeutic agents in a number of cell lines (7). A study by Alakhov *et al*. found that Pluronic P85 was successful in reducing the IC_{50} of cisplatin by 50% in SKVLB ovarian carcinoma cells (9). Similar results were obtained by our group for carboplatin in the DHD/K12/TRb rat colorectal carcinoma cell line (11). In these studies with non-MDR cells, although the modest improvement of cell toxicity *in vitro* may appear insignificant, the current study presents compelling evidence that even a small difference *in vitro* leads to a considerable, significant reduction of tumor volume *in vivo* compared with free drug.

In this study, we attempted to determine whether Pluronic P85 or L61 could improve the response of rat colorectal adenocarcinoma (DHD/K12/TRb) to carboplatin administered *via* direct intratumoral injection. This treatment scheme was evaluated *in vivo* by examining two pharmacodynamic endpoints: tumor size and histopathology. Our results provide considerable insight into the control of tumor growth with this treatment. No difference was seen between saline and Pluronic P85 treatment, suggesting that P85 itself at the relatively high dose of 10.0 mg/ml is not toxic to the tumor cells or the animal. In

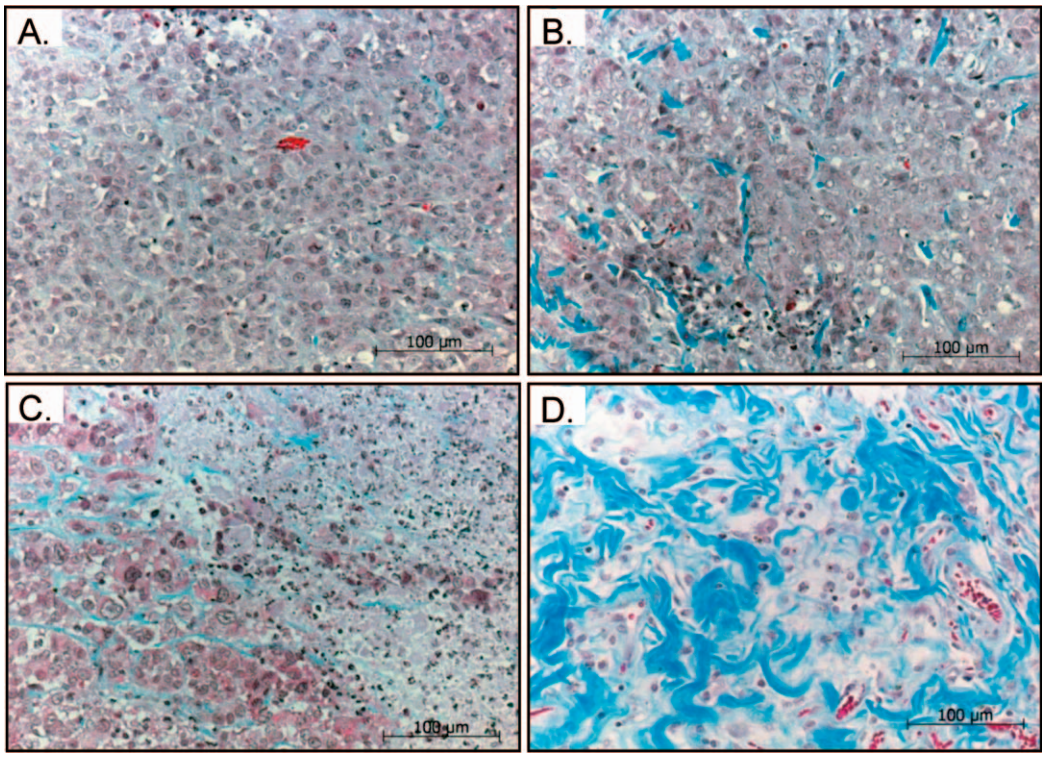

Figure 4. Representative histology images of subcutaneous rat tumors 4 wks after selected treatments (MTC stain). Magnification: ×200. (A) Saline control. The area is populated with rapidly proliferating viable tumor cells. (B) P85 control. Viable tumor cells have similar appearance to saline control. (C) Low-dose CPt and P85. The region shows extensive necrotic debris (top right) with an area of viable tumor (bottom left). (D) High-dose CPt. In completely treated tumors, widespread collagen deposition and increased vascularization were noted in the former location of each tumor. Figure is available in color online.

contrast, significantly decreased tumor volume was seen between treatments that received 1.0 mg/ml CPt with P85 compared with CPt alone. These results suggest that a simple addition of P85 to a carboplatin chemotherapy regimen may significantly enhance the treatment outcome.

We also showed that L61 injection (10.0 mg/ml) alone significantly reduced the tumor volume relative to saline, but no difference was seen between treatment groups that received L61 and CPt with L61. These results suggest an inherent toxicity of L61 at the administered dose, which is consistent with recently published work (29). Finally, at higher CPt doses (7.5 mg/ml), results showed that CPt has the dominant cytotoxic effect, with any additional effects of either Pluronic being negligible. However, one important finding at the high drug dose was the considerable toxicity of the combination of CPt and P85. Although neither agent alone exhibited significant observable systemic toxicity, together they resulted in significantly decreased survival (with no animals surviving the treatment course). During the course of the treatment, extreme weight loss and excessive urination were noted in this group, the latter of which may indicate renal damage.

A number of explanations are possible for the effects of Pluronic P85 or L61. The delay in *in vitro* release may indicate an association between carboplatin and Pluronic. Delivery of carboplatin in Pluronic solutions may lead to

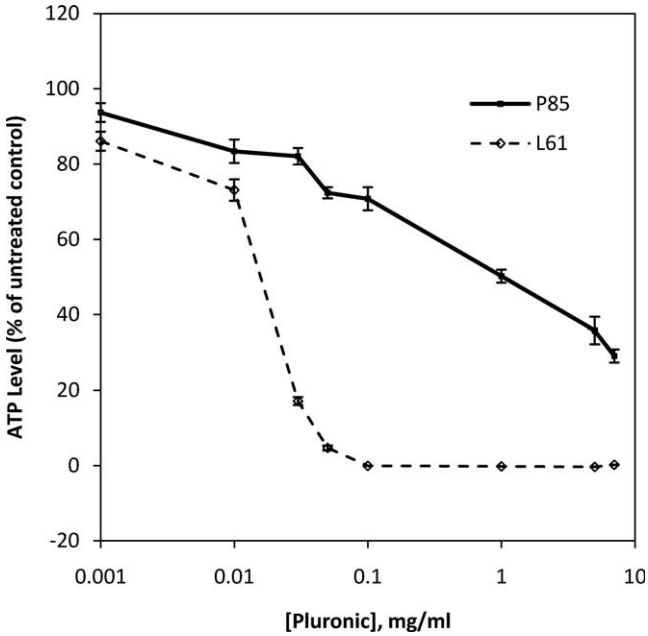

Figure 5. Intracellular ATP concentration (mean ± SEM) after treatment with either Pluronic P85 or L61 relative to untreated control. Significant differences ($P < 0.05$) were seen between all treatment conditions relative to untreated control, except at 0.01 mg/ml.

association of the hydrophilic drug with the corona of Pluronic micelles or the hydrophilic domains of the unimers, both of which may lead to a decreased immediate availability of drug to the tumor and an extended presence of the drug in the tumor vicinity. According to Batrakova *et al*., the availability of free drug decreases when Pluronic concentration is above the critical micelle concentration (CMC), due to the formation of Pluronic micelles (12). The Pluronic concentration in our *in vivo* study was 2.17×10^{-3} *M*, which is significantly higher than the previously measured CMC of P85 (6.5×10^{-5} *M*) or of L61 (1.1×10^{-4} *M*) and should result in a solution of both micelles and unimers (30). The subsequent sustained release of carboplatin from these micelles could then expose tumor cells to toxic drug concentrations for a longer time, inducing a greater decrease in cell viability than the shorter drug exposure of the free drug. This assumption is in agreement with results from our *in vitro* release studies, which showed that both P85 and L61 have a prolonged zero-order release phase of carboplatin. The delayed release from conditions in the presence of P85 also may be due to a strong association of drug with the polymer. A similar argument also can be applied to the L61 group, since it has relatively fewer units of PEO (2/PEO segments), which would lead to a weaker association with the drug. L61 is relatively hydrophobic and tends to form lamellar aggregates with larger size and lower stability (31), possibly explaining why the drug was released faster.

Both L61 and P85 may sensitize the tumor cells to the cytotoxic effects of carboplatin, leading to higher efficacy of the drug in the presence of Pluronic. Pluronic has been shown to decrease the activity of the Pgp transporter (12, 14), increase the permeability of the cell membrane, deplete intracellular GSH/GST (7, 15), reduce drug sequestration within cytoplasmic vesicles (28), and promote apoptotic signaling (8). To investigate one possible mechanism of sensitization, our study measured levels of intracellular ATP following exposure of cells to a range of Pluronic concentrations. Our results suggest that although both P85 and L61 lead to a significant reduction in ATP, P85 showed a milder effect. P85 partially depletes the intracellular ATP levels, which may cause cell stress and weaken the cells' ability to respond to the toxic effects of drug. However, L61 may lead to a complete depletion of intracellular ATP-induced cell death. Any combination of these effects would be expected to improve the efficiency of carboplatin treatment. In all likelihood, the increased treatment efficacy in our study may be due to a balance between slower drug release and increased cell susceptibility due to different degrees of intracellular ATP reduction.

While the results of using Pluronic as part of an intratumoral chemotherapy regimen are promising, interpretation of the results are suggestive, because the effect has only been shown in a single tumor model. The subcutaneous, solid tumor model of a naturally metastatic colorectal cancer line may exhibit somewhat different physiologic properties than naturally occurring tumors. In addition, repeated direct intratumoral injection of free drug or drug/polymer aggregates may not be clinically feasible and may lead to a different outcome than systemic or regional administration of the same therapy. Nonetheless, the inhibitory effects of tumor growth justify further study into the combination of Pluronic P85 and carboplatin or other antineoplastic agents, particularly in extended release drug delivery formulations.

Conclusions

The current study examined the efficacy of carboplatin/Pluronic complexes delivered locally to a subcutaneous allograft of colorectal carcinoma in BDIX rats. With the combination of P85 and carboplatin, tumor growth was significantly delayed compared with tumors receiving the free drug, and the improved drug efficacy was confirmed by histologic analysis. The inhibition of tumor growth was well correlated to the modulation of carboplatin release of Pluronic and its effects on intracellular ATP expression. Our results suggest that P85 is effective in increasing the cytotoxic effects of antineoplastic agents and improving inhibition of tumor growth with chemotherapy, whereas L61 itself is toxic to cells because it completely depletes intracellular ATP needed for normal cellular function. Future study will provide additional information about whether these results can be extended to other tumor types and MDR tumors.

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