Research Article

Theme: Clinical and Commercial Translation of Drug Delivery Systems

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Examination of Gossypol-Pluronic Micelles as Potential Radiosensitizers

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Abstract. Chemoradiotherapy, the combination of chemotherapy and radiotherapy to treat cancer, has the potential to enhance local therapeutic effects and simultaneously treat systemic disease. However, chemoradiotherapy may also enhance normal tissue effects leading to both acute and late toxicities. Furthermore, subtherapeutic chemoradiotherapy may result in aggressive tumor repopulation. Tumor-specific radiosensitizing chemotherapy may yield a synergistic therapeutic effect and avoid augmentation of normal tissue toxicity. In this study, the radiosensitizing effects of gossypol were investigated. Also, Pluronics were studied for gossypol solubilization and co-radiosensitization effects. Gossypol inhibits Bcl-2 and Bcl-X_L, antiapoptotic proteins that are overexpressed in various cancer cells. Pluronic micelles (P85, F88, L35, and P123) effectively encapsulated gossypol, raising its water solubility by more than 1000-fold. Cytotoxic, anticlonogenic, and radiosensitizing effects were evaluated to characterize gossypol and Pluronic combinations. Gossypol and P85 had the strongest antiproliferative effect on A549 human lung adenocarcinoma cells in a cell viability assay. The IC₅₀ value was seven times lower than gossypol only treatment (330±70 nM vs 2400±400 nM, (mean±SE)). Gossypol and P85 showed significant inhibition of clonogenic survival, approximately 30% inhibition, compared to treatment with gossypol alone. An experimental sequencing study demonstrated greater inhibition of clonogenic survival when drug treatment followed radiation compared to a sequence of drug treatment followed by radiation. These results suggest that Pluronic micelles readily solubilize gossypol and that the combination of gossypol and P85 may augment the therapeutic effects of ionizing radiation.

KEY WORDS: chemoradiotherapy; gossypol; Pluronic; polymeric micelles; radiosensitization.

INTRODUCTION

Surgery, radiotherapy, and chemotherapy are the three major therapies for cancer treatment. Often, a combination of therapies is necessary to increase therapeutic efficacy (1). The addition of chemotherapy to radiotherapy may result in increased local therapeutic effects of radiation while simultaneously addressing systemic disease. Consequently, chemoradiotherapy has emerged as standard first-line treatment for an array of malignancies. However, chemoradiotherapy can also augment normal tissue injury resulting in severe acute side effects. Consequently, a critical issue in the design of novel chemoradiotherapies is the development of combinations that have the capacity to enhance antitumor effects while

minimizing unwanted normal tissue injury. Tumor-specific radiosensitizing chemotherapy is expected to yield synergistic antitumor effects and minimize side effects (2). Thus, therapeutic agents with both independent, tumor-specific cytotoxic effects and radiosensitizing effects are expected to be excellent candidates for combined modality therapies.

Gossypol, a polyphenolic compound extracted from cotton seed (3), has been reported to radiosensitize various cancer cells (4–8). Gossypol inhibits the effects of antiapoptotic proteins Bcl-2 and Bcl-X_L (3–8), which are commonly overexpressed in cancer cells. Although gossypol is expected to enhance radiation-induced apoptosis of cancer cells via this mechanism, gossypol is highly hydrophobic and poorly water soluble, requiring a drug solubilization strategy for IV administration. Previous research has studied gossypol in the context of oral applications in clinical and preclinical studies, but gastrointestinal disturbances have been reported (3,7,9).

Pluronics were selected in this study to solubilize gossypol and enhance its anticancer effects by membrane disruption. Pluronics are triblock copolymers of propylene oxide (PO) and ethylene oxide (EO). They are known to solubilize hydrophobic compounds in the micellar state and have been proven to be effective solubilizers of polyphenols, such as resveratrol, for IV administration (10). Our previous

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study confirmed that Pluronic F68 (poloxamer 188) solubilized gossypol up to 5.4 ± 0.1 mg/mL in water (11). Pluronics have been reported to have selective membrane-perturbing effects on cancer cells, according to their PO:EO ratio and chain length (12,13). According to those reports, Pluronics have been categorized into four groups: group I—high HLB, large molecular weight; group II—intermediate HLB, intermediate molecular weight; group IIIa—low HLB, small molecular weight; and group IIIb—low HLB, large molecular weight. Pluronics in groups I and IIIa were reported to have a membrane solidification effect, whereas Pluronics in groups II and IIIb have a membrane fluidization effect (12). P85, one of the Pluronics in group II, was reported to inhibit respiration in mitochondria, deplete ATP, and decrease P-glycoprotein ATPase activity in both multidrug resistant (MDR) cells and non-MDR cells (12,13). More recently, Pluronic L10 was reported to radiosensitize Gli36 human glioma cells by inhibiting heat shock protein (Hsp) 90 and 70 (14). Hsp 90 and 70, molecular chaperone proteins that are overexpressed in various cancer cells, contribute to the conformational maturation of oncogenic survival signaling proteins and inhibit the apoptotic pathway to protect cancer cells from death (15–18). Therefore, Hsp 90 and 70 are considered novel targets for cancer treatment (19–21). Several reports suggest that Hsp 90 inhibitors show radiosensitizing effects by inhibiting radiation-induced cell survival signals, cell cycle checkpoint activation, and DNA double strand break repair (22–27). However, inhibition of Hsp 90 is reported to trigger the upregulation of Hsp 70. Therefore, dual inhibition of Hsp 90 and 70, such as that exhibited by Pluronic L10, is considered to be more attractive for cancer therapy (28–32). In this study, F88, P85, L35, and P123 were selected as representative Pluronics in group I, II, IIIa, and IIIb, respectively. The effects of Pluronics type, amount, and gossypol concentration on cancer cell viability, clonogenic survival, and radiosensitization were evaluated with the aim of producing a novel and potent micellar radiosensitizer for cancer treatment.

MATERIALS AND METHODS

Materials

Racemic gossypol was purchased from LKT Laboratories, Inc. (St. Paul, MN). Pluronic P85, F88, L35, P123 were kindly provided by BASF Corp. (Florham Park, NY). A549 human lung adenocarcinoma cells were purchased from ATCC (Manassas, VA). CellTiter-Blue® Cell Viability Assay kit was purchased from Promega (Madison, WI). Crystal Violet was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were obtained from Thermo Fisher Scientific Inc. (Fairlawn, NJ) and were of analytical grade.

Methods

Preparation of Gossypol-Loaded Pluronic Micelles

Gossypol and Pluronics (P85, F88, L35, and P123) were dissolved in 2.0 mL of acetone in a round-bottom flask. Acetone was evaporated under reduced pressure at 60°C. After the evaporation, 1.0 mL of 0.9% NaCl solution was

added to rehydrate gossypol and Pluronics. Subsequently, the solution was centrifuged at 13,000 rpm for 5 min to remove insoluble gossypol and filtered through a 0.2 μ m sterile syringe filter. Gossypol-loaded Pluronic micelles were prepared at 1:9, 1:19, 1:99, and 1:332 of gossypol:Pluronic weight ratios (i.e., gossypol-loading ratios of Pluronic micelles at 10, 5, 1, and 0.3%, respectively).

Physicochemical Evaluation of Gossypol-Loaded Pluronic Micelles

Z-average diameter and polydispersity index (PDI) of gossypol-loaded Pluronic micelles at 25°C were measured using a Zetasizer Nano-ZS (Malvern Instruments, UK) at a fixed angle of 173°. The loading ratio of gossypol in the prepared gossypol-loaded Pluronic micelles was measured using a reverse-phase Shimadzu Prominence HPLC system (Shimadzu, Japan). Twenty microliters of gossypol-Pluronic micelle solution was dissolved in 980 μ L of mobile phase (3:1 acetonitrile:aqueous phosphoric acid (1%)). Ten microliters of the dissolved solution was injected into a Zorbax RX-C8 analytical column (4.6 mm $\times$ 250 mm, particle size 5 μ m, Agilent) with a flow rate of 1.0 mL/min, a run time of 12 min, and column oven temperature at 40°C. The separation was conducted in an isocratic mode mobile phase. Gossypol was detected at 373 nm, and its retention time was 8.5 min. The level of gossypol showed linearity at 0.21–210 μ g/mL with the limit of detection at 0.2 μ g/mL.

Cell Viability Assays

A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. A549 cells were maintained at 37°C under an atmosphere of 5% CO₂ in a humidified incubator. A549 cells were seeded at 1000 cells/well on 96-well plates and incubated for 24 h. Various ratios (1:9, 1:19, 1:99, and 1:332) of gossypol-loaded Pluronic micelles were evaluated at gossypol concentrations of 1, 2, 5, 10, and 50 μ M. After 72 h of incubation, cell viability was determined using a CellTiter-Blue® Cell Viability Assay kit according to manufacturer's instructions. IC₅₀ values were calculated from obtained data with use of Graphpad Prism6® software. Gossypol dissolved in DMSO was used as a control. Experiments were performed in triplicate.

Clonogenic Survival Assays

Effect of Gossypol on A549 Cell Clonogenic Survival. Clonogenic survival assays were performed as previously described (33). Cell radiation was performed using a Shepherd & Associates Model 109 irradiator (San Fernando, CA) with a 137cesium source at a dose rate of 3.89 Gy/min. Lethally irradiated (30 Gy) A549 cells were used to ensure identical total cell seeding for all experiments (2500 cells in 2 mL media per well). The number of lethally radiated cells and unirradiated cells was as follows: 0 Gy, 2400:100; 2 Gy 2300:200; 4 Gy, 2200:300; 6 Gy 1500:1000; 8 Gy 0:2500. After seeding, cells were incubated for 24 h. After the 24-h incubation, gossypol (2–4 μ M) dissolved in DMSO was added to the wells (Day 1). Final DMSO level

Radiosensitizing Effects of Gossypol, Pluronic, and Gossypol-Loaded Pluronic Micelles. The seeding condition of A549 cells was the same as that described in section 2.2.4.1. After 24-h incubation, cells seeded on 6-well plates were treated with 2.0 mL of gossypol dissolved in DMSO, Pluronic, or gossypol-loaded Pluronic micelles (Day 1) and incubated for 24 h. The concentration of gossypol in the wells was fixed at 2.0 μ M, and the Pluronic level in the wells was fixed at 0.1 mg/mL, in correspondence with a 1:99 weight ratio of gossypol:Pluronic. One day after drug treatment, irradiation was carried out at doses of 0, 2, 4, 6, and 8 Gy (Day 2). Subsequent procedures were the same as those described in section 2.2.4.1. Survival fraction (SF) was calculated as (observed plating efficiency)/(plating efficiency at 0 Gy). A fitting curve was applied for the SF with a linear-quadratic equation: $SF = \exp(\alpha D + \beta D^2)$, where D is radiation dose (33). From the equation of fitting curve, radiation dose at 50% cell kill was calculated. Subsequently, the sensitizer enhancement ratio (SER) was calculated as radiation dose without drug/radiation dose with drug.

Effects of Treatment Sequence on Gossypol and P85 Inhibition of Clonogenic Survival. Clonogenic survival assays were performed as described in section 2.2.4.2. The concentration of gossypol and P85 level were fixed at 2.0 μ M and 0.1 mg/mL, respectively. Drug treatment was performed 48, 24 and 1 h prior to radiation as well as 1, 24 and 48 h after radiation.

Statistical analysis was performed using Student's *t* tests. Differences were deemed statistically significant if the two-tailed *p* value was less than 0.05.

Physicochemical Evaluation of Gossypol-Loaded Pluronic Micelles

Table I. Physicochemical Properties of Gossypol-Loaded Pluronic Micelles

Pluronic	P85			F88			L35			P123		
	Theoretical loading ratio (%)	Size (nm)	PDI	Actual loading ratio (%)	Size (nm)	PDI	Actual loading ratio (%)	Size (nm)	PDI	Actual loading ratio (%)	Size (nm)	PDI
Gossypol:pluronic	1:9	17.2±0.6	0.06±0.01	11.8±0.2	38.4±0.2	0.11±0.01	12.2±0.2	120±5	0.10±0.01	6.4±0.4	67±1	0.270±0.008
	1:19	21±2	0.09±0.02	6.3±0.4	34±4	0.21±0.02	6.1±0.1	130±10	0.14±0.01	3.9±0.3	41±10	0.24±0.02
	1:99	21±3	0.31±0.06	1.0±0.1	37±2	0.6±0.1	1.12±0.04	59.1±0.6	0.09±0.01	1.0±0.1	19.3±0.1	0.04±0.02
	1:332	19.1±0.6	0.31±0.01	0.40±0.04	17.6±0.1	0.54±0.03	0.33±0.04	58±2	0.12±0.02	0.37±0.04	18.2±0.1	0.026±0.003
PDI polydispersity index												

loading. Gossypol-loaded L35 micelles were much larger than gossypol-loaded P85 and F88, (60 to 130 nm); particle diameter increased with drug loading. L35 was the only formulation (1:9 of gossypol:L35) that showed markedly lower gossypol-loading efficiency compared to the theoretical loading ratio. These data suggest that the low molecular weight of L35 limits gossypol-loading efficiency at high gossypol concentration. Gossypol-loaded P123 micelles ranged in size from 20 to 70 nm. Micelle size increased with drug loading. In summary, Pluronic micelles, with the exception of gossypol-L35 micelles at a 1:9 ratio of gossypol:L35, efficiently encapsulated gossypol.

Pluronics increased the water solubility of gossypol up to 12 ± 1 mg/mL in our study (P85: 9.4 ± 0.4 , F88: 9.9 ± 0.1 , L35: 10 ± 1 , P123: 12 ± 1 mg/mL). Gossypol is known to be extremely water insoluble with a maximum solubility of less than 0.01 mg/mL (34). Pluronics increased the solubility of gossypol in water more than 1000-fold. In summary, Pluronic micelle solubilization may be sufficient for intravenous infusion of gossypol. This formulation may permit more compelling preclinical and clinical examination of gossypol (34).

Cell Viability Assay *In Vitro*

The IC_{50} value of gossypol was 2.4 ± 0.4 μ M for A549 cells. L35 did not significantly affect the IC_{50} values for A549 cells (Fig. 1). F88 slightly decreased IC_{50} values (2-fold) as the level of Pluronic increased. In contrast, P85 and P123 decreased IC_{50} values by 7- and 5-fold, respectively, as the level of Pluronic increased (Fig. 1). The strongest cytotoxicity was obtained using gossypol and P85 at 1:332 of gossypol:P85. Group II Pluronics with intermediate to high hydrophobicity, such as P85, show cell membrane fluidization effects (12), which cause cell membrane instability and loss of homeostasis. Moreover, P85 is reported to inhibit respiration in mitochondria of cancer cells by affecting the respiratory chain complexes I and IV (13). Respiratory inhibition causes impairment of mitochondrial function, consequently inducing the release of cytochrome C, and onset of apoptosis (13). Thus, gossypol and P85 may induce apoptosis due to Bcl-2 and Bcl-X_L inhibition and mitochondrial respiration

Fig. 1. Cytotoxicity of gossypol and Pluronics against human lung adenocarcinoma A549 cells (open circle P85, open square F88, open diamond L35, open triangle P123. $N=3$)

Fig. 2. Gossypol inhibition of A549 clonogenic survival (* $P<0.0001$. $N=3$)

inhibition, respectively. On the other hand, Pluronics in group I and IIIa do not have any effects on mitochondrial membranes, and Pluronics in group IIIb have less perturbing effect to mitochondrial membrane than Pluronics in group II (12). Thus, P85 (Pluronics in group II) showed higher cytotoxicity than other Pluronics.

Fig. 3. **a** Effect of Pluronics on A549 cell clonogenic survival (Pluronics at 0.1 mg/mL. $N=3$). **b** Effect of Pluronics-solubilized gossypol on A549 cell clonogenic survival (G gossypol at 2 μ M; * $P<0.05$. $N=3$)

Clonogenic Assay *In Vitro*

The effect of gossypol on clonogenic survival is shown in Fig. 2. The inhibitory effects of gossypol are dose-dependent and increase sharply above 2 μ M.

Fig. 4. **a** Effect of gossypol on A549 radiosensitivity (black circle gossypol at 2 μ M in DMSO, open circle XRT only. $N=3$). **b** Effect of Pluronics (0.1 mg/mL) on A549 radiosensitivity (black circle P85, black square F88, black diamond L35, black triangle P123, open circle XRT only. $N=3$). **c** Effect of Pluronics-solubilized gossypol on A549 radiosensitivity (gossypol at 2 μ M, Pluronics at 0.1 mg/mL: black circle gossypol+P85, black square gossypol+F88, black diamond gossypol+L35, black triangle gossypol+P123, open circle XRT only. $N=3$)

Table II. The SER Values of Gossypol Alone, Pluronics Alone, and Pluronics-Solubilized Gossypol

Gossypol alone	0.84			
	P85	F88	L35	P123
Pluronics	1.01	0.98	0.83	0.83
Gossypol/Pluronics	0.89	0.93	0.84	0.9

The effect of different Pluronics alone on A549 clonogenic survival is shown in Fig. 3a. The effect of both DMSO- and Pluronics-solubilized gossypol on A549 clonogenic survival is shown in Fig. 3b. Gossypol at 2 μ M and Pluronics alone at 0.1 mg/mL did not significantly inhibit A549 clonogenic survival. Similarly, gossypol (2 μ M) solubilized in F88, L35, and P123 failed to show statistically significant inhibition of A549 clonogenic survival. Only when gossypol was solubilized in P85 was inhibition of clonogenic survival observed.

To assess potential radiosensitizing effects of gossypol, different Pluronics, and different Pluronics-solubilized gossypol, A549 cells were treated with these agents for 24 h and then subjected to ionizing radiation (0–8 Gy). As shown in Fig. 4, no radiosensitization was observed, contrasting with earlier data on Pluronic L10 (14). The

Fig. 5. Effect of gossypol, Pluronic P85, and Pluronic P85 solubilized gossypol on A549 radiosensitivity. A549 cells were treated **a** 1 h or **b** 48 h prior to radiation. (gossypol at 2 μ M, P85 at 0.1 mg/mL, black circle gossypol, black square P85, black diamond gossypol+P85, open circle XRT only. $N=3$)

SER value of gossypol alone was 0.84. Also, the SER value of P85, F88, L35, and P123 were 1.01, 0.98, 0.83, and 0.83, respectively. Similarly, the SER value of P85, F88, L35, and P123-solubilized gossypol were 0.89, 0.93, 0.84, and 0.90, respectively (Table II). These data confirmed mathematically that no radiosensitization was observed in those groups.

To assess if potential radiosensitization was dependent on the duration of cell exposure to gossypol, cells were treated for 1 or 48 h with gossypol, Pluronic P85 or gossypol-containing Pluronic P85 micelles prior to radiation. Similar to 24 h pretreatment, no significant radiosensitization was observed at 8 Gy with 1 or 48-h pretreatment (Fig. 5a, b). SER values for 1-h pretreatment with DMSO-solubilized gossypol (0.95) and 48-h

Table III. The SER Values of Gossypol Alone, P85 Alone, and P85-Solubilized Gossypol

	Interval (h)	Gossypol	P85	P85-solubilized gossypol
Pretreatment	1	0.95	1.42	1.14
	24	0.84	1.01	0.89
	48	0.72	0.80	0.92
Post-treatment	1	1.06	1.23	1.86
	24	1.01	1.21	1.20
	48	1.12	1.42	1.55

pretreatment with DMSO-solubilized gossypol (0.72), P85 (0.80), and P85-solubilized gossypol (0.92) showed no radiosensitization. SER values for 1-h pretreatment with P85 and P85-solubilized gossypol showed slight radiosensitization (1.42 and 1.14, respectively).

To determine if therapy sequence impacted clonogenic survival, A549 cells were first subjected to ionizing radiation (0–8 Gy) then treated with control media, gossypol in DMSO, Pluronic P85, or Pluronic P85 micelles containing gossypol (1, 24, and 48 h after radiation). As seen in Fig. 6, post-radiation treatment of A549 cells with P85 micelles containing gossypol resulted in a statistically significant augmentation of radiation cell kill at 8 Gy. No such radiosensitization was observed with gossypol alone or P85 alone. The SER values of the post-treatment group document greater radiosensitization than those of the pretreatment group (Table III). The highest SER value recorded was for P85-solubilized gossypol 1 h following cell radiation (1.86). In general, gossypol treatment following radiation appeared to be more effective in augmenting radiation cell kill. Although the molecular mechanisms for this unique pattern of radiosensitization are still under further investigation, the results of clonogenic assays reflect cell killing, as opposed to inhibition of cell proliferation as assessed in the cell viability assays. Thus, tumor cell killing may be facilitated by a combination of radiation and gossypol-P85 treatment.

CONCLUSION

Gossypol was successfully solubilized by P85, F88, L35, and P123 Pluronic micelles using a solvent evaporation method. P85 solubilized gossypol showed the strongest cancer cell cytotoxicity in this study. Gossypol (2 μ M) alone, Pluronics (0.1 mg/mL) alone, and gossypol (2 μ M) in F88, L35, and P123 micelles did not show anticlonogenic effects. Only gossypol (2 μ M) solubilized in P85 micelles showed anticlonogenic effects. The anticlonogenic effects of gossypol were concentration dependent. The combination of gossypol and P85 augmented radiation cell kill uniquely when drug treatment followed cell radiation. In summary, gossypol-loaded P85 micelles may be an attractive candidate for combination with ionizing radiation, and these data will help guide further examination. Additional studies are needed to clarify the mechanism by which gossypol-loaded P85

Fig. 6. Effect of gossypol, Pluronic P85, and Pluronic P85 solubilized gossypol on A549 radiosensitivity. Radiated cells were treated **a** 1 h, **b** 24 h, or **c** 48 h after radiation (gossypol at 2 μ M, P85 at 0.1 mg/mL, black circle gossypol, black square P85, black diamond gossypol+P85, open circle XRT only. $N=3$)

micelles radiosensitize cancer cells and evaluate the performance of this novel nanoparticle *in vivo*.

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