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Injectable (–)-Gossypol-loaded Pluronic P85 Micelles for Cancer Chemoradiotherapy

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Abstract

Purpose—The goal of tumor-specific chemoradiotherapy is to achieve synergistic anticancer effects with clinically acceptable toxicity. Our previous studies showed that Pluronic P85 augments radiation cancer cell killing of (±)-gossypol *in vitro*. In this study, radiosensitizing effect of (–)-gossypol, more potent Bcl protein inhibitor, with Pluronic P85 was investigated.

Materials and methods—The inhibitory effect of (–)-gossypol solubilized Pluronic P85 with 0–8 Gy of radiation on clonogenic survival rate of A549 human lung adenocarcinoma cells was investigated *in vitro*. Anticancer effect of (–)-gossypol solubilized Pluronic P85 with fractionated radiation of 15 Gy was assessed by A549 tumor bearing mice.

Results—(–)-gossypol loaded Pluronic P85 was found to be a more potent radiosensitizer *in vitro*. Pluronic P85 increased the anti-proliferative activity of (–)-gossypol against A549 cells (82±42 versus 190±60 nM). In addition, the combination of P85 and (–)-gossypol effectively reduced clonogenic survival of A549 cells: (11±5%) compared to (–)-gossypol and P85 alone (62±27% and 93±13%, respectively), and enhanced radiation cancer cell killing. *In vivo*, P85 (200 mg/kg/day) and (–)-gossypol (15 mg/kg/day) could be safely injected intravenously over 5 days and enhanced radiation-related tumor control in an A549 xenograft model.

Conclusion—Pluronic P85 and (–)-gossypol act as a novel dual agent radiosensitizer and holds promise as a chemoradiotherapeutic strategy.

Keywords

polymeric micelles; gossypol; pluronic; radiosensitizer; chemoradiotherapy

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DECLARATION OF INTERESTS

No potential conflicts of interest were disclosed.

INTRODUCTION

Chemoradiotherapy has the potential to increase local therapeutic effects of radiation while simultaneously affecting distant metastasis. Although several groups have confirmed that chemoradiotherapy is effective in clinical studies (Dai et al. 2015, Furuse et al. 1999, Hahn et al. 2005, Kiura et al. 2003), severe, acute side effects and aggressive tumor repopulation caused by incomplete tumor control remain major issues (Furuse et al. 1999, Hahn et al. 2005, Kiura et al. 2003, Withers et al. 1988). Thus, tumor-specific radiosensitizing chemotherapy has gained attention, aiming for potent anti-cancer activity and low side effects (Werner et al. 2012). Therapeutic agents targeting over-expressed molecules in tumor cells that are not present in normal tissue may be effective for tumor-specific targeted therapy. Moreover, therapeutic agents that exhibit both cytotoxic and radiosensitizing effects are expected to achieve enhanced anti-cancer efficacy.

Based upon this concept, we have focused on gossypol, a polyphenolic compound extracted from cotton seed (Wang et al. 2009). Gossypol was first used in China as a male oral contraceptive, and later it was found to have antitumor effects (Wang et al. 2009). The antitumor activity of gossypol is due to down regulation of Bcl-2 and Bcl-XL (Anderson et al. 2014, Makoska et al. 2008). These two proteins are commonly overexpressed in cancer cells and are considered attractive therapeutic targets. Moreover, the importance of these proteins as anti-cancer targets is highlighted by the observation that these proteins influence the therapeutic activity of several clinically used cytotoxic agents (Baggstrom et al. 2011, Huang et al. 2010, Liu et al. 2012, Oltersdorf et al. 2005, Wang et al. 2009, Wang et al. 2013). Several studies have evaluated the anticancer effects of gossypol against various cancer cells and in tumor models (Gilbert et al. 1995, Gunassekaran et al. 2011, Huang et al. 2010, Liu et al. 2012, Oltersdorf et al. 2005, Wang et al. 2013). Recently, inhibition of antiapoptotic such as Bcl-2 and Bcl-XL has been reported to have radiosensitizing effects (Kasten-Pisula et al. 2007, Moretti et al. 2010, Xu et al. 2005, Zerp et al. 2009). Although gossypol has encouraging anticancer and radiosensitizing effects, activity in clinical trials has not been compelling. In addition, gossypol is hydrophobic in nature and has poor water solubility, hampering the investigation of parenteral routes of administration as an alternative to oral therapy and this has limited investigational progress (Baggstrom et al. 2011, Wang et al. 2009, Xu et al. 2005).

Pluronic are ABA triblock copolymers of propylene oxide (PO) and ethylene oxide (EO) and Pluronic P85 has tumor-specific anticancer effects due to membrane perturbation and ATP depletion (Alakhova et al. 2010, Batrakova et al. 2003). Pluronic have been widely studied for drug solubilization for parenteral routes of administration, fulfilling strict requirements in safety, stability, and solubility. Pluronic have recently been studied as a class of tumor-specific radiosensitizers, reducing pro-survival heat shock protein 70 and 90 levels (Perera et al. 2013). More recently, Pluronic have been shown to be very effective in solubilizing gossypol in water, reaching ca. 10 mg/mL, and Pluronic P85 has demonstrated enhancement of the radiosensitization achieved with ($\pm$)-gossypol (Tomoda et al. 2015). Given that ($-$)-gossypol is more potent than ($\pm$)-gossypol due to a higher affinity for Bcl-2 and Bcl-XL proteins (Benz et al. 1990, Wang et al. 2009), ($-$)-gossypol is a better candidate for drug development. Accordingly, ($-$)-gossypol was solubilized by Pluronic P85 micelles

and the radiosensitizing effects of this combination has been investigated *in vitro* and *in vivo*.

MATERIALS AND METHODS

2.1. Materials

(-)-gossypol was a kind gift from Dr. Michael Dowd (U.S. Department of Agriculture, Washington D.C.) Pluronic P85 (P85) was 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.

2.2. Methods

2.2.1. Preparation of (-)-gossypol-loaded P85 micelles—P85 (19.8 mg) and (-)-gossypol (0.10 mg) were dissolved in 2.0 mL of acetone in a cylindrical glass tube. Acetone was evaporated under reduced pressure at 60 °C. After evaporation, 1.0 mL of 0.9% NaCl solution was added to rehydrate P85 and (-)-gossypol. The aqueous solution was centrifuged at 13,000 rpm for 5 min to remove precipitate and filtered through a 0.2 µm sterile syringe filter.

2.2.2. Analysis of size, polydispersity index (PDI) of (-)-gossypol-loaded P85 micelles, and water solubility of (-)-gossypol—Z-average diameter and PDI of (-)-gossypol-loaded P85 micelles at 25 °C were measured using a Zetasizer Nano-ZS (Malvern Instruments, UK) at a fixed angle of 173°. The solubilization of (-)-gossypol by P85 micelles was measured using a reverse-phase Shimadzu Prominence HPLC system (Shimadzu, Japan). One hundred µL of (-)-gossypol-P85 micelle solution was dissolved in 900 µL of mobile phase (3:1 acetonitrile:aqueous phosphoric acid (1%)). Ten µL of the dissolved solution was injected into a Zorbax RX-C8 analytical column (4.6 mm ×250 mm, particle size 5 µm, Agilent) at a flow rate of 1.0 mL/min, a run time of 12 minutes, and a column oven temperature of 40 °C. (-)-gossypol was detected at 373 nm. Retention time of (-)-gossypol was 8.5 minutes. The level of (-)-gossypol was linear from 0.19 to 190 µg/mL, and the limit of detection was 0.19 µg/mL.

2.2.3. *In vitro* cell viability assay—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 overnight. (-)-Gossypol-loaded P85 micelles were diluted and evaluated at gossypol concentrations of 0.25, 0.5, 1, 2, 5, 10, and 50 µM. After incubation for 72 hrs, cell viability was determined using a CellTiter-Blue® cell viability assay kit according to manufacturer instructions. The IC₅₀ value was calculated from obtained data with use of Graphpad Prism6® software. (-)-Gossypol dissolved in DMSO was used as a control. Experiments were performed in triplicate to represent the data as mean ± SE.

2.2.4. *In vitro* clonogenic assay—The experimental procedure was similar to previously described methods (Tomoda et al. 2015). Briefly, a feeder layer of A549 cells was prepared by radiation at 30 Gy using a ^{137}Cs irradiator (JL Shepherd Model 109 Irradiator, JL Shepherd & Associates, CA). The feeder layer and non-irradiated A549 cells were seeded on 6-well plates to a total of 2500 cells/well (Day 0). After seeding, cells were incubated overnight. Then, the A549-seeded wells were irradiated at doses of 0, 2, 4, 6 and 8 Gy. Within 1 hour after radiation, (–)-gossypol-loaded P85 micelles were added to the wells (Day 1). The concentration of (–)-gossypol in the wells was fixed at 1.0 μM , and the P85 level in the wells was fixed at 21.7 μM . The cultured medium was refreshed once at day 8 and incubated again for 3 days. At day 11, the medium was removed and cells were washed once with PBS. A549 cells were then stained with a 0.5% crystal violet/methanol solution for 15 min at 37 °C, and the number of colonies was counted using a cutoff of 50 aggregated cells. Plating efficiency (PE) was calculated as (colony count)/(inoculated non-irradiated cell number). Clonogenic survival of A549 cells was defined as (PE/PE of non-treatment). The survival fraction (SF) was calculated as (PE)/(PE at 0 Gy) of each treatment. A fitting curve was applied for the SF with a linear-quadratic equation: $\text{SF} = \exp(\alpha D + \beta D^2)$, where D is radiation dose (Franken et al. 2006). The sensitizer enhancement ratio (SER) was calculated as radiation dose at 50% cell kill without drug/radiation dose at 50% cell kill with drug.

2.2.5. *In vivo* radiosensitization by (–)-gossypol and Pluronic P85—Female athymic nude mice ages 6–8 weeks were purchased from Harlan Laboratories (Madison, WI). A549 cells were harvested from sub-confluent cultures after trypsinization. Mice were anesthetized with 1.5% isoflurane/oxygen; this state was maintained with 1% isoflurane/oxygen. Mice were subcutaneously inoculated with A549 cells on the right flank (100 μL , 2×10^6 cells/animal). After reaching a tumor volume of 150 mm^3 , mice were randomly divided into 6 groups (n=4): (1) radiation with (–)-gossypol-loaded P85 micelles ((–)-gossypol and P85 at 15 and 200 mg/kg/day), (2) (–)-gossypol-loaded P85 micelles ((–)-gossypol and P85 at 15 and 200 mg/kg/day), (3) radiation with P85 micelles (200 mg/kg/day), (4) P85 micelles (200 mg/kg/day), (5) radiation with saline, and (6) saline alone. Treatment consisted of radiation therapy (3 Gy) followed by drug/vehicle infusion (200 μL /20 g mice body weight) daily for 5 consecutive days. Tumor volume was calculated as $0.5 \times a \times b^2$ with “a” as the larger diameter of the tumor and “b” as the smaller diameter of the tumor. Body weight and tumor diameter were recorded for up to ca. 1 month. All mice used for this study were euthanized either when tumor volume reached 400% initial tumor volume or on day 32. All animal experiments were approved by UW-Madison’s Institutional Animal Care and Use Committee and conducted in accordance with institutional and NIH guidance.

2.2.6. Statistical analysis—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.

RESULTS AND DISCUSSION

The IC_{50} value of (–)-gossypol was 190 ± 60 nM for A549 cells, an IC_{50} value that is ca. 13 times lower than the value for (±)-gossypol (2400 ± 430 nM) (Tomoda et al. 2015). Moreover, Pluronic P85 acting as a unimeric species at 1700 nM (i.e. below its CMC value of $7.5 \times$

10^{-5} M) decreased the IC_{50} value of (–)-gossypol by 2.3-fold to 82 ± 42 nM (Fig. 1). These anticancer mechanisms of (–)-gossypol and Pluronic P85 work in concert to inhibit the proliferation of A549 cancer cells and were expected to enhance radiation cancer cell killing.

The effects of (–)-gossypol, P85, and the combination of (–)-gossypol and P85 on the clonogenic survival of A549 cells are shown in Figs. 2 and 3. The effect of (–)-gossypol on A549 clonogenic survival was dose-dependent and resulted in a complete loss of clonogenic survival of A549 cells at $2.0 \mu\text{M}$. The effect of (–)-gossypol on clonogenic survival of A549 cells was much stronger than that of (±)-gossypol: 0.99 ± 0.04 at $2 \mu\text{M}$ (Tomoda et al. 2015). P85 did not significantly inhibit A549 clonogenic survival (0.93 ± 0.08), whereas (–)-gossypol at $1.0 \mu\text{M}$ reduced A549 clonogenic survival to 0.7 ± 0.1 . Notably, the combination of (–)-gossypol at $1.0 \mu\text{M}$ and P85 at $21.7 \mu\text{M}$ dramatically reduced A549 clonogenic survival: 0.11 ± 0.02 .

To assess radiosensitizing effects of (–)-gossypol, P85, and the combination of (–)-gossypol at $1.0 \mu\text{M}$ and P85 at $21.7 \mu\text{M}$, A549 cells were subjected to ionizing radiation (0–8 Gy) followed by drug treatment *in vitro*. As shown in Fig. 4, neither (–)-gossypol or P85 alone significantly radiosensitized A549 cells. This result for (–)-gossypol was surprising because previous studies have suggested (–)-gossypol acts as a potent radiosensitizer (Moretti et al. 2010, Xu et al. 2005, Zerp et al. 2009). On the other hand, the combination of (–)-gossypol at $1.0 \mu\text{M}$ and P85 at $21.7 \mu\text{M}$ showed a statistically significant enhancement of radiation cell kill in comparison to the radiation control. The SER value confirmed that the P85-solubilized (–)-gossypol augmented radiation cell kill (1.31), whereas individual treatment of (–)-gossypol alone and P85 alone only slightly augmented radiosensitivity (1.07 and 1.13, respectively).

Radiosensitization by (–)-gossypol, P85, and the combination of (–)-gossypol and P85 was evaluated in an A549 xenograft model. After tumors became palpable, mice were irradiated at 3 Gy for 5 days with or without (–)-gossypol and P85 at 15 and 200 mg/kg, respectively. P85 played a dual role as a solubilizer for (–)-gossypol and as a radiosensitizer. For intravenous injection of (–)-gossypol, P85 at 20 mg/mL assembled into P85 micelles with ca. 10% wgt drug/wgt polymer, and with an average hydrodynamic diameter of 17.8 ± 0.4 nm, resulting in a final level of (–)-gossypol at ca. 1.6 ± 0.04 mg/mL in sterile water. (–)-gossypol and P85 at 15 and 200 mg/kg, respectively, was safely injected into mice, noting body weight change < 10% and an absence of death in mice (data not shown).

Radiosensitization by the combination of (–)-gossypol and P85 is shown in Fig. 5. P85 alone had little effect on tumor growth. Treatment with the combination of P85 and (–) gossypol exerted considerable tumor growth delay as did all combinations that included radiation therapy. The most significant tumor growth delay was seen with radiation in combination with P85 solubilized (–) gossypol consistent with *in vitro* data.

CONCLUSIONS

Pluronic P85 increased the anti-proliferative activity of (–)-gossypol against A549 cells, and P85/(–)-gossypol micelles effectively reduced clonogenic survival of A549 cells: ($11 \pm 5\%$)

compared to (–)-gossypol and P85 ($62 \pm 27\%$ and $93 \pm 13\%$, respectively) and enhanced radiation cell killing of A549 cancer cells. *In vivo*, P85 (200 mg/kg/day)/(–)-gossypol (15 mg/kg/day) micelles were safely injected intravenously and enhanced the antitumor efficacy of radiation therapy in an A549 xenograft model. While P85 probably cannot act as a long circulating carrier for (–)-gossypol, it can readily solubilize (–)-gossypol and potentiate its radiosensitizing effects. Thus, Pluronic P85 as a membrane-active ABA block copolymer acts as a biological response modifier in the context of radiosensitization, expanding the scope of applications beyond inhibition of p-glycoprotein and reversal of the MDR phenotype *in vivo*. In summary, Pluronic P85 and (–)-gossypol micelles act as a novel dual agent radiosensitizer, suggesting a promising tactic for cancer chemoradiotherapy.

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Fig. 1. IC₅₀ value of (±)-gossypol, (±)-gossypol + P85 (1:332), (-)-gossypol, and (-)-gossypol + P85 (1:199) (*: P<0.05, N=3)

Fig. 2.
Effect of (-)-gossypol on A549 clonogenic survival (N=3)

Fig. 3.
Effects of P85, (-)-gossypol, and (-)-gossypol + P85 on A549 cell clonogenic survival (*: $P < 0.0005$, **: $P < 0.005$, $N=3$)

Fig. 4. Effects of P85, (-)-gossypol, and (-)-gossypol + P85 on A549 cell radiosensitivity (black circle: (-)-gossypol at 1 μ M in DMSO, black square: P85, black diamond: (-)-gossypol + P85, open circle: radiation only) (*: $P < 0.005$, $N = 3$)

Fig. 5.

Effect of (-)-gossypol (15 mg/kg) and P85 (200 mg/kg) micelles and radiation on tumor growth in an A549 xenograft mouse model (black circle: (-)-gossypol + P85 + radiation, white circle: (-)-gossypol + P85, black diamond: P85 + radiation, white diamond: P85, black square: saline + radiation, white square: saline, *: $P < 0.05$, $N=4$).