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ORIGINAL ARTICLE

Radiosensitization of hormone-refractory prostate cancer cells by gossypol treatment

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Summary

Purpose: *Many drugs have been tested to increase the sensitivity of prostate cancer cells to radiotherapy. Gossypol, a natural polyphenolic compound extracted from the cotton plant, is one of the agents the efficacy of which has been investigated in the treatment of prostate cancer for this purpose. The main aim of this study was to investigate the best gossypol application with irradiation, when gossypol was applied either sequentially (24 h before and after irradiation) or concurrently in PC-3 hormone-refractory and radioresistant prostate cancer cells.*

Methods: *The XTT viability assay was used to evaluate the cytotoxicity of different concentrations of gossypol in PC-3 cells. Irradiation was applied to PC-3 cells via 6 MV photon linear accelerator and delivered 24 h before, 24 h after radiation or at the same time with gossypol administration.*

Results: *Gossypol caused radiosensitization of PC-3 cells that are known to be radioresistant, with high Bcl-2 levels. Among different applications of gossypol and irradiation (before, after and concurrent) in prostate cancer cells, the best results were observed by the application of gossypol 24 h before irradiation.*

Conclusion: *Our study suggests that gossypol represents a promising novel anticancer treatment for radiosensitization of human hormone-refractory prostate cancer cells.*

Key words: Bcl-2, gossypol, PC-3 prostate cancer cells, radiosensitization, radiotherapy

Introduction

Prostate cancer is one of the most common malignancies affecting mainly elderly men in the developed world [1]. In advanced disease stages, androgen deprivation therapy remains the standard of care for these patients. However, the disease progresses to a hormone-refractory state within approximately 2 years [2].

Although there is no randomized trial for the treatment of early-stage prostate cancer, it is understood that there is no difference between radical prostatectomy and definitive radiation therapy both in biochemical recurrence and in disease-free survival [3]. However, results from several trials have shown that local tumor control is directly associated with the radiation dose which requires above 70 Gy in order to ensure local control, due to the cellular characteristics of prostate cancer treated with radiotherapy [4]. Increased cure rates may be achieved either by using advanced radiotherapy techniques or increasing doses of radiation or using some agents to overcome radioresistance of tumor cells in recent years. Therefore, many agents were tested for radiosensitization of prostate cancer cells in order to enhance both the efficacy of radiotherapy and lower its doses [5].

Overexpression of Bcl-2/ Bcl-X_L antiapoptotic proteins is observed in 80-100% of hormone-refractory prostate cancer [6,7]. Overexpression of these proteins is associated with decreased apoptosis and results in resistance to chemo- and radiotherapy [8]. Thus, new treatment approaches are needed in this area.

Gossypol is a natural polyphenolic compound extracted from the cotton plant (*Gossypium species*) and tropical tree (*Thespesia populnea*) [9]. Recently, gossypol was reported to have potent anticancer activities in many types of malignancies, including prostate cancer [10]. Gossypol was shown to be a potent inhibitor of Bcl-2/ Bcl-

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X_L, however the exact mechanisms responsible for inhibition of cell growth and stimulation of apoptosis have not been elucidated [11,12]. In the literature, there are studies demonstrating that gossypol increases the sensitivity of prostate cancer cells to conventional cytotoxic treatment without causing any significant toxicity [13,14].

Prostate cancer is a genetically and phenotypically heterogeneous disease. This may be a consequence of mutations of different cell types resulting in different malignant evolution pathways. PC-3 cell line is derived from bone metastasis of prostate cancer cells which are androgen receptor-negative and highly aggressive. From the literature it is well known that PC-3 cells exert higher expression levels of the anti-apoptotic protein Bcl-2, leading to greater resistance to cytotoxic agents and radiation [15,16]. Thus, in this study, the antitumor activity of racemic gossypol - which is the main form of the agent found in nature - with irradiation by 3 different treatment approaches in human hormone-refractory and radioresistant prostate cancer cells, PC-3, was investigated.

Methods

Cell lines and reagents

Human hormone-refractory and radioresistant PC-3 prostate cancer cell line was obtained from ICLC (Genova, Italy). Cells were grown as monolayers and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK), and maintained at 37° C in a humidified atmosphere with 5% CO_2. Growth and morphology were monitored and cells were passaged when they had reached 90% confluence. Cell culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Gossypol (>98% purity) was purchased from Sigma Chemical Co, USA. A stock solution of gossypol (10^{-2}M) was prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co, USA). The DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to the tumor cells. The final dilutions were made immediately before use, and new stock solutions were made for each experiment. All other chemicals, unless mentioned, were purchased from Sigma. All the experimental results were compared with the results of untreated controls taken as reference.

Viability assay

In order to investigate the cytotoxic effect of gossypol in prostate cancer cells, PC-3 cells were exposed to different increasing concentrations of gossypol at 24, 48, 72 h and XTT cell viability assay was performed (Roche Applied Science, Mannheim, Germany).

Briefly, cells (3×10^4cell/well) were seeded into 96-well flat-bottomed microtiter plates containing 200 µL of growth medium in the presence or absence of increasing concentrations of gossypol and increasing doses of radiation treatment at 37° C in 5% CO_2 after verifying cell viability by trypan blue dye exclusion test using Cellometer automatic cell counter (Nexcelom Inc., USA). After an incubation period of indicated time intervals, 100 µL of XTT were added to each well and plates were incubated at 37° C for another 4 h. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, USA). The mean of triplicate experiments for each dose was used to calculate the IC_{50} value.

Irradiation

Irradiation was delivered to PC-3 cells at Ege University, School of Medicine Radiation Oncology Department. The irradiation-alone group received 4-16 Gy and the gossypol plus irradiation group received 8 Gy via 6 MV photon linear accelerator. One and 5 µM concentrations of gossypol and 8 Gy irradiation dose were selected for this set of experiments. These were doses below IC_{50} levels for both gossypol and radiotherapy. Irradiation was delivered to cancer cells from 1 cm distance "bolus" from the posterior field of the wells.

Statistical analysis

Data were analyzed by using GraphPad PRISM 5.0 software (San Diego, CA, USA). Data were analyzed using Student's *t*-test to compare the results in two groups. A p-value<0.05 was considered significant.

Results

PC-3 cells cytotoxicity after gossypol exposure

A time- and dose-dependent cytotoxicity was observed in PC-3 cells by gossypol exposure.

There were parallel decreases in the percentage of cell viability in a time- and dose-dependent manner. The time to highest cytotoxicity was at 72 h.

As shown in Figure 1 there was a 38, 53, and 65% decrease in PC-3 cells viability exposed to 5, 10, and 20 µM of gossypol, respectively, as compared to the untreated control. The IC_{50} value of gossypol at 72 h was calculated from the dose-response curve and was 9.3 µM.

PC-3 cells cytotoxicity after irradiation

Cell death in PC-3 cells rose with increasing doses of irradiation.

As shown in Figure 2, at 72 h there was a 37,40,52, and 55% decrease in the proliferation of PC-3 cells when exposed to 4,8,12, and 16 Gy, respectively, as compared to the untreated controls. The IC_{50} value of PC-3 cells at 72 h was 10.5 Gy.

Optimal time to gossypol administration in relation to irradiation

Gossypol sensitized PC-3 cells to irradiation at most when administered 24 h before irradiation. When 1 μM gossypol was applied 24 h before irradiation with 8 Gy, no significant cytotoxicity was observed at 24 h, but 71% cytotoxicity was observed at 48 h and 76% at 72 h. Similarly, with 5 μM gossypol given 24 h before irradiation with 8 Gy, no significant cytotoxicity was seen at 24 h, but 73% cytotoxicity was observed at 48 h and 82% at 72 h (Figure 3). In contradistinction to monotreatment with either gossypol or irradiation, the percentage of cytotoxicity was found close to each other at 48 and 72 h by sequential treatment. Thus, as Figure 3 clearly shows, adding gossypol to PC-3 cells 24 h before irradiation significantly enhanced irradiation-induced cytotoxicity as compared to concurrent administration or 24 h after irradiation ($p<0.01$). Although, treating cells with irradiation and gossypol at the same time or with gossypol 24 h after irradiation showed enhancement of cytotoxicity as compared to controls this was not statistically significant (Figures 4,5).

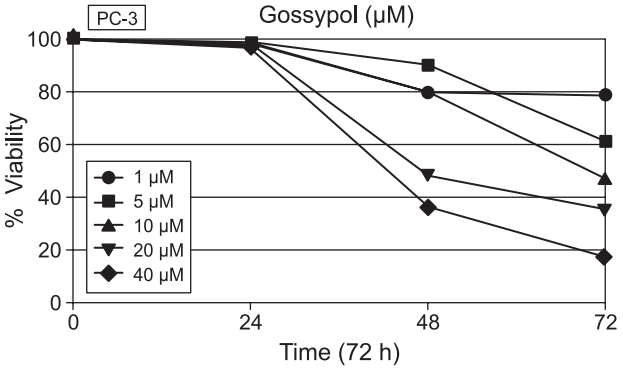

Figure 1. Cytotoxic effect of gossypol in PC-3 hormone-refractory prostate cancer cells. The effect was in a time-and dose-dependent manner. IC_{50} value of gossypol at 72 h was 9.3 μM.

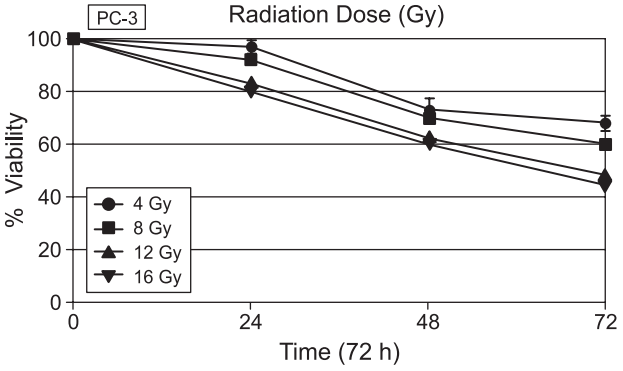

Figure 2. Cytotoxic effect of radiotherapy in PC-3 hormone-refractory prostate cancer cells. The effect rose as the dose increased.

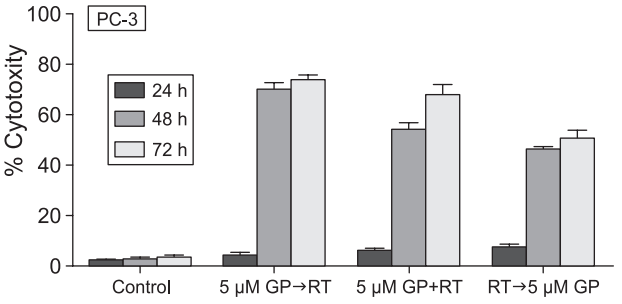

Figure 3. Cytotoxic effect of gossypol (GP) plus irradiation when GP (5 μM) was applied either sequentially (24 h before or after irradiation) or concurrently. GP sensitized PC-3 cells to irradiation most when applied 24 h before irradiation ($p < 0.01$).

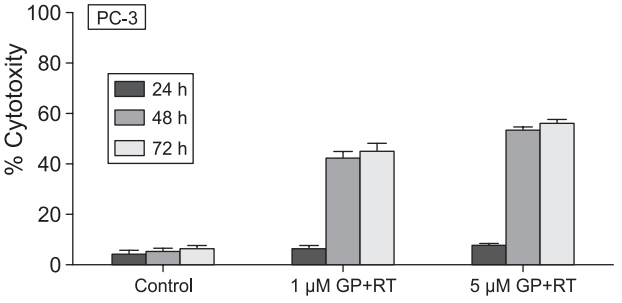

Figure 4. Treating cells with irradiation and gossypol (GP) 1 and 5 μM) at the same time showed potentiation of cytotoxicity as compared to control but without statistical significance ($p>0.05$).

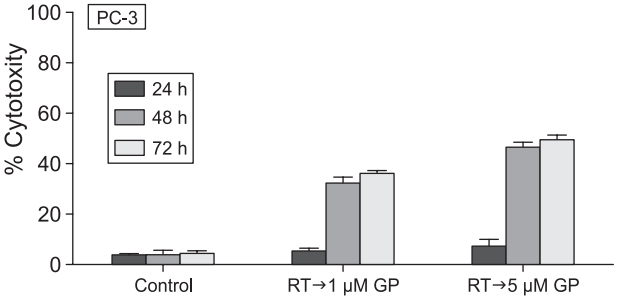

Figure 5. Treating cells with irradiation 24 h before gossypol (GP) exposure (1 and 5 μM) showed potentiation of cytotoxicity as compared to control but without statistical significance ($p>0.05$).

Discussion

Radiation therapy is used to treat all stages of localized prostate cancer [16]. However, both clinical and radiobiological evidence indicate that prostate cancer cells can be relatively resistant to radiation [17-19]. Radioresistance markedly impairs the efficacy of radiotherapy and involves antiapoptotic signal transduction pathways that prevent radiation-induced cell death [15,20]. PC-3 is a hormone-refractory human prostate cancer cell line, which is resistant to current chemotherapy and radiation therapy. It has significantly high levels of Bcl-2 and Bcl-X_L that might contribute to PC-3 cells' resistance to the cytotoxic effect of chemotherapy and radiotherapy [15].

Gossypol has been shown to have potent anticancer activities in many types of malignancies. Multiple intracellular pathways and molecular targets have been proposed for its antitumor activity, such as inhibiting cellular energy metabolism, direct toxicity to mitochondria, modulation of cell cycle regulatory protein Rb and cyclin D1, including inhibition of protein kinase C activity [21], and antiangiogenesis [22,23]. Gossypol has been reported as a potent small molecule inhibitor of both Bcl-2 and Bcl-X_L inducing strong apoptosis in several cancer cell lines with high levels of Bcl-2/Bcl-X_L [11,23]. Various preclinical and clinical studies have shown that gossypol is well tolerated with acceptable clinical safety [21].

Gossypol has also been used for enhancing the antitumor activity of conventional cytotoxic agents in many type of human cancers. This topic is widely studied for overcoming drug resistance. In a study by Bauer et al. cisplatin-resistant cells seemed to depend on wild-type p53 and Bcl-X_L for survival, and BH3 mimetic agents, such as gossypol, might be useful adjuncts to overcome cisplatin resistance in head and neck cancer cells. In that study, gossypol was an efficient inducer of apoptosis, but it is worth mentioning that the degree of apoptosis was considerably higher in tumor cell with wild-type p53 and high levels of Bcl-X_L [13]. These results have also been supported by some other studies on different types of cancer cells [24,25]. These recent data strongly support the evidence that Bcl-2/Bcl-X_L may very likely be the major molecular targets for many types of human cancers, including prostate cancer.

In addition to these findings, several recent studies showed that Bcl-2 overexpression increases the angiogenic potential of cancer cells by increasing angiogenic factors such as vascular endothelial growth factor. Xu et al. showed that antiangiogenesis might be one of the routes involved in the *in vivo* antitumor activity of gossypol in combination with radiation in hormone-refractory prostate cancer [23]. Moreover, Karaca et al. have recently showed that gossypol treatment significantly decreased the secretion of some pivotal angiogenic molecules in PC-3 cells [22].

In our study, we have demonstrated that treatment of PC-3 cells with gossypol significantly reduced the radioresistance of PC-3 cells. Among 3 different treatment approaches, the most positive results were observed by administering gossypol 24 h before irradiation. Although it is clear that exposure to gossypol before irradiation significantly sensitizes cancer cells to undergo apoptosis, it remains to be elucidated whether gossypol- mediated radiosensitization is through inhibition of Bcl-2/Bcl-X_L or other molecular targets in the apoptotic pathway. Furthermore, another point needing explanation is the difference between the therapeutic effect of gossypol and irradiation and why it is best to give gossypol before irradiation. This may somehow be related to the inhibition of antiapoptotic molecules, which cause radioresistance, by gossypol.

In conclusion, our study suggests that gossypol represents a promising novel anticancer agent for molecular targeted radiosensitization of human hormone-refractory prostate cancer. We are currently planning detailed studies to elucidate the pathways involved in radiosensitization of prostate cancer cells by gossypol. Further *in vitro* and *in vivo* studies of gossypol in combination with radiation therapy may prove this approach as a treatment option for hormone-refractory prostate cancer, especially with high levels of Bcl-2/Bcl-X_L.

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