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ORIGINAL ARTICLE

7-Geranyloxcycoumarin enhances radio sensitivity in human prostate cancer cells

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Abstract

Background Prostate cancer is the second most prevalent and the fifth deadliest cancer among men worldwide. To improve radiotherapy outcome, we investigated the effects of 7-geranyloxycoumarin, also known as auraptene (AUR), on radiation response of prostate cancer cells.

Methods and results PC3 cells were pretreated with 20 and 40 μM AUR for 24, 48 and 72 h, followed by X-ray exposure (2, 4 and 6 Gy). After 72 h recovery, cell viability was determined by alamar Blue assay. Flow cytometric analysis was performed to assess apoptosis induction, clonogenic assay was carried out to investigate clonogenic survival, and the expression of *P53, BAX, BCL2, CCND1* and *GATA6* was analyzed by quantitative polymerase chain reaction (qPCR). Cell viability assay indicated that toxic effects of radiation was enhanced by AUR, which was also confirmed by increased numbers of apoptotic cells and reduced amount of survival fraction. The qPCR results demonstrated significant induction of *P53* and *BAX*, while the expression of *BCL2, GATA6,* and *CCND1* was significantly downregulated.

Conclusion The findings of the present study indicated, for the first time, that AUR improved radio sensitivity in prostate cancer cells, and thus, has the potential to be used in future clinical trials.

Keywords Auraptene · Radiation · Prostate cancer · Radiosensitizer

Introduction

Prostate cancer is considered as the second most prevalent neoplasm and the fifth leading cause of cancer death among men worldwide, with a total of 1,414,259 registered cases and 375,304 related deaths in 2020 [1]. The use of ionizing radiation (IR) is an effective strategy for the treatment of many types of cancer [2, 3]. Nevertheless, utilization of IR as a single therapeutic modality is accompanied by a range of side effects such as fatigue, distress, anxiety, and depression [4]. Another obstacle in cancer radiotherapy is the limited capacity of adjacent healthy tissues to tolerate applied IR. Therefore, designing new approaches to maximize anticancer efficiency at the lowest doses of IR along with the least amounts of injuries to non-target nearby tissues is desired [5]. Radiosensitizers are chemicals or pharmaceutical agents that make cancer cells more vulnerable to IR-induced damages. So far, many such compounds have shown promising results in pre-clinical evaluation, however, their safety, efficacy and practicability need to be demonstrated [6]. Based on their structures, radiosensitizers can be

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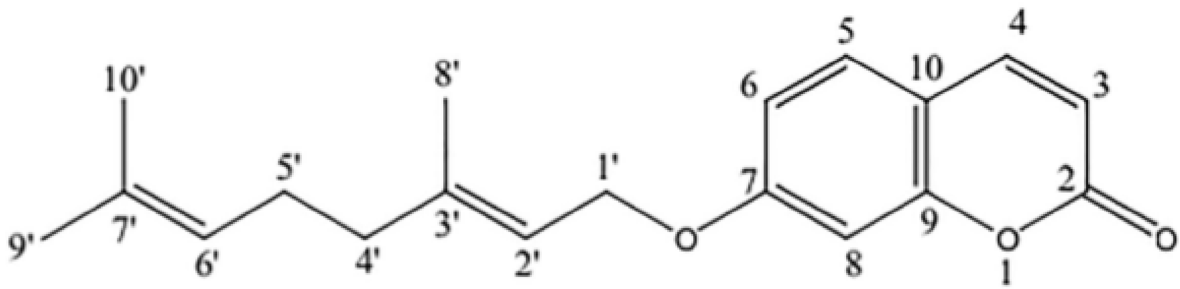

Fig. 1 Chemical structure of 7-geranyloxcycoumarin (AUR).

classified into three categories as follows; small molecules such as free radicals and pseudo substrates, macromolecules like miRNAs and proteins, and nanomaterials such as nano formulated drugs [7]. Various mechanisms are involved in the radio sensitization of tumor cells, including suppression of endogenous radioprotective substances, formation of cytotoxic agents, inhibition of DNA repair biomolecules, and alteration of signaling pathways [8].

Natural products with preventive and anticancer properties are potential candidates for reducing radiotherapy-induced side effects [9]. Coumarins are a group of natural compounds that induce antitumor effects at low concentrations, therefore, could act as potential agents in adjuvant therapies [10]. 7-Geranyloxycoumarin (Fig. 1), also known as auraptene (AUR), exerts anticancer effects by interfering with various signaling pathways related to cytokines, growth factors, and/or apoptosis [11]. In addition, AUR has the potential to improve radio sensitivity of gastric and colon carcinoma cells [12, 13].

Considering the anticancer properties of AUR, in single use or in combination with therapeutic modalities, we hypothesized that AUR could act as a radiosensitizer and enhance the efficacy of IR in human prostate cancer cells. Hence, the effects of AUR, alone and in combination with IR, was investigated on the viability of cells by alamar Blue assay at first. Then, flow cytometry analysis with annexin V and propidium iodide (PI) was performed to assess apoptosis induction, and clonogenic assay was carried out to investigate clonogenic survival. Finally, the expression of genes involved in IR-induced apoptosis was analyzed by quantitative polymerase chain reaction (qPCR).

Materials and methods

Synthesis of AUR

Synthesis of AUR was done through a reaction between 7-hydroxycoumarin (1 M) and trans geranyl bromide (1.5 M) in the presence of 1,8-diazabicyclo (5.4.0) undec-7-ene (2 M), as previously described [14]. In order to purify AUR, column chromatography (petroleum ether/ethyl acetate 9:1 v/v) was performed (mp = 62.7–63.4 °C). ^{1}H and ^{13}C NMR were used to confirm the AUR structure (Table 1). Dimethyl sulfoxide (DMSO) (Merck, Germany) was used as solvent to prepare different stock solutions of AUR, and final concentrations were prepared with complete culture medium just before treatment. To note, untreated cells and cells

Table 1 ^{1}H and ^{13}C NMR spectra of AUR [25]

^{1}H-NMR data ($CDCl_3$, 500 MHz)[a]		^{13}C-NMR data ($CDCl_3$, 125.7 MHz)	
H	AUR	C	AUR
2	-	2	161.2
3	6.24 d (9.6)	3	112.9
4	7.63 d (9.6)	4	143.4
5	7.36 d (7.2)	5	128.6
6	6.85 dd (7.2, 2.0)	6	113.2
7	-	7	162.1
8	6.82 d (2.0)	8	101.5
9	-	9	155.8
10	-	10	112.3
1'	4.59 d (7.0)	1'	65.4
2'	5.47 t (7.0)	2'	118.3
3'	-	3'	142.3
4'	2.13 m	4'	39.4
5'	2.15 m	5'	26.4
6'	5.08 q (7.0)	6'	123.5
7'	-	7'	131.9
8'	1.75 m	8'	16.7
9'	1.65 m	9'	25.6
10'	1.59 q (7.0)	10'	17.6
11'	-	11'	-
12'	-	12'	-
13'	-	13'	-
14'	-	14'	-
15'	-	15'	-

[a] J values are in parenthesis and reported in Hz; chemical shifts are given in ppm

treated with 0.4% v/v DMSO were considered as controls in all experiments.

Cell culture

Human prostate cancer cell line (PC3) was purchased from Pasteur Institute of Iran (Tehran, Iran). Cells were maintained in RPMI 1640 medium (Biowest, France) supplemented with 10% fetal bovine serum (Biowest, France) and 1% penicillin-streptomycin (Biowest, France). Cells were incubated at 37°C in a humidified environment of 95% air and 5% CO_2.

Cell viability assay

To determine viability of cells, alamar Blue assay was performed according to the following procedure; First, cells were cultured in 96-well plates and incubated overnight. Then, for single administration of AUR, cells were treated with 5, 10, 20, 40, or 80 µM AUR and incubated for 48, 72, 96, 120, and 144 h as distinguished groups. For AUR + IR combinatorial treatment, cells were pretreated with 20 and 40 µM AUR for 24, 48, and 72 h, followed by exposure to 2, 4, and 6 Gy of X-ray (Elekta Compact™ linear accelerator, Crawley, United Kingdom) and 72 h recovery time. For viability assessment, alamar Blue reagent (0.01 mg/ml, Sigma-Aldrich, Germany) was added to each well (18µL/well) followed by 3 h of incubation. Then, the optical density (OD) was measured by a microplate reader (Biotek, USA) at 600 nm, and cell viability (%) was calculated by the following equation:

$$Cell\ viability\,(\%) = 100 - [(OD_{treat} - OD_{control})/(OD_{blank} - OD_{control}) \times 100]$$

Detection of apoptosis

After single and combinatorial administrations of AUR, induction of apoptosis was investigated. Briefly, untreated cells and cells treated with 40 µM AUR, alone and in combination with 4 Gy IR, as well as their relevant DMSO controls, were collected and washed with chilled phosphate buffered saline. Then, a staining protocol with fluorescein isothiocyanate - Annexin V and PI (MabTag, Germany) was applied according to the manufacturer's instruction. Finally, cells were assessed by a flow cytometer (BD FACS-Calibur) using FL1 and FL2 filters, and data were analyzed with FlowJo software version 7.6.

Clonogenic assay

To assess clonogenic survival upon combinatorial treatment, cells were cultured in 96-well plates and treated with 40 µM AUR, while 0.4% DMSO and untreated cells were considered as controls. After 72 h, cells were exposed to 4 Gy IR followed by 24 h recovery. Then, cells were harvested, counted and seeded onto 12-well plates and incubated for 8–10 days for the colony development. Afterwards, colonies were fixed with methanol (Merck, Germany) and stained by Giemsa (Labtron, Iran) followed by rinsing with distilled water. Stained colonies were manually counted under an inverted microscope (Labomed). A colony was defined to consist of at least 50 cells. The experiment was carried out in triplicate and three independent experiments.

Plating efficiency (PE) and survival fraction (SF) were calculated using the following formulas:

$$PE\,(\%) = number\ of\ colonies\ formed\,/\,number\ of\ cells\ seeded \times 100$$

$$SF = number\ of\ colonies\ formed\,/\,[number\ of\ cells\ seeded \times PE]$$

qPCR for gene expression analysis

Upon treatment of cells with AUR alone and in combination with IR, the expression of candidate genes was evaluated by qPCR. In summary, total RNA was collected from all experimental groups using an RNA extraction kit (Denazist Asia, Iran) based on the manufacturer's protocol. Then, cDNA synthesis was carried out (Parstous, Iran), and the fidelity of amplified cDNAs was validated by PCR using *TBP* primers (Table 2). The Rotor-Gene 6000 detection system (Qiagen, Germany) was used to perform qPCR for *P53, BAX, BCL2, CCND1* and *GATA6* by SYBR green mix and primers listed in Table 2. PCR cycling conditions were as 94 °C for 5 min, [95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s] 38 cycles.

Table 2 Primer sequences and product length used in the current study

Gene name	5'→3' Forward primer	5'→3' Reverse primer	Product size (bp)
TBP	ACAACAGCCTGCCACCTTA	GAATAGGCTGTGGGGTCAGT	120
P53	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG	123
BCL2	GATGACTGAGTACCTGAACCG	CAGAGACAGCCAGGAGAAATC	124
BAX	GGACGAACTGGACAGTAACATGG	GCAAAGTAGAAAAGGGCGA-CAAC	150
CCND1	TGAAGGAGACCATCCCCCTG	TGTTCAATGAAATCGTGCGG	151
GATA6	GCCAACTGTCACACCACAAC	AGTTGGAGTCATGGGAATGG	125

Statistical analysis

Statistical analysis was performed by GraphPad Prism 7.01 (San Diego, CA, USA), and one-way ANOVA and Tukey tests were used for multiple comparison. The data are presented as the mean ± standard deviation (mean ± SD), and significant differences were defined as p values < 0.05, 0.01, 0.001 and 0.0001.

Results

AUR enhanced radiation response of PC3 cells

To investigate the effects of AUR and AUR + IR, PC3 cells were treated and viability was evaluated by alamar Blue assay. As presented in Fig. 2-a, AUR reduced cell viability in a time- and concentration-dependent manner, and significant ($p < 0.01$) decrease in viability of cells was only observed upon treatment with 80 µM AUR. Accordingly, 40 µM AUR, a concentration at which cell viability was around 80% in all 5 time points, was chosen for combinatorial treatment.

To evaluate the effects of AUR on radiation response of cells, 24, 48 and 72 h pretreatment with 40 µM AUR was followed by IR exposure (2, 4 and 6 Gy), and viability was assessed upon 72 h recovery. Results revealed a substantial increase in the toxicity of applied IR in almost all groups, as compared to the control samples treated with 0.4% DMSO + 2, 4, and 6 Gy (Fig. 2-b). In particular, viability was significantly ($p < 0.0001$) decreased when cells were

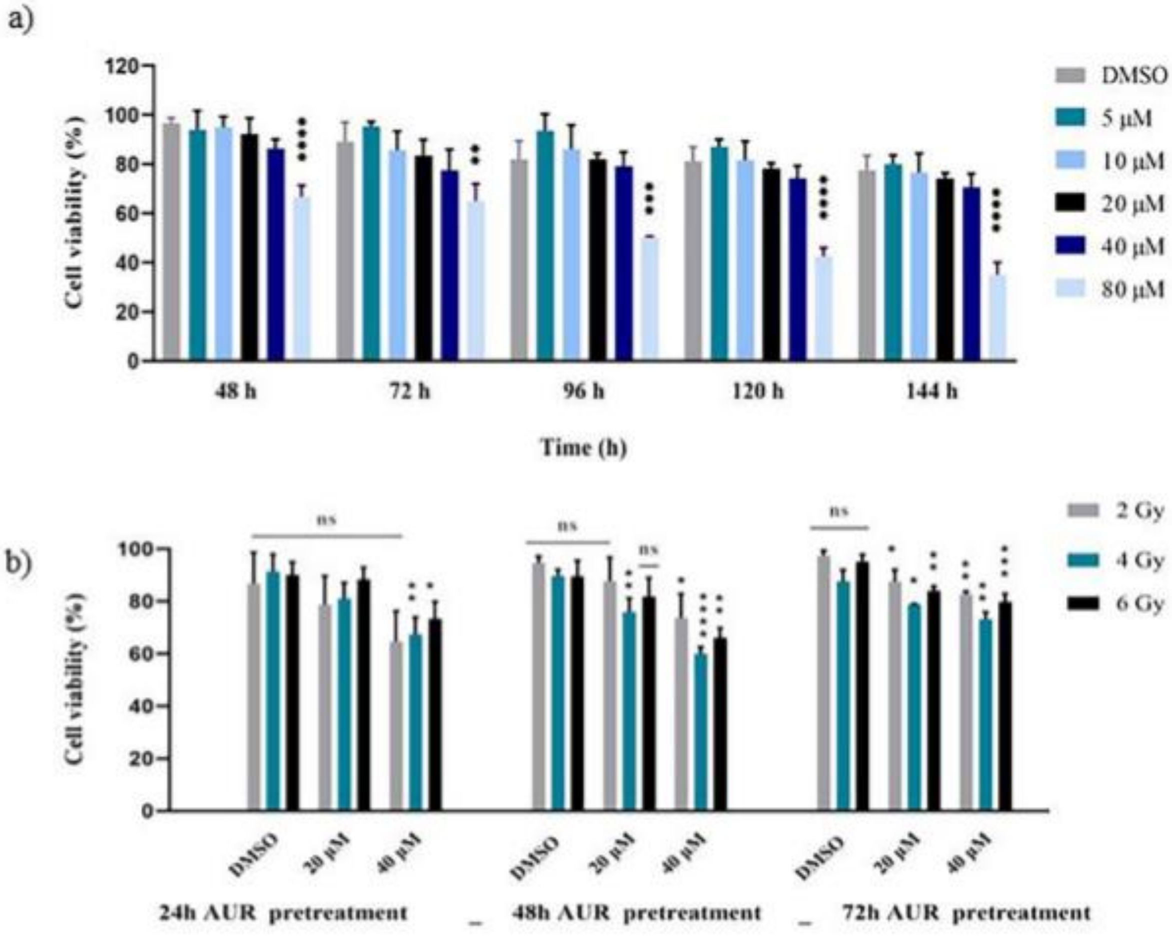

Fig. 2 (a) Cytotoxicity of 0.4% DMSO and different concentrations of AUR on PC3 cells after 48, 72, 96, 120, and 144 h treatment. (b) Viability of PC3 cells upon pretreatment with 0.4% DMSO and 20 and 40 µM AUR, followed by X-ray exposure at 2, 4, and 6 Gy. All experiments were carried out in triplicate for at least 3 times, and results are presented as a mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

pretreated with 40 µM AUR for 48 h followed by 4 Gy radiation exposure.

Alterations in the density and morphology of PC3 cells after single and combinatorial treatments confirmed results of viability assay. As shown in Fig. 3, number of attached and alive cells was reduced after single administration of AUR and IR. However, when cells were treated with AUR + IR, not only density of cells decreased considerably, but also cells were aggregated and granulated.

Combination of AUR and IR increased PC3 cell apoptosis

To investigate cell apoptosis induced by AUR + IR, flow cytometry was carried out after Annexin V and PI staining. Figure 4-a-g demonstrates alive, apoptotic and necrotic cells upon single and combinatorial treatments. As shown, after treatment with 40 µM AUR, 73.8%, 7.9%, and 15.9% of cells were alive, early apoptotic, and late apoptotic, respectively. In the case of AUR + IR treatment, 63.6%, 4.8% and 24.5% of cells were detected as alive, early apoptotic and late apoptotic, respectively, which was different form the percentage of cells in the relevant DMSO + IR treatment.

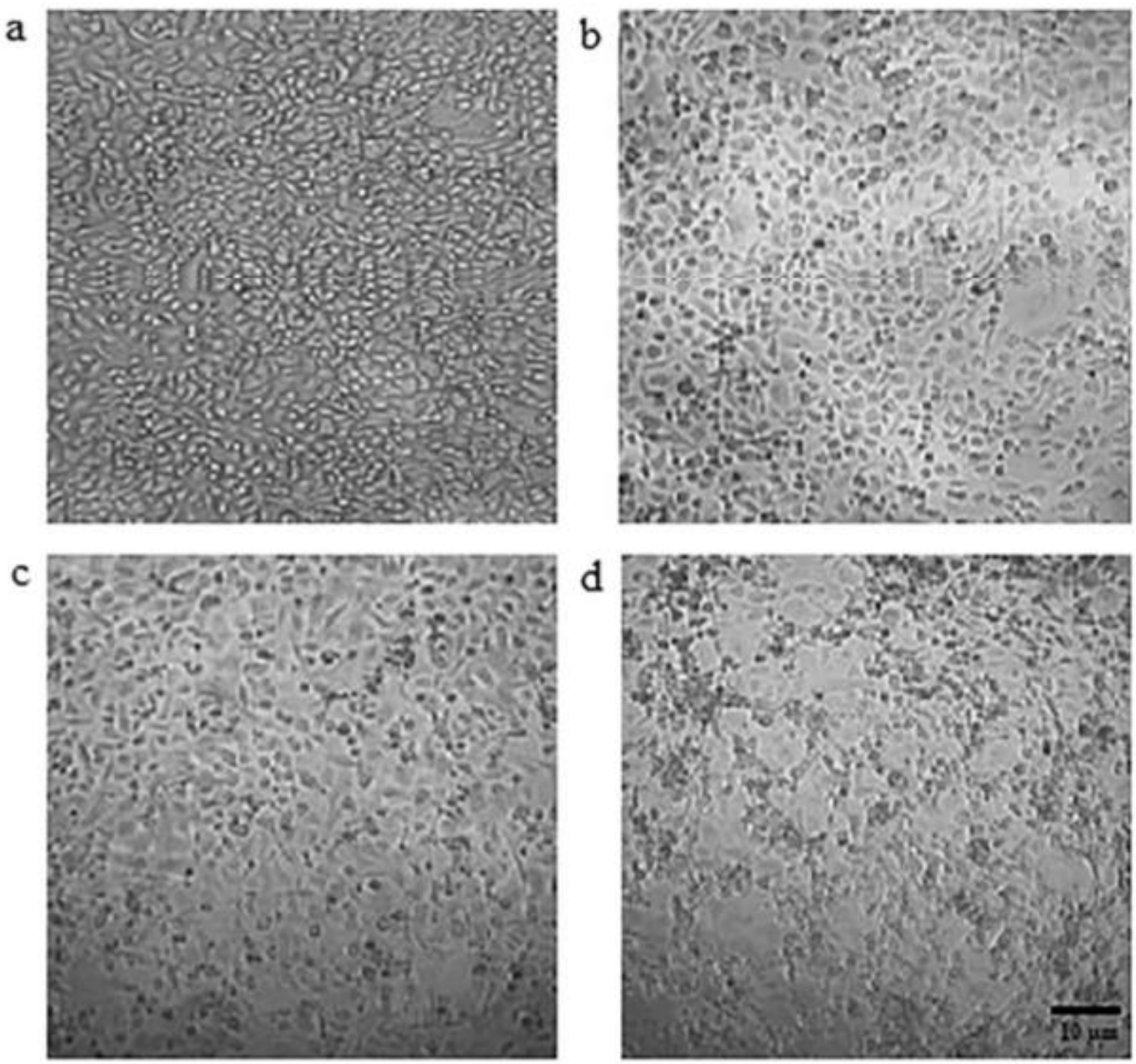

Fig. 3 Phase contrast photomicrographs of (a) untreated PC3 cells and cells treated with (b) 40 µM AUR, (c) 4 Gy IR, and (d) 40 µM AUR + 4 Gy IR.

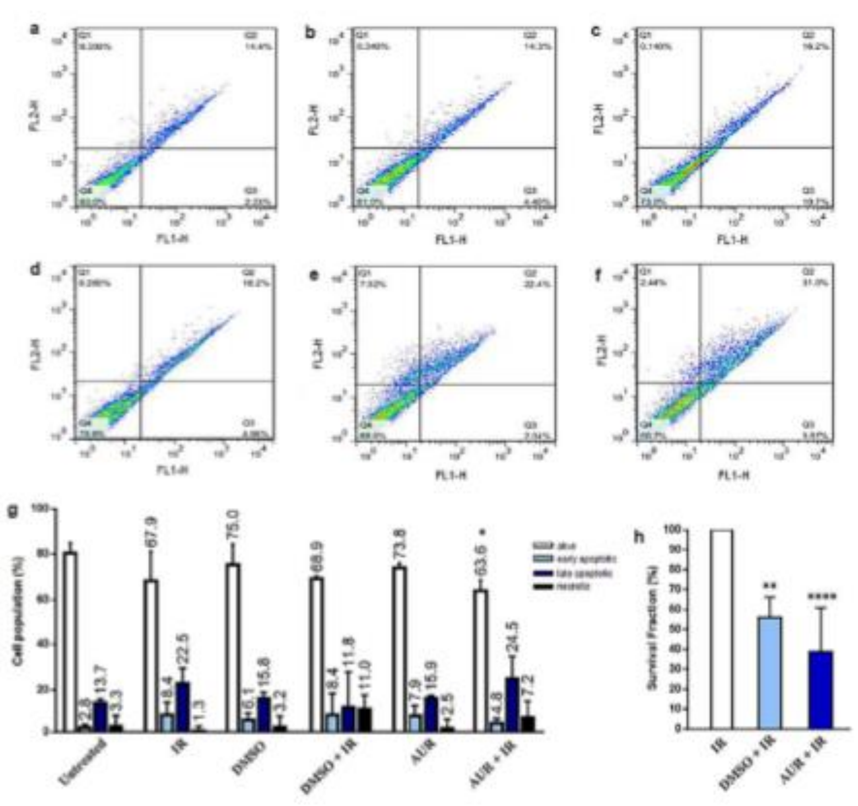

Fig. 4 Representative Annexin V and PI dot plots for (**a**) untreated cells and cells treated with (**b**) 0.4% DMSO, (**c**) 40 µM AUR, (**d**) 4 Gy IR, (**e**) DMSO + IR and (**f**) AUR + IR. (**g**) Amounts of alive, early apoptotic, late apoptotic, and necrotic cells in different treatment groups after flow cytometry analysis. (**h**) Clonogenic survival of PC3 cells upon coadministration of AUR and IR. Apoptosis detection was performed two times and clonogenic assay was carried out in triplicate and three independent experiments. Results are presented as mean ± SD. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$.

Coadministration of AUR and IR reduced clonogenic survival

To further study radio sensitizing effects of AUR, untreated cells and cells treated with 0.4% DMSO and 40 µM AUR were exposed to 4 Gy IR and assessed for colony formation during 8–10 days. As presented in Fig. 4-h, pretreatment with AUR significantly ($p < 0.0001$) reduced survival fraction of irradiated PC3 cells in comparison with untread and DMSO controls.

Combinatorial treatment altered the expression of genes involved in IR-induced apoptosis

To unravel the mechanisms involved in AUR + IR effects, the expression of *P53, BAX, BCL2, CCND1* and *GATA6* was investigated (Fig. 5). Results revealed that single use of AUR and IR increased *P53* expression, however, up regulation of this gene was more significant ($p < 0.0001$) after combinatorial treatment. Likewise, when compared with other treatments, overexpression of *BAX* was more considerable ($p < 0.0001$) upon AUR + IR treatment. Regarding anti-apoptotic gene *BCL2*, obtained findings revealed significant ($p < 0.0001$) down regulation upon combinatorial use of AUR and IR. In addition, AUR alone and in combination with IR reduced *CCND1* expression to lower levels ($p < 0.0001$), when compared with other treatments. Last but not least, significant ($p < 0.0001$) induction in *GATA6* expression was detected upon IR exposure, although such change was not observed after AUR + IR treatment.

Discussion

Prostate cancer is the second most prevalent cancer in men worldwide, accounting for 13.5% of all diagnosed cancer cases in males [15]. Despite many advancements in the diagnosis and treatment of prostate cancer, this neoplasm is considered as the fifth cause of cancer-related death among men [1, 16]. Although radiotherapy is a major modality for cancer treatment, there are still intractable challenges to enhance IR effects on cancer cells and simultaneously minimize side effects to nearby healthy tissues. To overcome this obstacle, one strategy is the enhancement of radiation response in cancer cells. In this regard, radiosensitizers have attracted a lot of attention, as they make cancer cells more susceptible to IR-induced damages via inhibition of

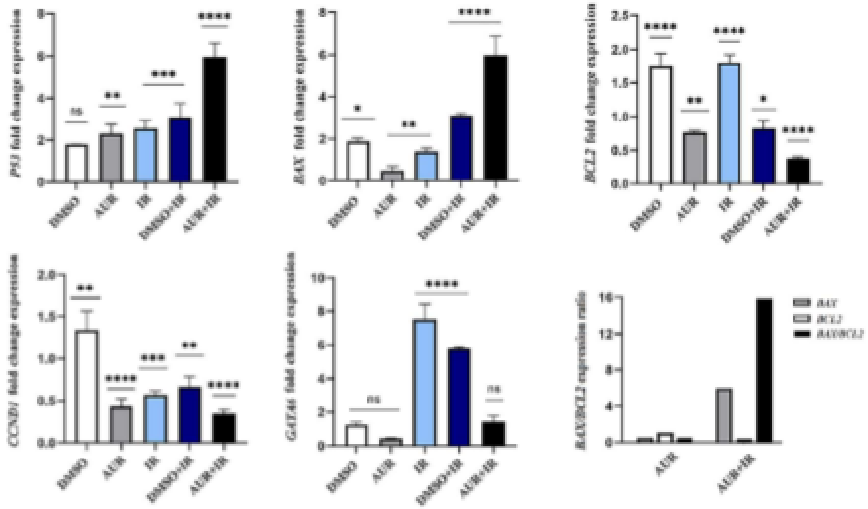

Fig. 5 (**a**) Expression levels of *P53*, *BAX*, *BCL2*, *CCND1* and *GATA6* in DMSO, AUR, IR, DMSO + IR, and AUR + IR treatment groups. (**b**) Alteration of *BAX/BCL2* expression ratio after AUR and AUR + IR treatment. Results are presented as mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

signaling pathways and interfere with biomolecules to name a few [8].

AUR is a natural coumarin with valuable pharmaceutical properties. For instance, anticancer activity of AUR has been demonstrated in glioblastoma, colon, and breast carcinoma cells [17–19]. Since this agent induces anticancer effects at low concentrations, it could be utilized as an adjuvant for radiotherapy [9–11]. Recent studies have reported synergistic activity of AUR with other therapeutic modalities, such as chemotherapy, thermal therapy and radiotherapy [12, 13, 20]. Studying the effects of AUR on human colon adenocarcinoma cells revealed improvement in radiation response, which was mediated by alterations in *P21* and *GATA6* expression [20]. Furthermore, radio sensitizing effects of AUR have been reported in vivo, and down regulation of *CCND1* and *CD44*, along with involvement of PI3K-AKT-mTORC signaling pathway and caspase-3 activity were confirmed in AUR + IR treatment [12]. Findings of the present study are in consistent with previous reports, and indicate that AUR is a potent agent that enhances the cytotoxic effects of radiation on prostate cancer cells.

Exposure of cells to IR induces various alterations in cell growth, intercellular communication and transcriptome that are all associated with cellular morphological phenotypes [21]. For instance, increased volume, decreased projected area and changes in cellular roundness have been reported in endothelial cells, osteoblasts and bladder carcinoma cells upon X-ray irradiation [22, 23]. In consistency, we observed morphological changes, in the forms of granulation and aggregation, upon irradiation of prostate cancer cells. In addition, it has been reported that pretreatment of colon cancer cells with AUR potentiated granulation, detachment, and apoptotic morphology of irradiated cells [20], which is also similar to our observations.

The most frequently used method to quantitatively evaluate radiation response of cells is clonogenic survival assay, which is based on the capacity of single cells to grow to colonies [24]. Regarding PC3 cells, it has been demonstrated that inhibition of NF-κB and treatment with 5-thiocyanato-2′-deoxyuridine enhanced sensitivity and reduced clonogenic survival upon IR exposure [25, 26]. Likewise, findings of the present study indicated that pretreatment of PC3 cells with AUR improved radio sensitivity and decreased clonogenic survival.

In order to determine the underlying mechanisms of AUR + IR action, the expression of genes involved in IR-induced apoptosis was evaluated. P53, a tumor suppressor protein, is activated by radiation-induced DNA damages, and regulates the expression of genes involved in cell cycle arrest and apoptosis such as *BAX* and *BCL2* [27]. While a homodimer of *BAX* induces apoptosis, a *BAX-BCL2* heterodimer leads to the survival of cells [28]. AUR has the potential to alter the expression of *P53*, *BAX*, and *BCL2* [29–33]. In addition, single administration of IR induced changes in the pattern of *P53*, *BAX*, and *BCL2* expression [34, 35]. Present results revealed overexpression of *P53* upon single use of AUR and IR, and such increase was more considerable when cells were treated with AUR + IR. Furthermore, combinatorial treatment induced positive and negative regulatory effects on *BAX* and *BCL2* expression, respectively. Accordingly, *BAX* up regulation and *BCL2* suppression probably led to the formation of more *BAX-BAX* homodimers rather than *BAX-BCL2* heterodimers, which could explain, to some extent, the increased number of apoptotic cells after AUR + IR treatment. CCND1 is an important regulator of the cell cycle, and its overexpression is associated with prostate cancer progression [36]. Down regulation of *CCND1* resulted to a longer duration of G_1 phase, and thus, reduced proliferation of cancer cells [37–39]. Previously, decreased expression of *CCND1* was demonstrated after AUR + IR treatment of colon cancer cells [12]. Similarly, our findings revealed that AUR alone and in combination with IR considerably reduced *CCND1* expression in prostate cancer cells. GATA6 is a transcriptional regulator with tumor suppressive action. A high level of *GATA6* expression, which has been reported in various cancers, was associated with tumor progression [40]. It has been shown that irradiation of cancer cells induced *GATA6* expression [41]. On the other hand, pretreatment with AUR followed by X-ray exposure prevented such alteration to be observed [20]. Our results are in agreement with previous reports, since up regulation of *GATA6* was detected in irradiated PC3 cells, while such change was not detected after AUR + IR treatment.

Conclusion

The findings of the present study indicated, for the first time, that AUR improved radiation effects on prostate cancer cells. In case future research on animal models confirms AUR potential, this natural coumarin could be used as a radiosensitizer in clinical trials.

Authors' Contributions Yasaman Abolhassani: Methodology, Investigation, Software, Analysis and interpretation of data, Writing- Original Draft. Sara Mirzaei: Methodology & Investigation. Masoud Nejabat: Writing – Review & Editing. Seyedehsaba Talebian: Methodology & Investigation. Hamid Gholamhosseinian: Radiotherapy. Mehrdad Iranshahi: AUR Synthesis. Fatemeh B. Rassouli, and Khadijeh Jamialahmadi: Supervision, Conceptualization and Design, Funding Acquisition.

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Data Availability All data generated or analyzed during this study are included in this article.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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