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Auraptene Attenuates Malignant Properties of Esophageal Stem-Like Cancer Cells

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Abstract

The high incidence of esophageal squamous cell carcinoma has been reported in selected ethnic populations including North of Iran. Low survival rate of esophageal carcinoma is partially due to the presence of stem-like cancer cells with chemotherapy resistance. In the current study, we aimed to determine the effects of auraptene, an interesting dietary coumarin with various biological activities, on malignant properties of stem-like esophageal squamous cell carcinoma, in terms of sensitivity to anticancer drugs and expression of specific markers. To do so, the half maximal inhibitory concentration values of auraptene, cisplatin, paclitaxel, and 5-fluorouracil were determined on esophageal carcinoma cells (KYSE30 cell line). After administrating combinatorial treatments, including nontoxic concentrations of auraptene + cisplatin, paclitaxel, or 5-fluorouracil, sensitivity of cells to chemical drugs and also induced apoptosis were assessed. In addition, quantitative real-time polymerase chain reaction was used to study changes in the expression of tumor suppressor proteins 53 and 21 (*P53* and *P21*), cluster of differentiation 44 (*CD44*), and B cell-specific Moloney murine leukemia virus integration site 1 (*BMI-1*) upon treatments. Results of thiazolyl blue assay revealed that auraptene significantly ($P < .05$) increased toxicity of cisplatin, paclitaxel, and 5-fluorouracil in KYSE30 cells, specifically 72 hours after treatment. Conducting an apoptosis assay using flow cytometry also confirmed the synergic effects of auraptene. Results of quantitative real-time polymerase chain reaction revealed significant ($P < .05$) upregulation of *P53* and *P21* upon combinatorial treatments and also downregulation of *CD44* and *BMI-1* after auraptene administration. Current study provided evidence, for the first time, that auraptene attenuates the properties of esophageal stem-like cancer cells through enhancing sensitivity to chemical agents and reducing the expression of *CD44* and *BMI-1* markers.

Keywords

auraptene, esophageal cancer, stem-like cancer cells, synergic effects

Abbreviations

AUR, auraptene; CSC, cancer stem cell; DBU, 1, 8-diazabicyclo [5.4.0] undec-7-ene; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; RPMI, Roswell Park Memorial Institute; 5-FU, 5-fluorouracil; QRT-PCR, quantitative real-time polymerase chain reaction; MTT, thiazolyl blue.

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Introduction

Esophageal squamous cell carcinoma (ESCC), the most common type of esophageal cancer, is malignant transformation of epithelial cells lining the esophagus. Different geographical distribution has been reported for ESCC incidence; the highest rates belong to countries located on the "esophageal cancer belt," extending from Northern Iran to North-Central China.[1] The mortality of ESCC mainly depends on nonspecific symptoms of the disease in early stages, and similar to other cancers of the digestive tract, development of drug-resistant cells negatively affects the outcome of conventional therapeutic

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modalities.[2,3] To design more effective strategies for cancer treatment, attention has been focused on identification of cancer stem cells (CSCs) in recent years, since accumulative evidence suggests that CSCs are responsible for cancer initiation, metastasis, and therapy resistance in a wide range of human malignancies.[4] Characterization of CSCs in different human tumors revealed that they could be detected by distinct molecular markers. In ESCC, for instance, CSCs have been introduced as cells positive for CD44, CD15,[5] CD133, CXCR4,[6] and CD90[7] antigens.

Auraptene (AUR), also known as 7-geranyloxycoumarin, is a natural prenyloxycoumarin found in plants belonging to Rutaceae and Apiaceae families. Various biological properties have been introduced for AUR including antibacterial, antiprotozoal, antifungal, antigenotoxic, anti-inflammatory, antioxidative, and immunomodulatory activities.[8] Importantly, dietary supplementation of AUR induced cancer chemopreventive effects in animal models of oral,[9] colon,[10-12] esophagus,[13] liver,[14,15] breast,[16] prostate,[17] and skin[18] cancers. Induction of glutathione S transferase activity, lipid peroxidation, modulation of inflammation, and suppression of superoxide generation have been introduced as mechanisms underlying AUR chemopreventive actions.[9,11,12,18] In addition, studies have demonstrated anticancer effects of AUR in a number of human cancer cell lines, except esophageal cancer cells or CSCs. For instance, AUR induced apoptosis in colon,[19] gastric,[20] and leukemia[21] cells; inhibited progression of renal[22] and breast[23] carcinoma cells; and prevented the reemergence of colon CSCs.[24]

Recent reports have demonstrated the importance of CSCs in the progression of esophageal cancers.[25-28] Nevertheless, current knowledge on natural or synthetic compounds that could selectively eliminate esophageal CSCs or attenuate their unfavorable properties is very limited. Accordingly, the aim of present study was to determine the effects of AUR on the characteristics of esophageal CSCs, including sensitivity to anticancer agents and expression of specific markers. To do so, KYSE30 cell line, highly enriched in stem-like cancer cells,[5] was used to evaluate the synergic effects of AUR on cisplatin, paclitaxel, and 5-fluorouracil (5-FU). Moreover, expression of apoptosis mediators, tumor suppressor proteins 53 and 21 (*P53* and *P21*), as well as CSC markers, cluster of differentiation 44 (*CD44*) and B cell-specific Moloney murine leukemia virus integration site 1 (*BMI-1*), was evaluated upon AUR or combinatorial treatments.

Materials and Methods

Preparation of AUR

Auraptene was synthesized as described previously.[29] Briefly, the reaction was carried out between 7-hydroxycoumarin (1 mol/L) and *trans*geranyl bromide (1.5 mol/L) in acetone at room temperature and in the presence of 1, 8-diazabicyclo [5.4.0] undec-7-ene (2 mol/L). Then, AUR was purified by column chromatography (petroleum ether/ethyl acetate 9:1 v/v) as white crystals (mp = 62.7°C-63.4°C). To prepare various concentrations of AUR, 2 mg of the crystal powder was dissolved in 100 μL dimethylsulfoxide (DMSO; Merck, Germany) and diluted with complete culture medium before experiments. To eliminate the effects of the solvent, equal amount of DMSO in all AUR concentrations (0.4% DMSO) was considered as the control treatment.

Culture of Cells

KYSE30 cells, obtained from Pasteur Institute (Tehran, Iran), were grown in Roswell Park Memorial Institute (RPMI) 1640/Ham F12 medium (Biowest, France) supplemented with 10% fetal bovine serum (FBS; Biowest, France), while HFF3 cells, purchased from Pasteur Institute, were cultured in Dulbecco's modified Eagle medium (Gibco, Scotland) supplemented with 10% FBS. Both cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and subcultured, when required, by 0.25% trypsin–1 mmol/L EDTA (Biowest, France).

In vitro *cytotoxicity Assay*

The thiazolyl blue (MTT) assay was used to determine the half maximal inhibitory concentration (IC_{50}) of AUR in both cell lines as well as the IC_{50} values of cisplatin, paclitaxel, and 5-FU in KYSE30 cells. To do so, cells were seeded, at a density of 5000 cell/well for KYSE30 cells and 8000 cell/well for HFF3 cells, in 96-well tissue culture plates (Falcon Becton–Dickinson, USA). After 24 hours, both cell types were incubated with increasing concentrations of AUR (10, 20, 40, and 80 μg/mL) and the relevant DMSO control, for 24, 48, and 72 hours. In addition, KYSE30 cells were treated with cisplatin (Mylan, UK, 2, 4, and 8 μg/mL), paclitaxel (Actavis, France, 2, 4, 8, and 16 μg/mL), and 5-FU (Ebewe Pharma, Austria, 2.5, 5, 10, and 20 μg/mL) for 24, 48, and 72 hours.

To study the synergy of AUR and anticancer agents, KYSE30 cells were treated with combinations of AUR and each drug: AUR (5, 10, and 20 μg/mL) + cisplatin (1, 2, and 4 μg/mL), + paclitaxel (1, 2, and 4 μg/mL), or +5-FU (2.5, 5, and 10 μg/mL) for 24, 48, and 72 hours. To note, the effect of each combination was evaluated using its relevant control (0.4% DMSO + drug).

For cytotoxicity assay, the MTT dye (ATOCEL, Austria) was dissolved in phosphate-buffered saline (5 mg/mL) and added to each well (20 μL/well), and the plates were incubated for 4 hours at 37°C. The reaction was then stopped by the addition of DMSO (150 μL/well) and optic densities of the wells were measured spectrophotometrically at 570 nm using an enzyme-linked immunosorbent assay plate reader (Awareness, USA).

Measurement of Apoptosis

Apoptosis was assessed in KYSE30 cells using fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit with propidium iodide (BioLegend, USA) according to the manufacturer's instruction. Briefly, following each combinatorial treatment, cells were collected, washed, and resuspended in a

Table 1. List of Primers, Their Sequence, and Product Length Used in the Current Study.

Gene Name	Forward (5′-3′)	Reverse (5′-3′)	Product Size, bp
GAPDH	GACCACTTTGTCAAGCTCATTTCC	GTGAGGGTCTCTCTCTTCCTCTTGT	151
P53	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG	123
P21	GGAAGACCATGTGGACCTGT	GGCGTTTGGAGTGGTAGAAA	146
CD44	CGGACACCATGGACAAGTTT	GAAAGCCTTGCAGAGGTCAG	176
BMI-1	CTGCAGCTCGCTTCAAGATG	CACACACATCAGGTGGGGAT	192

Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; P53 and P21, tumor suppressor proteins 53 and 21; CD44, cluster of differentiation 44; BMI-1, B cell-specific Moloney murine leukemia virus integration site 1.

staining buffer. Then, samples were stained with FITC-annexin V and propidium iodide for 15 minutes at room temperature in the dark, followed by the addition of binding buffer. Finally, the cells were analyzed by flow cytometry (BD FACSCalibur, USA) using FL1 and FL2 filters.

RNA Extraction, Complementary DNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction

Using RNX-plus (Cinnagen, Iran), the total cellular RNA was extracted from untreated cells and also KYSE30 cells treated with 20 μg/mL AUR (and its relevant DMSO control) as well as cells treated with combination of 20 μg/mL AUR + 1 μg/mL cisplatin, +1 μg/mL paclitaxel, or +2.5 μg/mL 5-FU (and their corresponding DMSO controls). To avoid DNA contamination, extracted RNAs were treated with RNase-free DNase I (Thermo Scientific, USA) followed by heat inactivation with EDTA. For complementary DNA (cDNA) synthesis, oligo-dT, deoxyribonucleoside triphosphates, RNase inhibitor, and M-MuLV reverse transcriptase (Thermo Scientific, USA) were used according to the manufacturer's protocol. The fidelity of amplified cDNAs was then confirmed by polymerase chain reaction (PCR) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primers, and final products were loaded on 1.5% agarose gel (Invitrogen, USA) for electrophoresis. Quantitative real-time polymerase chain reaction (QRT-PCR) was conducted in iQ5 real-time PCR detection system (Bio-Rad, USA) using SYBR green mix (Pars Toos, Iran). To note, GAPDH transcripts were used as internal control, and PCR efficiencies were calculated for all used primers from the given slopes of standard curves. The PCR cycling conditions were 95°C for 4 minutes (95°C for 15 s, 55°C for 15 s, 72°C for 15 s; 50 cycles) for *P53* and *P21* primers and 95°C for 4 min (95°C for 30 s, 59°C for 30 s, 72°C for 30 s; 40 cycles) for *CD44* and *BMI-1* primers. The primer sequences used are shown in Table 1.

Statistical Analyses

Significant level was ascertained by one-way analysis of variance, followed by Tukey multiple comparison tests using SPSS 19.0 software. Values were expressed as mean ± standard error of the mean, and $P < .05$ was considered to be statistically significant.

Table 2. The IC_{50} Values of AUR, Cisplatin, Paclitaxel, and 5-FU During 3 Consecutive Days.

	IC_{50}, μg/mL		
Treatments	24 h	48 h	72 h
AUR	80	72	76
Cisplatin	7.7	4.9	4.3
Paclitaxel	7.5	6	3.6
5-FU	>20	18	17

Abbreviations: AUR, auraptene; 5-FU, 5-fluorouracil; IC_{50}, half maximal inhibitory concentration.

Results

Auraptene Enhanced Toxicity of Cisplatin, Paclitaxel, and 5-FU

To study AUR effects on KYSE30 cells, it was first necessary to determine the IC_{50} of this coumarin during 3 consecutive days. In this regard, cells were treated with increasing concentrations of AUR for 24, 48, and 72 hours, and the IC_{50} values were calculated, as summarized in Table 2. Worth to mention, toxicity of AUR was also examined on human foreskin fibroblasts (HFF3 cell line), and the determined IC_{50} values were more than 80 μg/mL at all 3 time points (data not shown).

To assess the synergic effects of AUR on cisplatin, paclitaxel, and 5-FU, the IC_{50} values of each drug was also determined on KYSE30 cells after 24, 48, and 72 hours (Table 2). Later on, KYSE30 cells were treated with combination of AUR (5, 10, and 20 μg/mL) + cisplatin (1, 2, and 4 μg/mL), + paclitaxel (1, 2, and 4 μg/mL), or + 5-FU (2.5, 5, and 10 μg/mL), all in concentrations lower that their IC_{50}, for 3 continuous days. As presented in Figure 1, the results of MTT test indicated that nontoxic AUR significantly ($P < .05$) enhanced the cytotoxicity of anticancer drugs, specifically in the lowest concentration of each drug, and these effects were improved as time passed during combinatorial treatments. In case of AUR + cisplatin treatments, the highest synergic effect belonged to 20 μg/mL AUR, which increased the toxicity of 1 μg/mL cisplatin up to 22% and 32%, after 48 and 72 hours, respectively. To note, toxicity of 2 μg/mL cisplatin was also increased by 20 μg/mL AUR up to 15% and 26%, after 48 and 72 hours, respectively (Figure 1A). Evaluating synergic effects of AUR on paclitaxel revealed the same results; 72 hours after combinatorial treatments, 20 μg/mL AUR improved toxicity of 1 and 2 μg/mL paclitaxel up to 26% and

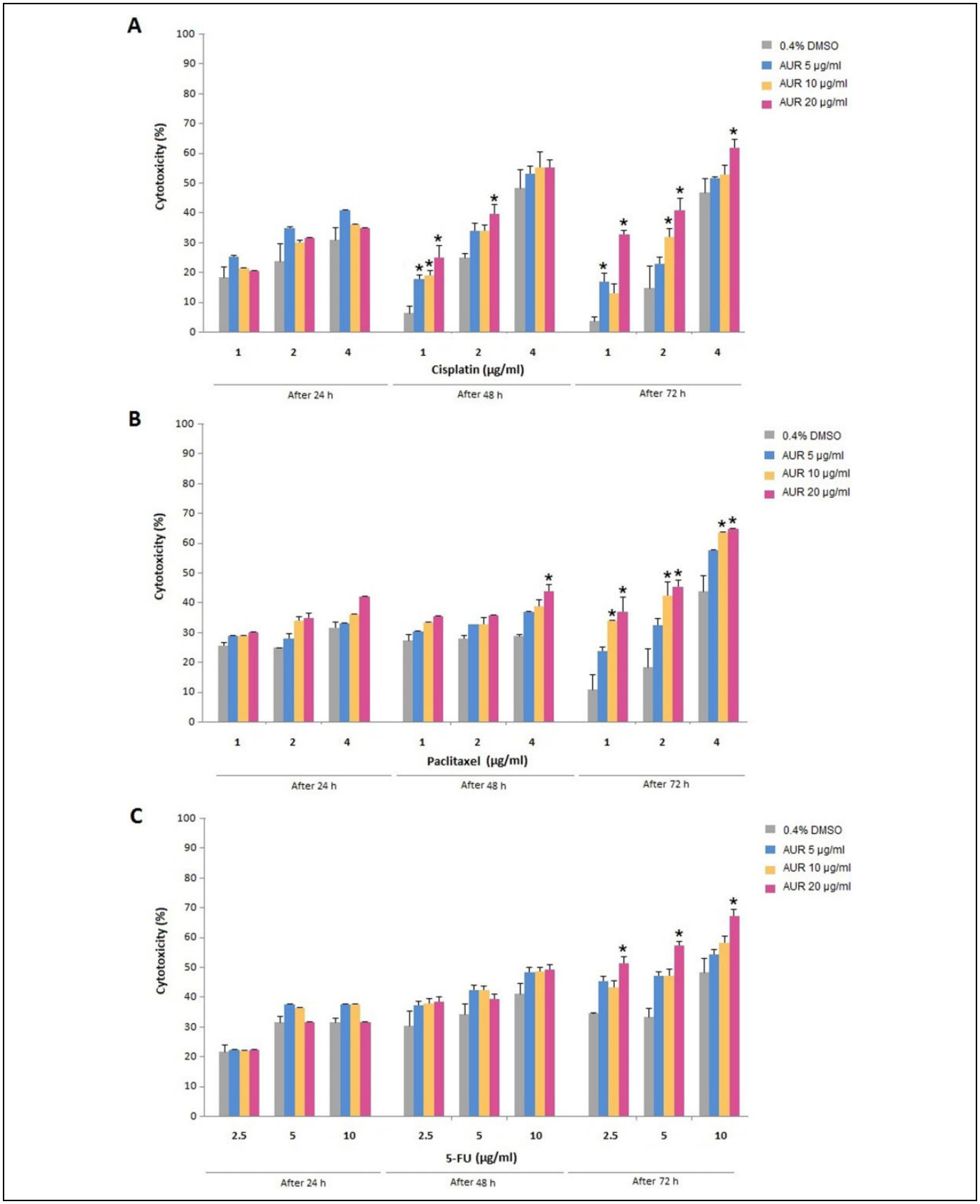

Figure 1. Effects of auraptene (AUR; 5, 10 and 20 µg/mL) and relevant dimethyl sulfoxide (DMSO) controls on the cytotoxicity of cisplatin (A), paclitaxel (B), and 5-fluorouracil (5-FU) (C) 24, 48, and 72 hours after combinatorial treatments.

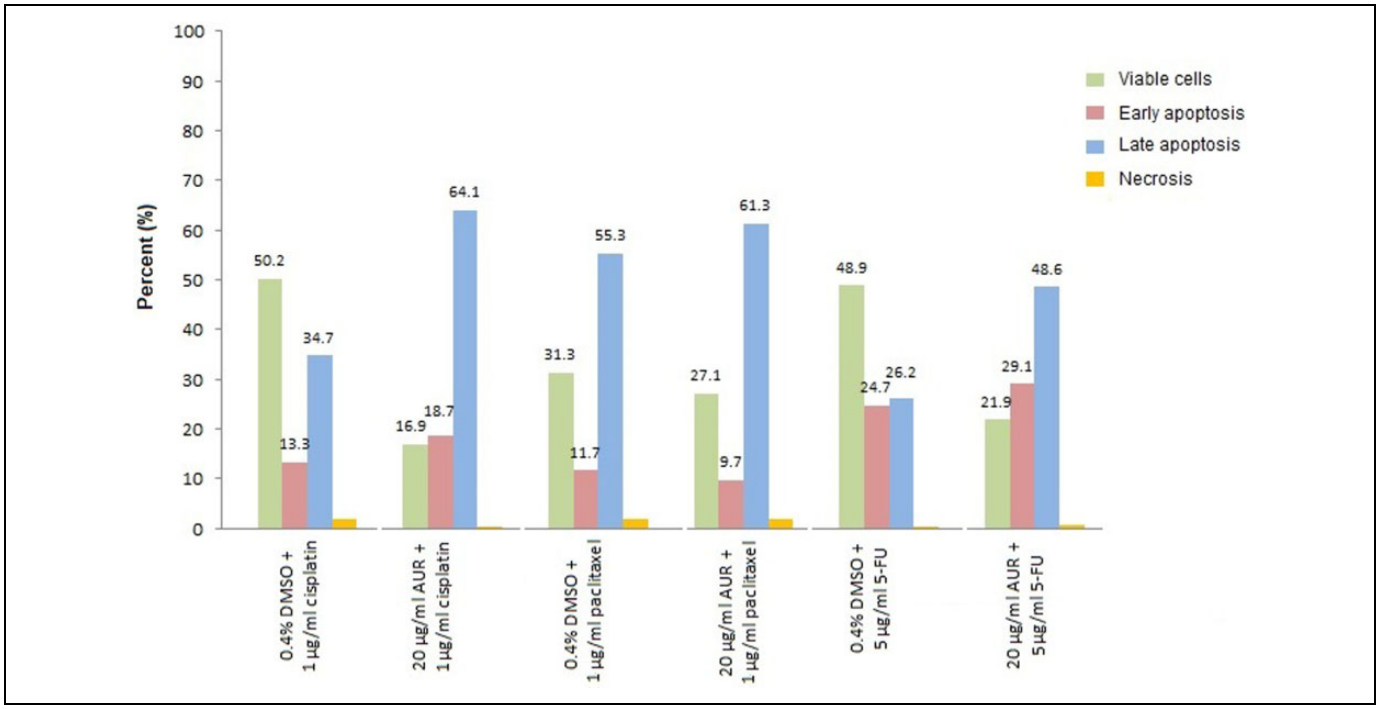

Figure 2. Apoptosis detected by fluorescein isothiocyanate (FITC)-annexin V and propidium iodide 72 hours after combinatorial treatments. Flow cytometry analysis differentiated alive and necrotic cells from early and late apoptotic cells. In comparison with relevant dimethyl sulfoxide (DMSO) controls, considerable increase was observed in the percentage of early and late apoptotic cells in auraptene (AUR) + cisplatin and AUR + 5-fluorouracil (5-FU) treatments.

27%, respectively (Figure 1B). Furthermore, our results revealed that after 72 hours, the cytotoxicity of 2.5 and 5 µg/mL 5-FU increased by 20 µg/mL AUR up to 19% and 24%, respectively (Figure 1C).

Synergic Effects of AUR Increased Apoptosis Induced by Anticancer Agents

To further study synergic effects of AUR on anticancer drugs, we evaluated apoptotic cells 72 hours after administration of our best combinatorial treatments: 20 µg/mL AUR + 1 µg/mL cisplatin, + 1 µg/mL paclitaxel, or + 5 µg/mL 5-FU. Figure 2 shows the effect of each combination on the percentage of alive and necrotic cells as well as early and late apoptotic cells in comparison with its relevant control (0.4% DMSO + drug). As presented, combination of AUR + cisplatin increased the percentage of early and late apoptotic cells up to 34.7%. In addition, 26.8% increase was observed in the percentage of early and late apoptotic cells upon coadministration of AUR + 5-FU. These observations support the results obtained from MTT assay. In case of AUR + paclitaxel treatment, despite experiments were repeated several times, changes in the percentage of apoptotic cells were not considerable (6%) in comparison with control treatment.

Overexpression of P53 and P21 in Combinatorial Treatments

To better elucidate the molecular mechanism of synergic activity, we studied the expression of *P53* and *P21*, genes involved in apoptosis induced by cisplatin, paclitaxel, and 5-FU. In this regard, KYSE30 cells were treated with combinations of AUR and each drug, using concentrations determined by MTT test and confirmed by apoptosis assay. Figure 3A shows relative fold changes in the expression of *P53* and *P21* over corresponding control treatments. As presented, the expression of *P21* significantly ($P < .05$) increased in all our combinatorial treatments. However, significant enhancement in *P53* expression was only observed in cells treated with AUR + 5-FU.

Auraptene Downregulated the Expression of CSC Markers CD44 and BMI-1

To study the effects of AUR on the expression of *CD44* and *BMI-1* in messenger RNA level, it was necessary to first determine the expression profile of these CSC markers in untreated KYSE30 cells. As presented in Figure 3B, we detected high level of *CD44* and *BMI-1* expression in KYSE30 cells by the use of specific primers. After administration of 20 µg/mL AUR for 48 and 72 hours, QRT-PCR analysis revealed significant ($P < .05$) downregulation of *CD44* after 48 hours as well as decrease in *BMI-1* expression at both time points (Figure 3C).

Conclusion

Cancers of the digestive tract are among the top 10 life-threatening malignancies worldwide. The high incidence of ESCC has been reported in selected ethnic populations

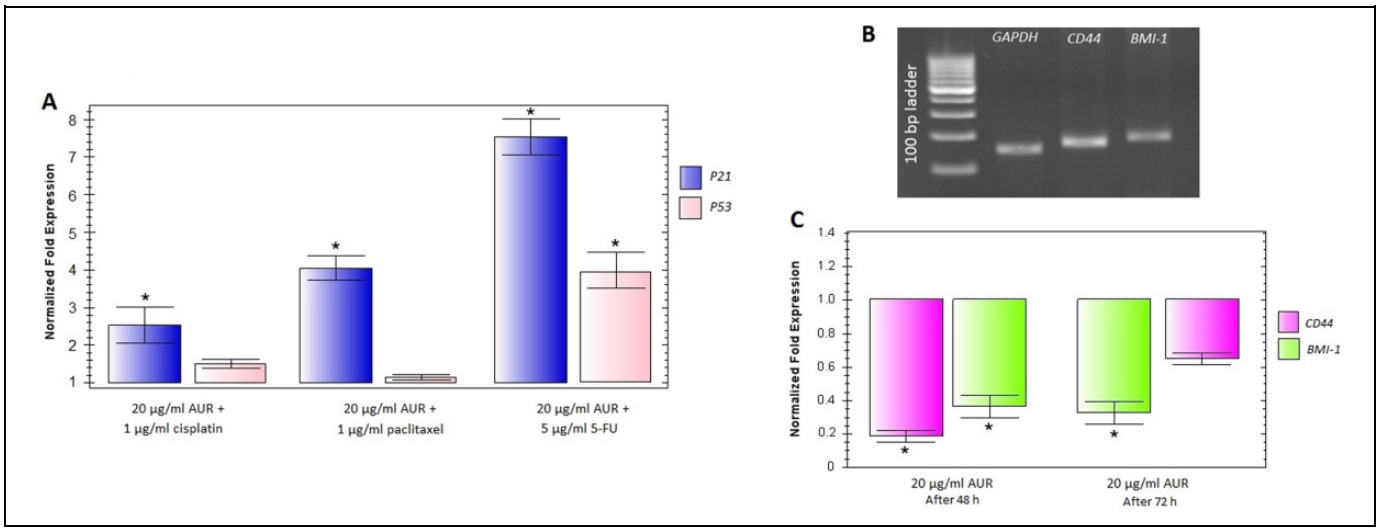

Figure 3. Quantitative real-time polymerase chain reaction (QRT-PCR) analysis of tumor suppressor proteins 53 and 21 (*P53* and *P21*) expression upon combinatorial treatments (A). To note, normalized values were plotted as relative fold change over relevant control treatments, including 0.4% dimethyl sulfoxide (DMSO) + 1 µg/mL cisplatin (for auraptene [AUR] + cisplatin), + 1 µg/mL paclitaxel (for AUR + paclitaxel), or + 5 µg/mL 5-fluorouracil (5-FU; for AUR + 5-FU). Expression pattern of cluster of differentiation 44 (*CD44*) and B cell-specific Moloney murine leukemia virus integration site 1 (*BMI-1*) in untreated KYSE30 cells (B). Changes induced by AUR (20 µg/mL) in the expression of *CD44* and *BMI-1*, after 48 and 72 hours (C). To note, normalized values were plotted as relative fold-change over relevant controls (0.4% DMSO for 48 and 72 hours, respectively).

including Caspian littoral region in Iran.[1] Despite advances in ESCC treatment, including surgical techniques, chemotherapy, and radiotherapy, the survival rate of ESCC is still very low. This is mainly due to the late detection of the disease, metastasis of malignant cells, and innate or acquired resistance to chemoradiotherapy. Progress in cancer cell biology suggests that achieving novel therapeutics and avoiding recurrence of malignant cells would be possible by targeting CSCs in many tumor types such as ESCC.[25] Accordingly, we examined the effects of AUR, the most abundant natural prenyloxycoumarin, on sensitivity of ESCC stem-like cells to anticancer drugs, as well as the expression of CSC markers involved in malignant properties, for the first time.

Auraptene is well characterized for its interesting and valuable pharmacological properties such as chemopreventive and anticancer effects. *In vitro* studies have suggested several mechanisms of action for AUR; it induced apoptosis in gastric cancer cells via suppression of mechanistic target of rapamycin (mTOR) pathways[20] and in leukemia cells through stimulation of caspase cascade.[21] Moreover, AUR inhibited progression of renal carcinoma cells by suppression of mitochondrial respiration[22] and controlled proliferation of breast cancer cells by modulating estrogen receptors[30] and changing the expression of genes related to cell cycle.[23] Among other key targets of AUR are matrix metalloproteinases[31] and P-glycoprotein[32] involved in metastasis and drug resistance of malignant cells, respectively. Nevertheless, current study is the only report indicating AUR effects on characteristics of esophageal stem-like cancer cells.

Results of MTT test revealed that nontoxic AUR significantly ($P < .05$) increased sensitivity of KYSE30 cells to cisplatin, paclitaxel, and 5-FU, drugs routinely used for ESCC treatment.[33] In addition, flow cytometrically detecting apoptotic cells confirmed the synergic effects of AUR on cisplatin and 5-FU. Previously, it has been reported that AUR enhanced tumor suppressive effects of all-trans retinoic acid in xenograft model of human skin cancer.[34] The present study, however, provides new evidence for synergic effects of AUR on anticancer agents using ESCC stem-like cells. One explanation for AUR activity, specifically when used in combination with paclitaxel, includes interaction with P-glycoprotein, due to the fact that AUR competitively interacts at the drug-binding site of this efflux pump.[32] Moreover, since cisplatin and 5-FU are substrates of other drug transporters, such as MRP2 and MRP5, respectively,[35,36] different mechanisms must be involved in synergy of AUR with these drugs.

To better evaluate the effects of AUR in combinatorial treatments, we examined the expression of genes involved in apoptosis induced by cisplatin, paclitaxel, and 5-FU. *P53*, a tumor-suppressor gene, acts as a sequence-specific DNA-binding transcription factor implicated in cellular responses to DNA damage.[37] *P21*, which is regulated by P53-dependent and independent pathways, encodes a multifunctional protein involved in cell cycle regulation, programmed death, and differentiation.[38] Studying the expression of *P53* and *P21* upon administration of AUR + drug combinations indicated significant ($P < .05$) upregulation of both genes in comparison with control treatments. Not only these results are in agreement with a previous study, which reported increased *P53* and *P21* expression in KYSE30 cells after cisplatin treatment,[39] but these results also confirm the effects of AUR on enhanced toxicity of anticancer drugs in these cells. Published studies have reported significant growth suppression in ESCC cell lines upon infection

with P53[40] or P21[41] recombinant adenoviral vectors. Accordingly, it is presumable that improved activity of anticancer drugs in our study might be, to some extent, due to enhanced expression of *P53* and *P21* in KYSE30 cells.

Due to the importance of CSC markers in the maintenance of stem-like cancer cell properties, the effect of nontoxic AUR was also examined on the expression of *CD44* and *BMI-1*, 2 common markers for gastrointestinal CSCs. CD44 is a transmembrane protein that integrates and transduces microenvironmental signals and, thus, affects regulation of genes involved in cell migration, proliferation, differentiation, and survival.[41] Reports have indicated the importance of CD44 as a prognostic marker in human malignancies, including esophageal cancers; CD44 expression has been correlated with clinicopathological features of ESCC and esophageal adenocarcinoma (EAC).[42-45] BMI-1, a polycomb group family member, acts as an oncoprotein regulating cell cycle events during tumorigenesis.[46] The elevated expression of BMI-1 was associated with advanced pathological stage, grade, and lymph node metastasis in ESCC and EAC.[47-49] Accordingly, CD44 and BMI-1 are considered as potential markers for esophageal cancer diagnosis as well as therapeutic targets for designing novel approaches.

We have previously introduced KYSE30 cells as a suitable model for studying ESCC stem-like cells highly positive for CD44 and CD15 markers.[5] In the present work, we detected high level of CD44 and BMI-1 transcripts in untreated KYSE30 cells and reported significant ($P < .05$) decrease in both markers upon AUR treatment. Similarly, downregulation of CSC markers, CD44 and CD166, has been reported in drug-resistant colon CSCs by AUR.[24] Interestingly, there are several reports indicating the importance of CD44 and BMI-1 in resistance of cancer cells to cisplatin, paclitaxel, and 5-FU. It has been revealed that downregulation of *CD44* in ovarian cancer cells increased chemosensitivity to cisplatin and paclitaxel,[50-52] and silencing *BMI-1* expression in $CD44^+$ nasopharyngeal stem-like cancer cells enhanced sensitivity to cisplatin.[53] In addition, *BMI-1* knockdown enhanced chemosensitivity to cisplatin in glioblastoma,[54] osteosarcoma,[55] lung,[56] and ovarian[57] cancer cells and effectively reversed resistance to 5-FU in gastric CSCs,[58] nasopharyngeal carcinoma cells,[59] and breast cancer cells.[60] In line with these reports, we observed enhanced toxicity of cisplatin, paclitaxcel, and 5-FU, as well as downregulation of *CD44* and *BMI-1*, in ESCC stem-like cells upon administration of nontoxic AUR. These results indicate great pharmaceutical value of AUR that could be used, in combination or alone, to negatively affect malignant properties of CSCs.

In summary, we reported enhanced chemosensitivity of ESCC stem-like cells upon administration of nontoxic AUR in combination with cisplatin, paclitaxel, and 5-FU. Detection of apoptotic cells, as well as overexpression of *P53* and *P21*, confirmed increased activity of anticancer agents mediated by AUR. Furthermore, decreased expression of CSC markers *CD44* and *BMI-1* in KYSE30 cells indicated attenuated malignant properties of these cells and could be considered as another proof for synergic effects of AUR. Based on current results and all studies reviewed here, it seems that AUR could gain enough validity to be used *in vivo* as a great candidate for ESCC combinatorial treatment and/or CSC-based therapy. However, to improve our knowledge about underlying mechanisms of AUR actions, and also introduce this coumarin as a suitable candidate for clinical studies, it is necessary to deeply understand the link between synergic effects of AUR, cell cycle control, and apoptosis. In addition, further investigations are required to determine AUR effects on other CSC properties of ESCC cells, such as radioresistance and metastasis.

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Declaration of Conflicting Interests

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