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<th>Journal:</th>
<th>Biochemistry and Cell Biology</th>
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<tr>
<td>Manuscript ID</td>
<td>bcb-2017-0146</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>19-Jun-2017</td>
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</tbody>
</table>
| Complete List of Authors: | Moussavi, Mahdi; Biotechnology, biology  
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| Is the invited manuscript for consideration in a Special Issue?: | N/A |
| Keyword:       | Hyperthermia, Auraptene, Colon adenocarcinoma |
Improved efficacy of hyperthermia by auraptene in human colon adenocarcinoma cells

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Abstract

Colon adenocarcinoma is one of the most common cancers worldwide, and resistance to current therapeutic modalities is a serious drawback in its treatment. Auraptene is a natural coumarin with considerable anticancer effects. The goal of present study was to introduce a novel combinatorial approach against colon adenocarcinoma cells. To do so, HT29 cells were pretreated with non-toxic auraptene and then hyperthermia was applied. Afterwards, viability of cells was assessed, changes induced in the cell cycle were analyzed, and expression pattern of candidate genes was studied. Results of MTT assay demonstrated significant ($p<0.01$) decrease in cell viability when 20 $\mu$g/ml auraptene was used for 72 h, heat shock was induced, and cells were recovered for 24 h. Flow cytometry analysis also indicated considerable changes in the distribution of cells between sub G$_1$/G$_1$ and G$_2$/M phases of cell cycle after combinatorial treatment. Real time RT-PCR studies revealed significant ($p<0.01$) up regulation of $P21$ in auraptene pretreated cells after heat shock induction, while no significant change was observed in $HSP27$ expression. Current findings not only indicate improved efficacy of hyperthermia by auraptene for the first time, also suggest that this coumarin could be used in future to achieve more effective therapeutic outcomes.

Keywords: Hyperthermia, Auraptene, Colon adenocarcinoma, Combinatorial approach.

Introduction

Colon adenocarcinoma is among the top causes of cancer related death around the world, with increasing rate of incidence in developing countries (Torre et al. 2015). Availability of screening methods such as colonoscopy allows the removal of precancerous lesions, and as the disease progress, surgical resection is applied alone or in combination with chemoradiotherapy (Siegel et al. 2016). Nevertheless, patients with late-staged colon adenocarcinoma still suffer from tumor recurrence and have poor survival, which is mainly because acquired resistance of malignant cells to anticancer modalities (Primrose et al. 2014).
To overcome limitations of conventional therapies, great deal of investigation has focused on
designation of novel and combined strategies, for instance, concomitant use of hyperthermia with
chemical drugs and/or ionizing radiation (Kolosnjaj-Tabi and Wilhelm, 2017). Thermal ablation of
tumors is a rapidly developing approach that preserves surrounding tissues by intra-procedural
monitoring. However, incomplete ablation, disease recurrence and inferior outcomes are draw backs
that decrease hyperthermia efficacy (Chu and Dupuy, 2014).

Auraptene (7-geranyloxycoumarin) is a well-known prenyloxycoumarin extracted from various plant
species, mainly those from Rutaceae and Apiaceae families. This natural coumarin has interesting
pharmacological and medicinal properties such as antioxidative, antigenotoxic and antibacterial
effects. Moreover, anticancer activities of auraptene have been attributed to its inhibitory effects on
breast, prostate, endometrium and liver carcinogenesis (Krishnan et al. 2009; Tang et al. 2007; Mori et
al. 2001; Hara et al. 2005; Sakata et al. 2004), and also apoptogenic activity toward human gastric and
leukemia cells (Moon et al. 2015; Jun et al. 2007). Regarding colon cancer studies, it has been shown
that auraptene administration induced chemopreventive effects in animal models (Hayashi et al. 2007;
Kohno et al. 2006, Tanaka et al. 2010), presented synergic effects with ionizing radiation (Moussavi
et al. 2017) and prevented the growth and sphere formation of chemotherapy-resistant colon cancer
cells (Epifano et al. 2013).

Currently, one of the main goals of anticancer studies is to deliberate or eradicate cancer cells by
innovative combinatorial strategies. Although improved efficacy of anticancer drugs and radiotherapy
has been reported in esophageal, cervical and colon carcinoma cells by auraptene (Saboor-Maleki et
al. 2016; Nabekura et al. 2008; Moussavi et al. 2017), it is not clear whether this coumarin could
induce similar effects on other therapeutic modalities. Accordingly, we aimed to introduce a novel
approach against colon adenocarcinoma cells by evaluating cytotoxic effects of hyperthermia alone
and in combination with auraptene. Upon assessment of cell viability, cell cycle analysis was carried
out using flow cytometry, and expression pattern of apoptosis and heat shock mediators (P21 and
HSP27) was investigated by real time reverse transcription polymerase chain reaction (RT-PCR).
Materials and methods

Preparation of auraptene

Auraptene [7-(E)-3, 7Dimethylocta-2,6-dienyloxy)-2H-chromen-2-one] was synthesized based on a previously described method (Askari et al. 2009). In summary, 7-hydroxy-coumarin (1 M) and trans-geranyl bromide (1.5 M) were reacted in acetone at room temperature, in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2M). Then, auraptene was purified as white crystals (mp = 62.7-63.4 °C) by column chromatography using petroleum ether/ethyl acetate (9:1, v/v), and its structure was confirmed by ¹H- and ¹³C- nuclear magnetic resonance.

To prepare different concentrations of auraptene, 2 mg of the crystal was dissolved in 100 µl dimethylsulfoxide (DMSO, Merck) and diluted with complete culture medium. To note, equal amount of DMSO in all auraptene concentrations (0.4% v/v) was considered as control treatment.

Culture and treatment of cells

HT29 cells were obtained from Pasteur Institute (Tehran, Iran) and cultured in Roswell park memorial institute (RPMI) 1640 (Biowest) supplemented with 10% fetal bovine serum (FBS, Biowest) and 1% (W/V) penicillin/streptomycin (Biowest). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and subcultured using 0.25 % trypsin-1 mM EDTA (Biowest).

To investigate toxic effects of hyperthermia in combination with auraptene, HT29 cells were pretreated with 10 and 20 µg/ml auraptene, as well as relevant DMSO control, for 24, 48 and 72 h, and then heat shock was applied by transferring cell culture plates into a water bath for 30 min at 51°C, followed by 12 and 24 h recovery in CO₂ incubator at 37°C.

Cell viability assay

To assess viability of cells upon combinatorial treatment with auraptene and hyperthermia, tetrazolium-based colourimetric (MTT) test was used. In this regard, MTT dye (Atocel) was dissolved in phosphate-buffered saline (PBS, 5 mg/ml) and added to each well (20 µl/well) by the end of
treatments. After 4 h of incubation in 37°C, the resulting formazan crystals were solubilized in 150 µl of DMSO, and absorptions were measured at 545 nm in ELISA plate reader (Awareness). To calculate the percentage of cell viability, the mean absorbance of auraptene or DMSO pretreated cells after heat shock induction was divided to the mean absorbance of untreated cells.

**Cell cycle analysis**

To determine changes induced in the cell cycle after combinatorial treatment, HT29 cells were stained with propidium iodide (PI, Sigma). Briefly, floating and attached cells in each treatment were collected and washed with cold PBS containing 5% FBS. Then, cell pellets were resuspended in a hypotonic buffer containing 50 µg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100, incubated for 15 min at 4 °C in the dark, and flow cytometrically analyzed using a FACSCalibur instrument (BD Biosciences).

**Gene expression studies**

To investigate effects of auraptene and hyperthermia on the expression of apoptosis and heat shock mediators, real-time RT-PCR was applied. In this regard, the total cellular RNA was extracted from treated cells and their relevant controls using RNX-plus (Cinnagen). After RNA samples were treated with DNase I (Thermo Scientific), cDNAs were synthesized using oligo-dT, dNTPs and M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. To confirm the fidelity of amplified cDNAs, GAPDH primers (forward: GACCACCTTGTCAAGCTCATTTCC and reverse: GTGAGGGTCTCTCTCTCTTTG) were used for PCR. Real-time RT-PCR was then performed in iQ5 real-time PCR detection system (Bio-Rad) using SYBR green mix (Pars Toos) and specific primers for P21 (forward: GGAAGACCATGTGGACCT GT and reverse: GGCGTTTGGAGTGGTAGAAA) and HSP27 (forward: AAGGATGGCGTG GTGGAGATCA and reverse: GAGGAAACTTGGGTGGG GTCCA) with the following program: 95°C for 4 min, [95°C for 15 s, 55°C for 15 s, 72°C for 15 s] (50 cycles). To note, PCR efficiencies were calculated for all used primers from the given slopes of standard curves, generated from serial dilutions of positive
controls. In all analysis, GAPDH transcripts were used as internal control, and normalized values were plotted as relative fold change over untreated cells.

**Statistical analysis**

The statistical significance was assessed by one way ANOVA and Tukey multiple comparison test using SPSS software. All data were presented as mean ± SD and p values less than 0.05 and 0.01 were considered significant for all comparisons.

**Results**

To assess cell viability upon combinatorial treatment, HT29 cells were pretreated with non-toxic auraptene for 3 consecutive days, and then subjected to hyperthermia. Since determined IC$_{50}$ of auraptene in HT29 cells was 39 µg/ml after 72 h (Moussavi et al. 2017), 10 and 20 µg/ml auraptene, and 0.4% DMSO (relevant control), were administrated in these experiments. As shown in Figure 1, a significant (p<0.01) decrease in cell viability was observed when 20 µg/ml auraptene was used for 72 h, and cells were recovered for 12 h after heat shock induction. More interestingly, percentage of viable cells decreased significantly (p<0.01) in all time points of auraptene (20 µg/ml) treatment when the recovery time after hyperthermia was 24 h.

Morphological observations indicated that in comparison with untreated HT29 cells, 51°C heat shock induced obvious alterations in cells morphology, while reduced number of attached and alive cells was only visible when cells were pretreated with 20 µg/ml auraptene for 72 h (Figures 2 A-D). In consistence with MTT results and morphological observations, flow cytometry analysis after PI staining revealed considerable changes in the cell cycle upon auraptene pretreatment and heat shock application (Figures 2 E-H). As presented, 48% and 39% of HT29 cells with no treatment were detected in sub G$_1$/G$_0$ and G$_2$/M phases of the cell cycle, respectively, while after hyperthermia, these amounts changed as to 40% and 45%. In DMSO pretreated cells, 69% and 19% of cells were present in sub G$_1$/G$_0$ and G$_2$/M phases, respectively, and when auraptene was administrated before hyperthermia, these amounts changed as to 83% and 12%.
To investigate the molecular mechanism underlying combinatorial effects of auraptene and hyperthermia, the expression pattern of *P21*, a gene involved in apoptosis induced by heat shock stress, and *HSP27*, which provides thermotolerance and cytoprotection under stress condition, was studied by real time RT-PCR. To do so, after HT29 cells were pretreated with 20 µg/ml auraptene for 72 h, heat shock was applied and cells were recovered for 24 h. Figure 3 presents relative fold changes in the expression of *P21* and *HSP27* over untreated cells. As shown, significant (*p*<0.05) increase in *P21* expression was detected upon hyperthermia, and more importantly, this upregulation was higher (*p*<0.01) when cells were pretreated with auraptene. Moreover, *HSP27* expression was increased upon heat shock application, and very interestingly, auraptene decreased *HSP27* expression, although it was not significant.

**Discussion**

Colon adenocarcinoma is one of the most common cancers worldwide with high mortality rate. Recurrence of colon cancer is common among patients, and developed resistance to current therapeutic modalities limits the efficacy of clinical outcomes (Primrose et al. 2014). Due to the presence of residual malignant cells after surgical resection and routine chemoradiotherapy, complete eradication of colon adenocarcinoma in advance stages is very complicated. Accordingly, a lot of effort has been devoted to design novel and more effective strategies against colon cancer cells. In present study, we reported on a new and interesting anticancer approach by combinatorial use of hyperthermia and auraptene, for the first time.

Hyperthermia is a novel therapeutic option that is usually applied as an adjunct to other treatments, and ongoing clinical trials are being done to better understand and improve this approach (Kolosnjaj-Tabi and Wilhelm, 2017). Auraptene, the most abundant prenyloxy coumarin present in nature, has valuable pharmaceutical activities in the field of cancer studies. For instance, it induced chemopreventive and anticancer effects in animal models and human cancer cell lines, and enhanced efficacy of conventional therapies through synergic act with chemical drugs (Genovese and Epifano, 2011).
In current attempt, we investigated whether hyperthermia in single or in combination with auraptene could induce more cytotoxic effects on HT29 colon cancer cells. Assessment of cell viability revealed that non-toxic auraptene significantly increased cytotoxicity of hyperthermia, specifically 24 h after heat shock induction. Although it has been previously indicated that auraptene improved cytotoxicity of anticancer drugs in skin, esophageal and cervical cancers (Kleiner-Hancock et al. 2010; Saboor-Maleki et al. 2016; Nabekura et al. 2008), and also enhanced toxicity of ionizing radiation in colon adenocarcinoma cells (Moussavi et al. 2017), this is the first report on improved efficacy of hyperthermia by this coumarin derivative.

Analysis of cell cycle revealed increased presence of HT29 cells in G_{2}/M phase after heat shock application, while combination of hyperthermia with auraptene resulted in considerable accumulation of cells in sub G_{1}/G_{0} phase. In consistence with our findings, G_{2}/M phase arrest was reported after hyperthermia in several cell types such as liver, lung, breast and renal cancer cells (Qi et al. 2015; Zhao et al. 2015; Yan et al. 2014; Lin et al. 2013). Same as present results, previous studies have also indicated growth controlling effects of auraptene on breast and gastric carcinoma cells by cell cycle arrest in sub G_{1} (Mousavi et al. 2015, Moon et al. 2015) and G_{0}-G_{1} phases (de Medina et al. 2010; Krishnan et al. 2009). Since down regulation of genes that promote G_{1} to S phase transition, such as cyclin D1, E2F1, CDC2 and UHRF1, has been attributed to anti-proliferative activity of auraptene (Krishnan and Kleiner-Hancock, 2012), observed effects of our combinatorial treatment on HT29 cells might be due to the changed expression of such cell cycle regulators.

In the investigation of mechanisms involved in our novel combinatorial approach, we analyzed the expression of P21 and HSP27 by real time RT-PCR. P21 is a key component of the cellular stress response that acts as a cyclin dependent kinase inhibitor and induces cell cycle arrest (Abbas and Dutta, 2009). Obtained results revealed a significant upregulation of P21 upon heat shock induction, and intriguingly, P21 overexpression was more efficient after combinatorial administration of auraptene and hyperthermia. In concordance with current results, upregulation of P21 in response to heat shock has been reported in colorectal, liver and retinoblastoma cancer cells (Wei et al. 2008; Choi et al. 2003; Jeon et al. 2016). Moreover, enhanced expression of P21 in auraptene pretreated...
cells is in agreement with published studies that reported auraptene-induced expression of cell cycle key inhibitors including *BAX*, *P21*, *P53* and *DDIT3* in various cancer cells (Mousavi et al. 2015, Saboor-Maleki et al. 2016, Moon et al. 2015, Krishnan and Kleiner-Hancock, 2012). In addition, since *P21* prevents cell cycle progression by inhibiting cyclin/CDK complexes, increased expression of *P21* upon combinatorial administration of auraptene and hyperthermia also explains, to some extent, accumulation of HT29 cells in subG1/G0 phase of the cell cycle.

*HSP27*, also known as *HSPB1*, acts as molecular chaperones in cells exposed to different stresses, including heat shock, to prevent aggregation of misfolded proteins and modulates cell death pathways through its anti-apoptotic activity (Garrido et al. 2006). Current findings demonstrated increased expression of *HSP27* upon heat shock application, while down regulation of this gene was observed by our combinatorial approach, although not significant. Similarly, heat shock-inducible *HSP27* expression has been observed in melanoma, lymphoma and colon and pancreatic adenocarcinoma cells (Coss et al. 2003; Rashmi et al. 2003; Tabuchi et al. 2008; Schafer et al. 2012). In line with our findings, increased thermally-induced apoptosis in melanoma and colon cancer cells has been reported upon *HSP27* knock down (Wang et al. 2016; Chen et al. 2007). Accordingly, it is presumable that improved toxicity of hyperthermia in auraptene pretreated cells might also be due to slight down regulation of *HSP27* in HT29 cells.

In conclusion, present attempt provided evidence, for the first time, on improved efficacy of hyperthermia by auraptene in colon adenocarcinoma cells. Since acquired resistance to conventional modalities is a major challenge in treatment of colon cancer, auraptene could serve as a potent agent in future combinatorial therapies. However, further research is necessary to define more precisely the mechanism of hyperthermia effects in combination with auraptene on colon and other kinds of cancer cells.

**Acknowledgements**

This work was supported with grants from Iran National Science Foundation (INSF, No. 95849744) and Ferdowsi University of Mashhad (No. 38995).
References


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Figure legends

Figure 1: Comparison of cell viability between 18 groups of treatments. HT29 cells were treated with 10 and 20 µg/ml auraptene and 0.4% DMSO, as relevant control, for 24, 48 and 72 h, then heat shock was applied and cells were recovered for 12 and 24 h. ** \( p < 0.01 \), significant difference with DMSO control.

Figure 2: Morphological alterations and flow cytometric analysis of HT29 cells after various treatments. Phase contrast micrographs (A-D) and cell cycle distribution (E-H) of cells without any treatment (A and E), after hyperthermia application (B and F), and pretreated with 0.4% DMSO (C and G) or 20 µg/ml auraptene (D and H) followed by heat shock induction.

Figure 3: Gene expression pattern of \( P21 \) and \( HSP27 \) in HT29 cells after combinatorial treatment. To note, normalized values were plotted as relative fold change over no treatment. * \( p < 0.05 \), significant difference with untreated cells; ** \( p < 0.01 \), significant difference with untreated cells.