ARTICLE

Efficacy of hyperthermia in human colon adenocarcinoma cells is improved by auraptene

Mahdi Moussavi, Farhang Haddad, Maryam M. Matin, Mehrdad Iranshahi, and Fatemeh B. Rassouli

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 this study was to introduce a novel combinatorial approach for treatment against colon adenocarcinoma cells. To do so, HT29 cells were pretreated with nontoxic auraptene and then hyperthermia was induced. Afterwards, the viability of the cells was assessed, changes induced in the cell cycle were analyzed, and the expression patterns of candidate genes were studied. Results from the MTT assay demonstrated significant ($p < 0.01$) decreases in cell viability when 20 µg/mL auraptene was used for 72 h, heat shock was induced, and cells were allowed to recover for 24 h. Flow cytometry analysis also indicated considerable changes in the distribution of cells between the sub-G_1/G_1 and G_2/M phases of cell cycle after the combinatorial treatment. Real-time RT–PCR studies revealed significant ($p < 0.01$) up-regulation of *P21* in the cells pretreated with auraptene after heat shock, whereas no significant change was observed in *HSP27* expression. Our findings not only indicate, for the first time, that the efficacy of hyperthermia was improved by auraptene pretreatment, but also suggest that this coumarin could be used in the future to achieve more effective therapeutic outcomes.

Key words: hyperthermia, auraptene, colon adenocarcinoma, combinatorial approach.

Résumé : L'adénocarcinome du côlon est l'un des cancers les plus répandus à travers le monde et la résistance aux thérapies actuelles est un frein sérieux à son traitement. L'auraptène est une coumarine naturelle qui présente des effets anticancéreux importants. L'objectif de cette étude était de présenter une nouvelle approche combinatoire contre les cellules d'adénocarcinome du côlon. À cette fin, des cellules HT29 ont été prétraitées avec une dose non-toxique d'auraptène et elles ont ensuite été soumises à une hyperthermie. Puis, la viabilité des cellules a été évaluée, les changements apportés cycle cellulaire ont été analysés et le patron d'expression de gènes candidats a été étudié. Les résultats du dosage au MTT ont démontré une diminution significative ($p < 0,01$) de la viabilité cellulaire lorsque 20 µg/mL d'auraptène était utilisé pendant 72 h, qu'un choc thermique était induit et que les cellules étaient prélevées après 24 h. L'analyse en cytométrie de flux indiquait aussi que des changements considérables se produisaient dans la distribution des cellules entre les phases G_0/G_1 et G_2/M du cycle cellulaire après le traitement combiné. Des études en RT–PCR en temps réel ont révélé une régulation à la hausse significative ($p < 0,01$) de *P21* chez les cellules prétraitées à l'auraptène et l'induction du choc thermique subséquente, alors qu'aucune changement significatif de l'expression de *HSP27* n'était observé. Les données actuelles indiquent que non seulement l'hyperthermie améliore l'efficacité de l'auraptène et ce, pour la première fois, mais aussi que cette coumarine pourrait être utilisée dans une approche combinatoire plus efficace contre l'adénocarcinome du côlon. [Traduit par la Rédaction]

Mots-clés : hyperthermie, auraptène, adénocarcinome du côlon, approche combinatoire.

Introduction

Colon adenocarcinoma is among the top causes of cancer-related death worldwide, with increasing rates of incidence in developing countries (Torre et al. 2015). The availability of screening methods such as colonoscopy allows the removal of precancerous lesions, and as the disease progresses, surgical resection is applied alone or in combination with chemoradiotherapy (Siegel et al. 2016). Nevertheless, patients with late-stage colon adenocarcinoma still suffer from tumor recurrence and have a poor survival prognosis, which is mainly because of the acquired resistance of malignant cells to anticancer treatments (Primrose et al. 2014).

To overcome the limitations of conventional therapies, a great deal of investigation has focused on designing novel and com-

bined 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 intraprocedural monitoring. However, incomplete ablation, disease recurrence, and inferior outcomes are drawbacks that decrease the efficiency of hyperthermia treatment (Chu and Dupuy 2014).

Auraptene (7-geranyloxycoumarin) is a well-known prenyloxy-coumarin 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, the anticancer activities of auraptene have been attributed to its in-

M. Moussavi and F. Haddad. Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran.
M.M. Matin and F.B. Rassouli. Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran; Cell and Molecular Biotechnology Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.
M. Iranshahi. Department of Pharmacognosy and Biotechnology, Biotechnology Research Center, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.
Corresponding author: Fatemeh B. Rassouli (email: behnam3260@um.ac.ir).

hibitory effects on breast, prostate, endometrium, and liver carcinogenesis (Mori et al. 2001; Sakata et al. 2004; Hara et al. 2005; Tang et al. 2007; Krishnan et al. 2009), and also apoptogenic effects on human gastric carcinoma cells and leukemia cells (Jun et al. 2007; Moon et al. 2015). Regarding colon cancer studies, it has been shown that auraptene administration induced chemopreventive effects in animal models (Kohno et al. 2006; Hayashi et al. 2007; Tanaka et al. 2010), presented synergistic 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 eradicate cancer cells by innovative combinatorial strategies. Although improvements in the efficacy of anticancer drugs and radiotherapy in combination with auraptene has been reported with esophageal, cervical, and colon carcinoma cells (Nabekura et al. 2008; Moussavi et al. 2017; Saboor-Maleki et al. 2017), it is not clear whether this coumarin could induce similar effects in combination with other therapies. Accordingly, we aimed to evaluate a novel approach against colon adenocarcinoma cells by examining the 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 the expression patterns of apoptosis and heat shock mediators (*P21* and *HSP27*) were investigated by real-time reverse-transcription polymerase chain reaction (RT–PCR).

Materials and methods

Preparation of auraptene

Auraptene [7-((*E*)-3,7-dimethylocta-2,6-dienyloxy)-2*H*-chromen-2one] was synthesized based on a previously described method (Askari et al. 2009). In summary, 7-hydroxy-coumarin (1 mol/L) and *trans*-geranyl bromide (1.5 mol/L) were reacted in acetone at room temperature, in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (2 mol/L). 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 [1]H- and ^{13}C-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. Note that equal amounts of DMSO for all auraptene concentrations (0.4% *v/v*) were used as the control treatments.

Culture and treatment of cells

HT29 cells were obtained from Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute (RPMI) 1640 (Biowest) medium supplemented with 10% fetal bovine serum (FBS; Biowest) and 1% (*w/v*) penicillin–streptomycin (Biowest). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air, and subcultured using 0.25% trypsin-1 mmol/L EDTA (Biowest).

To investigate the cytotoxic effects of hyperthermia in combination with auraptene, HT29 cells were pretreated with 10 or 20 μg/mL auraptene, as well as the appropriate DMSO control, for 24, 48, and 72 h, and then heat shock was applied by transferring the cell culture plates into a water bath for 30 min at 51 °C, followed by 12 and 24 h recovery periods in a CO_2 incubator at 37 °C.

Cell viability assay

To assess the viability of cells upon combinatorial treatment with auraptene and hyperthermia, a 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) at the end of the 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 an ELISA plate reader (Awareness). To calculate the percentage of cell viability, the mean absorbance of auraptene- or DMSO-pretreated cells after the heat shock was divided by the mean absorbance of the untreated cells.

Cell cycle analysis

To determine the changes induced in the cell cycle after the combinatorial treatment, HT29 cells were stained with propidium iodide (PI; Sigma). Briefly, floating and attached cells from each treatment were collected and washed with cold PBS containing 5% FBS. Then, the 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 analyzed by flow cytometry using a FACSCalibur instrument (BD Biosciences).

Gene expression studies

To investigate the 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 the treated cells and their relevant controls using RNX-plus (Cinnagen). After the RNA samples were treated with DNase I (ThermoScientific), the cDNA was synthesized using oligo-dT, dNTPs, and M-MuLV reverse transcriptase (ThermoScientific) according to the manufacturer's protocol. To confirm the fidelity of amplified cDNAs, GAPDH primers (forward, GACCACTTTGT-CAAGCTCATTTCC; and reverse, GTGAGGGTCTCTCTCTTCCTCTTGT) were used for PCR. Real-time RT–PCR was then performed in an iQ5 real-time PCR detection system (Bio-Rad) using SYBR green mix (Pars Toos) and specific primers for P21 (forward, GGAAGAC-CATGTGGACCTGT; and reverse, GGCGTTTGGAGTGGTAGAAA) and HSP27 (forward, AAGGATGGCGTGGTGGAGATCA; and reverse, GAGGAAACTTGGGTGGGGTCCA) 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). Note that PCR efficiencies were calculated for all of the primers used, from the given slopes of standard curves, generated from serial dilutions of positive controls. In all analyses, GAPDH transcripts were used as the internal control, and the normalized values were plotted as relative fold-change compared with the untreated cells.
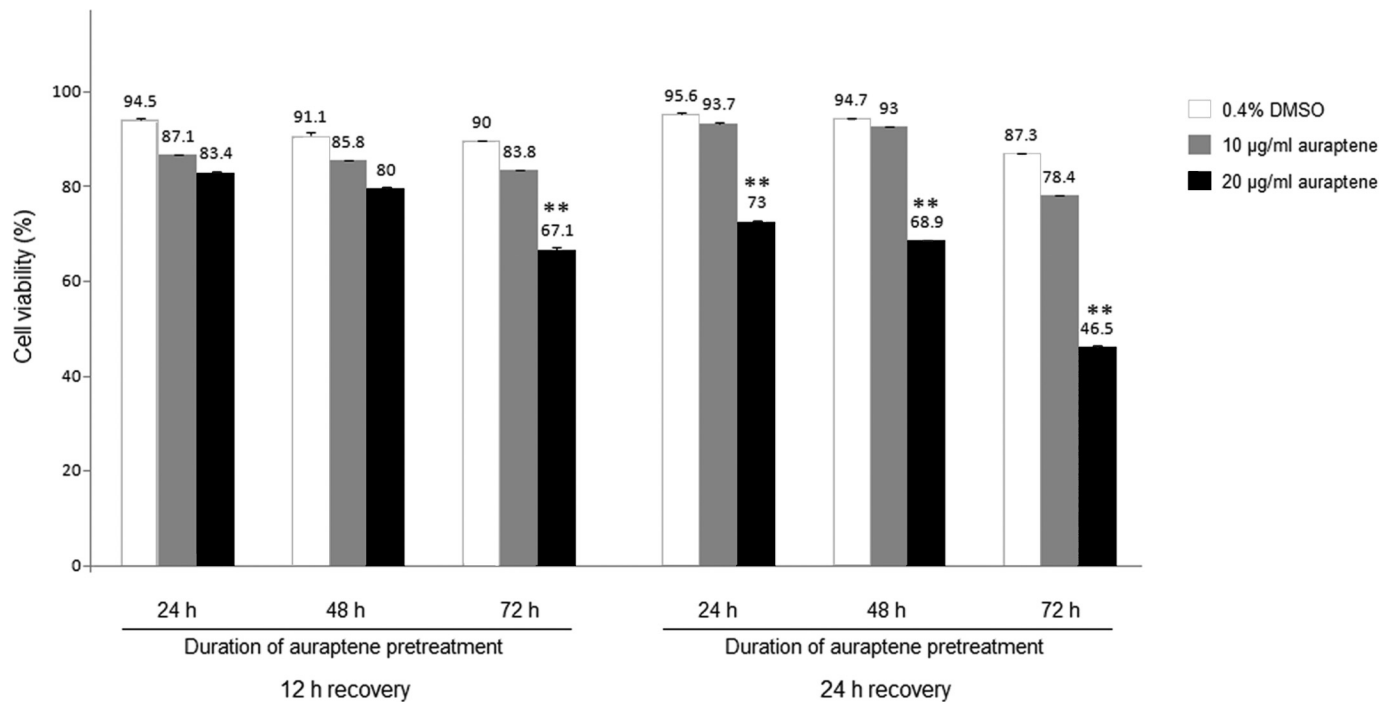
Statistical analysis

The statistical significance was assessed by one-way ANOVA and Tukey's multiple comparison test using SPSS software, version 19. All data presented are the mean ± SD, and *p* values less than 0.05 and 0.01 were considered statistically significant for all comparisons.

Results

To assess cell viability after the combinatorial treatment, HT29 cells were pretreated with nontoxic auraptene for 3 consecutive days, and then subjected to hyperthermia. Because the IC_{50} of auraptene in HT29 cells was determined to be 39 μg/mL after 72 h (Moussavi et al. 2017), we used 10 and 20 μg/mL auraptene, and 0.4% DMSO (relevant control) in these experiments. As shown in Fig. 1, a significant ($p < 0.01$) decrease in cell viability was observed when 20 μg/mL auraptene was used for 72 h, and the cells were allowed to recover for 12 h after heat shock. More interestingly, the percentage of viable cells decreased significantly ($p < 0.01$) for all of the 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, whereas a reduced number of attached and alive cells was only visible when cells were pretreated with 20 μg/mL auraptene for 72 h (Figs. 2A–2D). Consistent with the MTT results and morphological observations, flow cytometry analysis after PI staining revealed considerable changes in the cell cycle after auraptene pretreatment and heat shock (Figs. 2E–2H). As presented in Fig. 2, 48% and 39% of the untreated HT29 cells were detected in the sub-G_1/G_1 and G_2/M phases of the cell cycle, respectively, whereas after hyperthermia, these amounts changed

Fig. 1. Comparison of cell viability between 18 treatment groups: HT29 cells were treated with 10 or 20 μg/mL auraptene and 0.4% DMSO, as the relevant control, for 24, 48, and 72 h, then heat shock was applied and cells were allowed to recover for 12 and 24 h; **, $p < 0.01$ compared with the DMSO control.

to 40% and 45%. In the DMSO-pretreated cells, 69% and 19% of cells were present in sub-G_1/G_1 and G_2/M phases, respectively, and when auraptene was administrated before hyperthermia, these amounts changed to 83% and 12%.

To investigate the molecular mechanism underlying the 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 allowed to recover for 24 h. Figure 3 presents the relative fold-changes in the expression of *P21* and *HSP27* compared with the untreated cells. As shown, significant ($p < 0.05$) increases in *P21* expression were detected after hyperthermia, and more importantly, this up-regulation was higher ($p < 0.01$) when the cells were pretreated with auraptene. Moreover, *HSP27* expression was increased by heat shock, and very interestingly, auraptene decreased *HSP27* expression, although the decrease was not statistically significant.

Discussion

Colon adenocarcinoma is one of the most common cancers worldwide, with a 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). Owing to the presence of residual malignant cells after surgical resection and routine chemoradiotherapy, complete eradication of colon adenocarcinoma at the advanced stages is very difficult. Accordingly, a lot of effort has been devoted to design novel and more effective strategies against colon cancer. In this study, we reported, for the first time, a new and interesting anticancer approach by the combinatorial use of hyperthermia and auraptene.
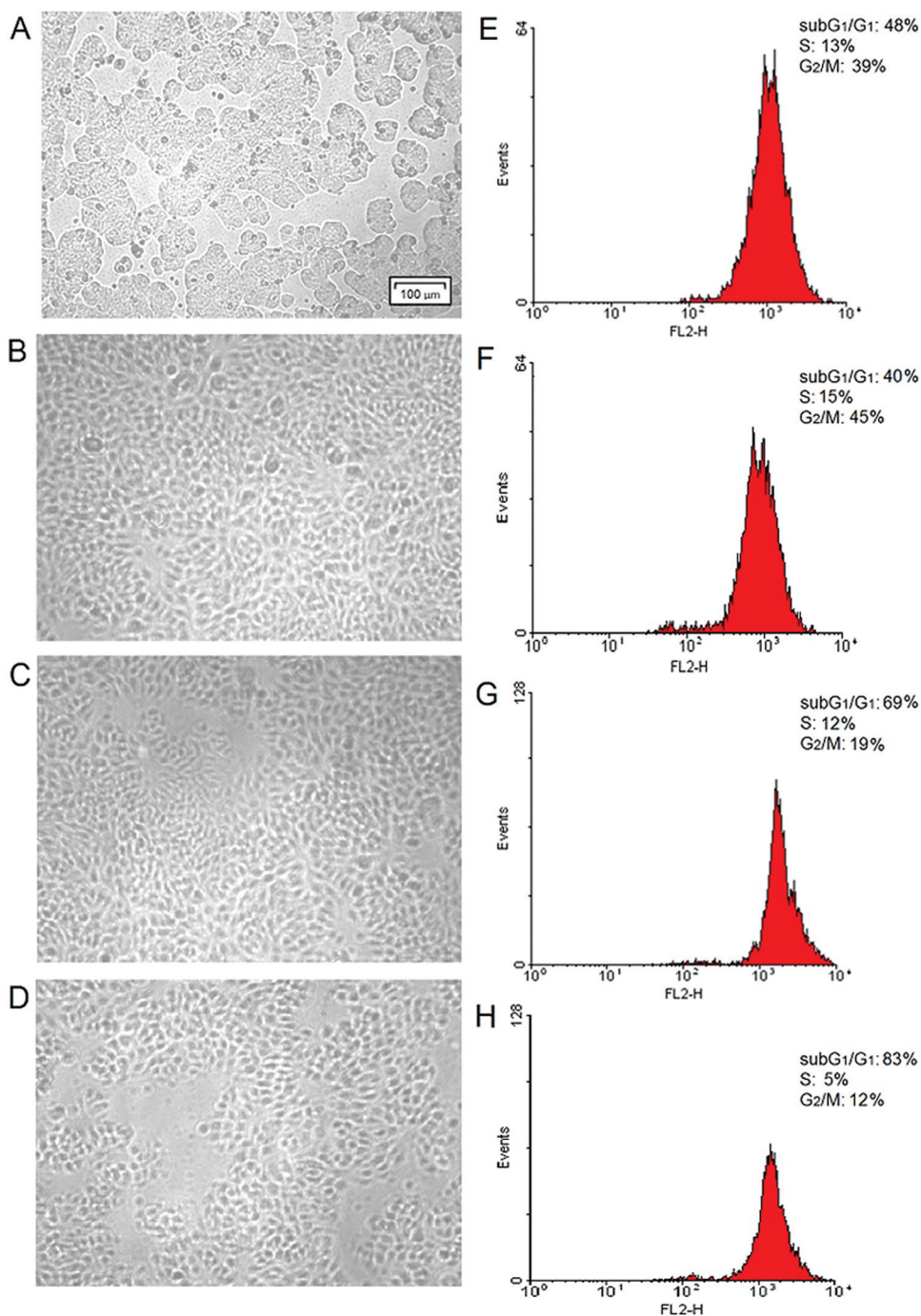
Hyperthermia is a novel therapeutic option that is usually applied as an adjunct to other treatments, and ongoing clinical trials are being done to better understand and improve this approach

(Kolosnjaj-Tabi and Wilhelm 2017). Auraptene, the most abundant prenyloxycoumarin 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 synergistic interactions with chemotherapeutic drugs (Genovese and Epifano 2011).

In our current attempt, we investigated whether hyperthermia alone or in combination with auraptene could induce a greater cytotoxic effect on HT29 colon cancer cells. The assessment of cell viability revealed that nontoxic auraptene significantly increased the cytotoxic effects of hyperthermia, specifically at 24 h after heat shock. Although it has been previously shown that auraptene improved the cytotoxicity of anticancer drugs in skin, esophageal, and cervical cancers (Nabekura et al. 2008; Kleiner-Hancock et al. 2010; Saboor-Maleki et al. 2017), and also enhanced the toxicity of ionizing radiation in colon adenocarcinoma cells (Moussavi et al. 2017), this is the first report on the improved efficacy of hyperthermia by this coumarin derivative.

The cell cycle analyses revealed the increased presence of HT29 cells in the G_2/M phase after heat shock, whereas the combination of hyperthermia with auraptene resulted in considerable accumulation of cells in sub-G_1/G_1 phase. Consistent with our findings, G_2/M phase arrest has been reported after hyperthermia in several cancer cell types, such as liver, lung, breast, and renal cancer cells (Lin et al. 2013; Yan et al. 2014; Qi et al. 2015; Zhao et al. 2015). Similar to our results, previous studies have also indicated the growth-controlling effects of auraptene on breast and gastric carcinoma cells by cell cycle arrest in the sub-G_1 (Moon et al. 2015; Mousavi et al. 2015) and G_0–G_1 phases (Krishnan et al. 2009; de Medina et al. 2010). Because the down-regulation of genes that promote G_1 to S phase transition, such as *cyclin D1*, *E2F1*, *CDC2*, and *UHRF1*, has been attributed to the anti-proliferative activity of auraptene (Krishnan and Kleiner-Hancock 2012), the observed effects from our combinatorial treatment on HT29 cells might be due to the changed expression of such cell cycle regulators.
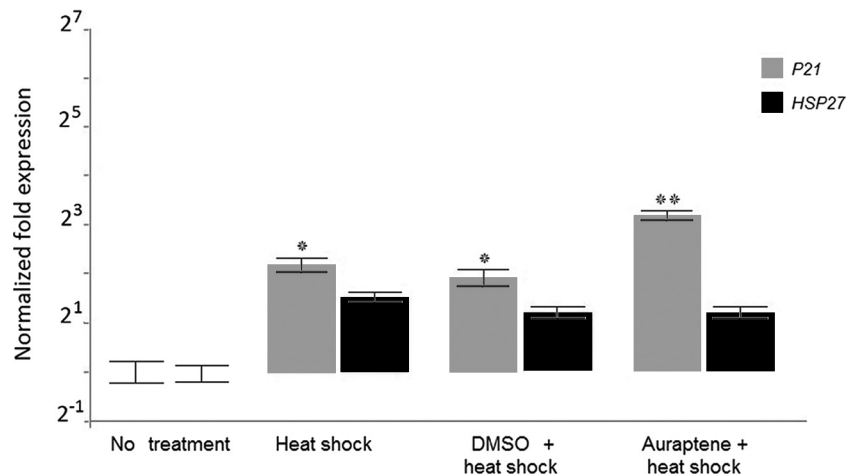
Fig. 2. Morphological alterations and flow cytometry analysis of HT29 cells after the various treatments. Phase contrast micrographs (A–D) and cell cycle distribution (E–H) of the untreated cells (A and E), after hyperthermia application (B and F), and pretreated with 0.4% DMSO (C and G) or 20 μg/mL auraptene (D and H) followed by heat shock. [Colour online.]

A

100 μm

B

C

D

E — Events — FL2-H
subG$_1$/G$_1$: 48%
S: 13%
G$_2$/M: 39%

F — Events — FL2-H
subG$_1$/G$_1$: 40%
S: 15%
G$_2$/M: 45%

G — Events — FL2-H
subG$_1$/G$_1$: 69%
S: 12%
G$_2$/M: 19%

H — Events — FL2-H
subG$_1$/G$_1$: 83%
S: 5%
G$_2$/M: 12%

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). Our results revealed a significant up-regulation of *P21* after heat shock, and intriguingly, *P21* overexpression was more efficient after the combined administration of auraptene and hyperthermia. In concordance with our results, up-regulation of *P21* in response to heat shock has been reported in colorectal, liver, and retinoblastoma cancer cells (Choi et al. 2003; Wei et al. 2008; Jeon et al. 2016).

Moreover, the enhanced expression of *P21* in cells pretreated with auraptene 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 (Krishnan and Kleiner-Hancock 2012; Moon et al. 2015; Mousavi et al. 2015; Saboor-Maleki et al. 2017). In addition, because *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, the accumulation of HT29 cells in the sub-G$_1$/G$_1$ phase of the cell cycle.

Fig. 3. Gene expression pattern of *P21* and *HSP27* in HT29 cells after the combinatorial treatment. Note that the normalized values were plotted as relative fold-change compared with the untreated cells; *, $p < 0.05$ and **, $p < 0.01$ compared with the untreated cells.

HSP27, also known as *HSPB1*, acts as a molecular chaperone in cells exposed to different stresses, including heat shock, to prevent aggregation of misfolded proteins, and also modulates cell death pathways through its anti-apoptotic activity (Garrido et al. 2006). Our findings demonstrated increased expression of *HSP27* after heat shock, while down-regulation of this gene was observed as a result of our combinatorial treatment, although this was not statistically significant. Similarly, heat-shock-inducible *HSP27* expression has been observed in melanoma, lymphoma, colon, and pancreatic adenocarcinoma cells (Coss et al. 2003; Rashmi et al. 2003; Tabuchi et al. 2008; Schäfer 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 (Chen et al. 2007; Wang et al. 2016). Accordingly, it is possible that the improved toxicity of hyperthermia in the cells pretreated with auraptene might also be due to a slight down-regulation of *HSP27* in the HT29 cells.

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

Acknowledgements

References

Abbas, T., and Dutta, A. 2009. p21 in cancer: intricate networks and multiple activities. Nat. Rev. Cancer, **9**: 400–414. doi:10.1038/nrc2657. PMID:19440234.

Askari, M., Sahebkar, A., and Iranshahi, M. 2009. Synthesis and purification of 7-prenyloxycoumarins and herniarin as bioactive natural coumarins. Iran. J. Basic Med. Sci. **12**: 63–69. doi:10.22038/ijbms.2009.5145.

Chen, F., Rezavi, R., Wang, C.C., and Harrison, L.E. 2007. Proteasome inhibition potentiates the cytotoxic effects of hyperthermia in HT-29 colon cancer cells through inhibition of heat shock protein 27. Oncology, **73**: 98–103. doi:10.1159/000120997. PMID:18337621.

Choi, E.K., Park, S.R., Lee, J.H., Chung, H.S., Ahn, H.E., Rhee, Y.H., et al. 2003. Induction of apoptosis by carboplatin and hyperthermia alone or combined in WERI human retinoblastoma cells. Int. J. Hyperthermia, **19**: 431–443. doi:10.1080/0265673021000017118. PMID:12850928.

Chu, K.F., and Dupuy, D.E. 2014. Thermal ablation of tumours: biological mechanisms and advances in therapy. Nat. Rev. Cancer, **14**: 199–208. doi:10.1038/nrc3672. PMID:24561446.

Coss, R.A., Storck, C.W., Daskalakis, C., Berd, D., and Wahl, M.L. 2003. Intracellular acidification abrogates the heat shock response and compromises

survival of human melanoma cells. Mol. Cancer Ther. **2**: 383–388. PMID:12700282.

de Medina, P., Genovese, S., Paillasse, M.R., Mazaheri, M., Caze-Subra, S., Bystricky, K., et al. 2010. Auraptene is an inhibitor of cholesterol esterification and a modulator of estrogen receptors. Mol. Pharmacol. **78**: 827–836. doi:10.1124/mol.110.065250. PMID:20702762.

Epifano, F., Genovese, S., Miller, R., and Majumdar, A.P. 2013. Auraptene and its effects on the re-emergence of colon cancer stem cells. Phytother. Res. **27**: 784–786. doi:10.1002/ptr.4773. PMID:22761031.

Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. 2006. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle, **5**: 2592–2601. doi:10.4161/cc.5.22.3448. PMID:17106261.

Genovese, S., and Epifano, F. 2011. Auraptene: a natural biologically active compound with multiple targets. Curr. Drug Targets, **12**: 381–386. doi:10.2174/138945011794815248. PMID:20955144.

Hara, A., Sakata, K., Yamada, Y., Kuno, T., Kitaori, N., Oyama, T., et al. 2005. Suppression of beta-catenin mutation by dietary exposure of auraptene, a citrus antioxidant, in N, N-diethylnitrosamine-induced hepatocellular carcinomas in rats. Oncol. Rep. **14**: 345–351. PMID:16012713.

Hayashi, K., Suzuki, R., Miyamoto, S., Shin-Ichiroh, Y., Kohno, H., Sugie, S., et al. 2007. Citrus auraptene suppresses azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ–*db/db* mice. Nutr. Cancer, **58**: 75–84. doi:10.1080/01635580701308216. PMID:17571970.

Jeon, T.W., Yang, H., Lee, C.G., Oh, S.T., Seo, D., Baik, I.H., et al. 2016. Electrohyperthermia up-regulates tumour suppressor Septin 4 to induce apoptotic cell death in hepatocellular carcinoma. Int. J. Hyperthermia, **32**: 648–656. doi:10.1080/02656736.2016.1186290. PMID:27269053.

Jun, D.Y., Kim, J.S., Park, H.S., Han, C.R., Fang, Z., Woo, M.H., et al. 2007. Apoptogenic activity of auraptene of *Zanthoxylum schinifolium* toward human acute leukemia Jurkat T cells is associated with ER stress-mediated caspase-8 activation that stimulates mitochondria-dependent or -independent caspase cascade. Carcinogenesis, **28**: 1303–1313. doi:10.1093/carcin/bgm028. PMID:17301064.

Kleiner-Hancock, H.E., Shi, R., Remeika, A., Robbins, D., Prince, M., Gill, J.N., et al. 2010. Effects of ATRA combined with citrus and ginger-derived compounds in human SCC xenografts. BMC Cancer, **10**: 394. doi:10.1186/1471-2407-10-394. PMID:20659317.

Kohno, H., Suzuki, R., Curini, M., Epifano, F., Maltese, F., Gonzales, S.P., and Tanaka, T. 2006. Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice. Int. J. Cancer, **118**: 2936–2942. doi:10.1002/ijc.21719. PMID:16395701.

Kolosnjaj-Tabi, J., and Wilhelm, C. 2017. Magnetic nanoparticles in cancer therapy: how can thermal approaches help? Nanomedicine, **12**: 573–575. doi:10.2217/nnm-2017-0014. PMID:28244818.

Krishnan, P., and Kleiner-Hancock, H. 2012. Effects of auraptene on IGF-1 stimulated cell cycle progression in the human breast cancer cell line, MCF-7. Int. J. Breast Cancer, **2012**: 502092. PMID:23320178.

Krishnan, P., Yan, K.J., Windler, D., Tubbs, J., Grand, R., Li, B.D., et al. 2009. Citrus auraptene suppresses cyclin D1 and significantly delays *N*-methyl nitrosourea induced mammary carcinogenesis in female Sprague–Dawley rats. BMC Cancer, **9**: 259. doi:10.1186/1471-2407-9-259. PMID:19640308.

Lin, Y., Liu, Z., Li, Y., Liao, X., Liao, S., Cen, S., et al. 2013. Short-term hyperthermia promotes the sensitivity of MCF-7 human breast cancer cells to paclitaxel. Biol. Pharm. Bull. **36**: 376–383. doi:10.1248/bpb.b12-00774. PMID:23229357.

Moon, J.Y., Kim, H., and Cho, S.K. 2015. Auraptene, a major compound of supercritical fluid extract of phalsak (*Citrus* Hassaku Hort ex Tanaka), induces apoptosis through the suppression of mTOR pathways in human gastric

cancer SNU-1 cells. Evid.-Based Complement. Altern. Med. **2015**: 402385. doi:10.1155/2015/402385.

Mori, H., Niwa, K., Zheng, Q., Yamada, Y., Sakata, K., and Yoshimi, N. 2001. Cell proliferation in cancer prevention; effects of preventive agents on estrogen-related endometrial carcinogenesis model and on an in vitro model in human colorectal cells. Mutat. Res., Fundam. Mol. Mech. Mutagen. **480–481**: 201–207. doi:10.1016/S0027-5107(01)00200-7. PMID:11506814.

Mousavi, S.H., Davari, A.S., Iranshahi, M., Sabouri-Rad, S., and Tayarani Najaran, Z. 2015. Comparative analysis of the cytotoxic effect of 7-prenyloxycoumarin compounds and herniarin on MCF-7 cell line. Avicenna J. Phytomed. **5**: 520–530. PMID:26693409.

Moussavi, M., Haddad, F., Rassouli, F.B., Iranshahi, M., and Soleymanifard, S. 2017. Synergy between auraptene, ionizing radiation, and anticancer drugs in colon adenocarcinoma cells. Phytother. Res. **31**: 1369–1375. doi:10.1002/ptr.5863. PMID:28675489.

Nabekura, T., Yamaki, T., and Kitagawa, S. 2008. Effects of chemopreventive citrus phytochemicals on human P-glycoprotein and multidrug resistance protein 1. Eur. J. Pharmacol. **600**: 45–49. doi:10.1016/j.ejphar.2008.10.025. PMID:18955043.

Primrose, J.N., Perera, R., Gray, A., Rose, P., Fuller, A., Corkhill, A., et al. 2014. Effect of 3 to 5 years of scheduled CEA and CT follow-up to detect recurrence of colorectal cancer: the FACS randomized clinical trial. J. Am. Med. Assoc. **311**: 263–270. doi:10.1001/jama.2013.285718.

Qi, D., Hu, Y., Li, J., Peng, T., Su, J., He, Y., and Ji, W. 2015. Hyperthermia induces apoptosis of 786-O cells through suppressing ku80 expression. PLoS One, **10**: e0122977. doi:10.1371/journal.pone.0122977. PMID:25902193.

Rashmi, R., Santhosh Kumar, T.R., and Karunagaran, D. 2003. Human colon cancer cells differ in their sensitivity to curcumin-induced apoptosis and heat shock protects them by inhibiting the release of apoptosis-inducing factor and caspases. FEBS Lett. **538**: 19–24. doi:10.1016/S0014-5793(03)00099-1. PMID:12633846.

Saboor-Maleki, S., Rassouli, F.B., Matin, M.M., and Iranshahi, M. 2017. Auraptene attenuates malignant properties of esophageal stem-like cancer cells. Technol. Cancer Res. Treat. **16**: 519–527. doi:10.1177/1533034616650119. PMID:27207438.

Sakata, K., Hara, A., Hirose, Y., Yamada, Y., Kuno, T., Katayama, M., et al. 2004. Dietary supplementation of the citrus antioxidant auraptene inhibits *N,N*-diethylnitrosamine-induced rat hepatocarcinogenesis. Oncology, **66**: 244–252. doi:10.1159/000078001. PMID:15218316.

Schäfer, C., Seeliger, H., Bader, D.C., Assmann, G., Buchner, D., Guo, Y., et al. 2012. Heat shock protein 27 as a prognostic and predictive biomarker in pancreatic ductal adenocarcinoma. J. Cell Mol. Med. **16**: 1776–1791. doi:10.1111/j.1582-4934.2011.01473.x. PMID:22004109.

Siegel, R.L., Miller, K.D., and Jemal, A. 2016. Cancer statistics, 2016. Ca-Cancer J. Clin. **66**: 7–30. doi:10.3322/caac.21332. PMID:26742998.

Tabuchi, Y., Takasaki, I., Wada, S., Zhao, Q.L., Hori, T., Nomura, T., et al. 2008. Genes and genetic networks responsive to mild hyperthermia in human lymphoma U937 cells. Int. J. Hyperthermia, **24**: 613–622. doi:10.1080/02656730802140777. PMID:18608577.

Tanaka, T., de Azevedo, M.B., Durán, N., Alderete, J.B., Epifano, F., Genovese, S., et al. 2010. Colorectal cancer chemoprevention by 2 β-cyclodextrin inclusion compounds of auraptene and 4′-geranyloxyferulic acid. Int. J. Cancer, **126**: 830–840. doi:10.1002/ijc.24833. PMID:19688830.

Tang, M., Ogawa, K., Asamoto, M., Hokaiwado, N., Seeni, A., Suzuki, S., et al. 2007. Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of the prostate (TRAP) and humane prostate carcinoma cells. Cancer Sci. **98**: 471–477. doi:10.1111/j.1349-7006.2007.00417.x. PMID:17284254.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. 2015. Global cancer statistics, 2012. Ca-Cancer J. Clin. **65**: 87–108. doi:10.3322/caac.21262. PMID:25651787.

Wang, H.X., Yang, Y., Guo, H., Hou, D.D., Zheng, S., Hong, Y.X., et al. 2016. HSPB1 deficiency sensitizes melanoma cells to hyperthermia induced cell death. Oncotarget, **7**: 67449–67462. doi:10.18632/oncotarget.11894. PMID:27626679.

Wei, Z.L., Zhao, Q.L., Yu, D.Y., Hassan, M.A., and Kondo, T. 2008. Enhancement of sodium butyrate-induced cell death by hyperthermia in HCT 116 human colorectal cancer cells. Anticancer Res. **28**: 1693–1700. PMID:18630528.

Yan, S.Y., Chen, M.M., Fan, J.G., Wang, Y.Q., Du, Y.Q., Hu, Y., and Xu, L.M. 2014. Therapeutic mechanism of treating SMMC-7721 liver cancer cells with magnetic fluid hyperthermia using Fe$_2$O$_3$ nanoparticles. Braz. J. Med. Biol. Res. **47**: 947–959. doi:10.1590/1414-431X20143808. PMID:25296356.

Zhao, P., Jiang, H., Su, D., Feng, J., Ma, S., and Zhu, X. 2015. Inhibition of cell proliferation by mild hyperthermia at 43 °C with Paris Saponin I in the lung adenocarcinoma cell line PC-9. Mol. Med. Rep. **11**: 327–332. doi:10.3892/mmr.2014.2655. PMID:25322761.