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Potent anti-angiogenic and cytotoxic effect of conferone on human colorectal adenocarcinoma HT-29 cells

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ABSTRACT

Background: Cancer is one of the leading causes of death worldwide, both in developed and developing countries. Of note, colorectal adenoma encompasses a high rate of gastrointestinal-associated cancer death in human being. Today, different strategies, including surgery approaches, photodynamic therapy, radiation and particularly natural compounds have been extensively used to manage tumor behavior in human body.

Methods: The objective of the present study was to elucidate the multilateral effects of conferone on HT-29 cell lines. In addition to cell cytotoxicity, the extent of lipid peroxidation, MDA formation, catalase, superoxide dismutase and intracellular ROS levels, as markers of oxidative stress, were also studied. P-glycoprotein-mediated cellular efflux effectiveness, anti-angiogenic and finally anti-migratory capacities of conferone-exposed HT-29 cells were monitored over a course of 72 h.

Results: It was found that, conferone mediated cell proliferation arrest and induced cell death through both apoptosis and necrosis phenomena. HT-29 cells, exposed to 20 μM conferone, under gone oxidative stress and total content of reactive oxygen species was increased in a time-dependent manner. Intracellular accumulation of rhodamine 123 and cell's swelling under iso- and hypo-osmotic conditions could be related to P-glycoprotein incorrect performance in the presence of conferone. A significant reduction in CD31 positive cells population and *in vitro* tubulogenesis of endothelial cells was also observed after incubation with conditioned medium collected from 72 h conferone-treated HT-29 cells. Conferone also precluded angiogenesis capability of treated HT-29 cells through an altered secretome profile, including vascular endothelial growth factor, Angiopoietin-1 and -2 factors. In addition to anti-angiogenic properties of conferone, a profound decrease in migration capability of HT-29 cells was also evident.

Introduction

At present, colon cancer is considered as a major cause of cancer-associate death in humans and a high rate of resistance to current chemotherapies has been reported as a main problem (Boring et al. 1994). Due to cancer's complex nature, different strategies, including surgery approaches, photodynamic therapy and radiation, have been extensively used while their efficiency varies according to tumor's type, stages and different locations (Seyed et al. 2014). With regard to multiple side effects originated by a variety of current chemotherapeutic drugs and low therapeutic effects of other methods, there are increasing attempts to develop new anti-cancer agents either by the application of natural

Abbreviations: P-gp, P-glycoprotein; VEGF, vascular endothelial growth factor; Ang-1, angiopoietin-1; Ang-2, angiopoietin- 2; EC, endothelial cell; VEGFR-1, - 2, vascular endothelial growth factor receptor-1, -2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered Saline; PI, propidium iodide; MDA, malonialdehyde; SOD, superoxide dismutases; IU, international unite; CAT, catalase; DCFH-DA, dichloro-dihydro-fluorescein diacetate; HBS, HEPES buffered saline solution; HUVECs, human umbilical vein endothelial cells; CM, conditioned media; TMB, 3,3′,5,5′-tetramethylbenzidine.

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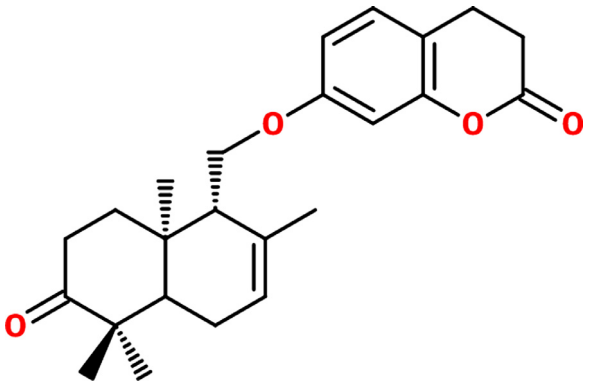

Fig. 1. The chemical structure of conferone.

bioactive or new synthetic compounds (Fauzi et al. 2011; Gottesman 2002). For a long history, it has been accepted that plants, as an appropriate alternative source, could prevent and exert suitable anti-carcinogenic effects for multiple types of cancer (Pan and Ho 2008). The genus *Ferula* from the family *Umbelliferae* (*Apiaceae*), which widely distributed from the Mediterranean region to central Asia, is a rich source of biologically active compounds (El-Razek et al. 2001). For example, various components such as sesquiterpene coumarins, terpene alcohols and other sesquiterpene derivatives such as conferone (Fig. 1) have been previously described (Bukreeva and Pimenov 1991; Iranshahi et al. 2003; Iranshahi et al. 2004). Some authorities acclaimed the inhibitory effects of sesquiterpene coumarins on p-glycoprotein (P-gp) which acts as a trans-membrane efflux pump (Beliveau 2006). P-gp is also seen in a wide variety of normal cells, particularly in secretory tissues (Chaudhary and Roninson 1991; Cordon-Cardo et al. 1989). Interestingly, a growing body of evidence introduced *MDR1* gene product, P-gp, a major cause for cancer treatment failure (Thiebaut et al. 1987).

Today, many possible mechanisms have been proposed to control tumor growth and metastasis. For example, angiogenesis, as a formation of new blood vessels from pre-existing network, is considered as an attractive approach to suppress a vast variety of tumors growth (Dormond and Rüegg 2003). Of note, a dynamic balance of angiogenic stimulators and inhibitors was orchestrated by a vast variety of predisposing factors (Mohammadi et al. 2015). Different growth factors, such as vascular endothelial growth factor (VEGF), Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2), have been identified as pro-angiogenic factors that are secreted by tumor cells to trigger normal endothelial cell (EC) growth through paracrine mechanisms via attachment to their corresponding receptors including vascular endothelial growth factor receptor-1, -2 (VEGFR-1,- 2) and Tie-2, respectively (Ciardiello et al. 2001; Rahbarghazi et al. 2012). Therefore, the many attempts have been conducted to control the signaling pathways of above mentioned ligands by either natural or synthetic compounds.

To our knowledge, there were no reports indicating the prohibitory effect of conferone on gastrointestinal-related tumors. In the current experiment, we scrutinized the inhibitory effect of conferone on cultured human colorectal adenocarcinoma cell line (HT-29 cells) in *in vitro* condition. The effect of conferone on cell cycle dynamic, proliferation rate and free radical production were also determined. In addition, P-gp pump-related cell pathologies under conferone treatment were further investigated.

Material and methods

For more detailed information please see provided supplementary materials (online).

Results

Conferone reduced HT-29 cell viability time dependently via apoptotic pathway

The treatment of HT-29 cells with different concentrations of conferone (10, 20, 30, 40 and 50 μM) at 24, 48 and 72 h of culture period showed a profound decrease in cell viability both dose- and time-dependently using colorimetric MTT assay (Fig. 2A). At the end of 24 h, the effective IC_{50} value found to be at level of 20 μM. Therefore, the 20 μM value was selected as the best effective dose of conferone with cytotoxic effect.

Annexin V/PI staining revealed a distribution of early-, late-apoptotic, and necrotic cells in cells exposed to IC_{50} value of conferone (20 μM) at three time points (Fig. 2B). Corroboration to our results, the rate of late apoptotic and necrotic cells were profoundly increased after 72 h of conferone treatment in which 0.07 and 0.11% early- and late-apoptotic cells of 24 h eventually reached to 5.39 and 32.1% after 72 h, respectively. In addition, the level of necrotic cell clearly shifted from 0.14 to 5.53% 72 h post-treatment.

Dual staining of HT-29 cells by AO/EB elucidated cells underwent both apoptotic and necrotic changes (Figs. 2C, D, E and F) (Kontek et al. 2014). We showed that conferone has potent capability to induce early (bright green dots in their nuclei indicating nuclear fragmentation marginalization in cell membrane) and late apoptotic (orange cytoplasm and nuclei with visible chromatin condensation) as well as necrotic (orange-stained cell nuclei, orange-red cytoplasm) changes through 72 h.

Dynamics of the cell cycle kinetics regulation affected by conferone

The predominant effect of conferone on inhibition of cell proliferation was also detected by Ki-67 expression (Fig. 3A). It was established that conferone attributed to the down-regulation of Ki-67 in treated cells as compared to control groups in which 72 h after treatment the percentage of Ki-67 in control cells scored 98.14% (Fig. 3A). The cell cycling kinetics analysis of malignant intestinal epithelial cells-being exposed to conferone also revealed a truly time-dependent decrease in the growth rate of exponentially growing cells accompanied by a prominent increase in percentage of G_0/G_1-phase cells and mild upward trend in percentage of S-phase cells whereas the cells at G_2/M phase declined through time (Fig. 3B). As the cells exposure time to conferone continued, the percentage of G_2/M phase reached from $6.09 \pm 1.07\%$ to 28.73 ± 8.3 at 72 h (Figs. 3B and C). Concurrent to Annexin V/PI staining result, the rate of apoptotic cells at sub-G_1 showed similar pattern of increase through time (Figs. 2B and C). Interestingly, a marked increase in the percentage of G_0/G_1 phase cells with concomitant decrease rate of cell at G_2/M stage, presumably suggests the inhibitory effect of conferone on HT-29 cell line cell cycle arresting at G_0/G_1 and S.

Conferone stimulated lipid peroxidation and generated intracellular free radicals

Our preliminary study showed a marked increases in amount of MDA level in cells being-exposed to conferone in which the level of MDA reached to 3.06-,3.59- and 1.35-folds compared to time-matched control group after 24, 48 and 72 h, respectively ($p_{24\ \text{control-treatment}} < 0.01$; $p_{48\ \text{control-treatment}} < 0.01$ and $p_{72\ \text{control versus treatment}} < 0.05$) (Fig. 3D). Further, the analysis of antioxidant enzymes such as SOD and CAT showed hyperactivity in a time-dependent manner in treated groups (Fig. 3D). SOD activity showed a significance difference only 72 h after treatment ($p < 0.05$) while CAT activity reached to marked significant levels at all-time points ($p_{(24,\ 48\ \text{and}\ 72)\ \text{control versus treatment}} < 0.01$)