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Comparing toxicity of galbanic acid, auraptene and umbelliprenin on adult T-cell leukaemia-lymphoma in normoxia and hypoxia

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ABSTRACT

Natural coumarins are valuable agents that induce anticancer effects and/or enhance sensitivity to therapeutic modalities. Galbanic acid (GBA), auraptene (AUR) and umbelliprenin (UMB) are coumarins derived from *Ferula* species with various pharmaceutical activities. The aim of the current research was to compare toxic effects of GBA, AUR, and UMB on human lymphoma cells in normoxia and hypoxia. In this regard, GBA and AUR were extracted from the roots of *F. szowitsiana* and UMB was derived from the roots of *F. persica,* all by thin-layer chromatography. MT-2 cells were treated with each agent for 3 consequent periods, while exposed to different O_2 contents (21% and 2%). By the end of each treatment, the viability of MT-2 cells was determined by resazurin dye-based colorimetric assay. Obtained results revealed that low doses of GBA (10 and 20 µM) induced significant ($p < 0.0001$) toxic effects in hypoxia. However, similar toxicity was observed when cells were treated with 40 µM AUR in normoxia and hypoxia. Notably, UMB was the only coumarin that exerted cytotoxic effects in all time points (48, 72 and 96 h) in normoxia and hypoxia, although its concentration was highest (80 µM). In conclusion, this is the first report indicating GBA was the most toxic coumarin against ATL cells in hypoxia, AUR induced similar effects in normoxia and hypoxia, and low toxicity of UMB was stable during the time and different O_2 contents. Future studies on other ATL cell lines are recommended to better evaluate the toxic effects of GBA, AUR and UMB *in vitro*.

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Introduction

Natural coumarins (2H-1-benzopyran-2-one) are valuable agents that have been used as anticancer agents or complementary treatments. Coumarin derivatives were widely extracted from plants belonging to the genus *Ferula* (Apiaceae), which are mainly distributed throughout Central and South-West Asia and the Mediterranean (1). *Ferula szowitsiana* is known as an ethnomedicinal plant with a wide spectrum of pharmacological activities, such as antioxidant, anti-inflammation and antimicrobial effects. *Ferula persica* is also a flowering and perennial herb that has been traditionally used for its laxative and carminative effects.

Sesquiterpene coumarins and sesquiterpene coumarin glycosides are abundant in *Ferula* species. Galbanic acid (GBA), auraptene (AUR) and umbelliprenin (UMB) are among the promising bioactive compounds from *Ferula* species (2). GBA ($C_{24}H_{30}O_5$, Figure 1-A) is a sesquiterpene coumarin with cancer chemopreventive, antiviral, antileishmanial and antibacterial effects (3). AUR ($C_{19}H_{22}O_3$, Figure 1-B) or 7-geranyloxycoumarin is a monoterpene coumarin that possesses antibacterial, antigenotoxic, antioxidative, anti-inflammatory and anticancer properties (4). UMB ($C_{24}H_{30}O_3$, Figure 1-C) or 7-prenyloxycoumarin has immunomodulatory and antioxidant activities, and its anticancer effects have been reported on various cancer cells (5).

Adult T-cell leukaemia-lymphoma (ATL) is a $CD4^+$ T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) (6). HTLV-1 is widespread in southwestern Japan, Africa, South America, the Caribbean Islands, and northeastern Iran, with an estimated 20 million persons affected globally (7). The seroprevalence of HTLV-1 is female-predominant and increases with age (8, 9). Upon a long latency period, 5 percent of infected people acquire ATL (10). ATL is divided into four main subtypes; acute, lymphomatous, chronic, and smoldering. The median survival time for smoldering and chronic types is about two years, while acute and lymphomatous types have a short median survival period, approximately 13 months (11). As ATL cells develop resistance to chemotherapy drugs (12), investigations are being carried out to introduce more effective agents with less unfavorable side effects.

Hypoxia is the deprivation of adequate oxygen and occurs when O_2 pressure is reduced to about 1 kPa, which

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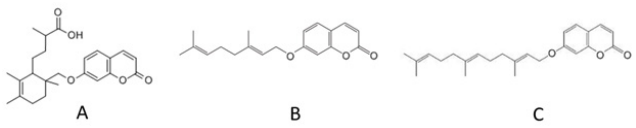

Figure 1. Chemical structure of GBA (A), AUR (B) and UMB (C) (3-5).

is ~7–10 mm Hg or ~1 % O_2. Hypoxia alters the regulation of cellular pathways by reducing reactive oxygen in different types of cells such as T lymphocytes (13). T cells, the revulsive cell type of the immune system, are commonly confronted with hypoxia in both health and disease states (14). For instance, during the development of T cells, thymocytes are present in relatively hypoxic regions in the thymus (15). In lymphoma, oxygen accessibility of cells influences the general appearance and clinical behavior of the disease (16). In this regard, it has been shown that severe oxygen deficiency transforms the hypoxic region into localized necrosis which is common in invasive lymphomas (17). On the other hand, the expression of chemokine CCL28 is induced by tumor-associated hypoxia, which can lead to tumor resistance and neoangiogenesis by inducing interference of regulatory T cells (18).

Besides ATL cells developing chemoresistance, hypoxia induces unpredictable effects on the viability of lymphoma cells. Both phenomena have made it crucial to find novel, preferably natural agents, that induce their toxic effects in different O_2 contents. Hence, the present study aimed to assess and compare the cytotoxicity of GBA, AUR, and UMB on ATL cells in normoxia and hypoxia.

Materials and Methods

Preparation of GBA and AUR

GBA (MW: 398.5 g/mol) and AUR (MW: 298.3 g/mol) were prepared as we previously described (19). In summary, after the roots of *F. szowitsiana* were air-dried and pulverized, the acetone (Merck) extract was prepared by macerating the powder in three changes of the solution for 48 h. Then, the combined solvent extract was vaporized to yield a viscous residual that was then dissociated by column chromatography on silica gel (5×50 cm). To do so, petroleum ether with different volumes of acetone, including petroleum ether (100), petroleum ether–acetone (95: 5), (90: 10), (85: 15), (80: 20), (75: 25), (70: 30), (60: 40), (50: 50) and acetone (100), were used. Finally, obtained fractions were compared by thin layer chromatography (TLC) on silica gel (Merck) using petroleum ether-ethyl acetate as a solvent and further purified on preparative TLC to achieve GBA and AUR.

Preparation of UMB

UMB (MW: 366.5 g/mol) was prepared as we previously reported (20). Briefly, the roots of *Ferula persica* were air-dried, triturated, and extracted with chloroform (Merck) by presoaking for 72 h. The extract was then subjected to preparative TLC on silica gel while petroleum ether-ethyl acetate (2:1) was used as a solvent. After fractions were deterged by chloroform, the pure UMB was identified by conventional spectroscopy.

Cell culture and treatment

In the present study, MT-2 cells were used as a human ATL cell line (Pasteur Institute, Tehran, Iran). Cells were cultured with RPMI 1640 (Biosera) complemented with 10% fetal bovine serum (Gibco) and incubated at 37°C with 5% CO_2 in the air. For subculture, the cell suspension was centrifuged at 125 ×g for 10 minutes, and then, the cell pellet was suspended in a fresh complete medium and divided into more culture flasks (SPL). For treatments, at first stock solutions of GBA, AUR and UB were prepared by dissolving crystals of each agent in dimethyl sulfoxide (DMSO, Merck), and then, final concentrations were obtained by diluting with complete culture medium immediately before each experiment. Afterward, MT-2 cells, at the density of 5×10^4 cell/well (96-well cell culture plates, SPL), were treated with final concentrations and incubated for 48, 72 and 96 h in a CO_2 incubator (Memmert) that provided 21% O_2 (normoxia). For hypoxia, cells were seeded and treated similarly but kept in a triple incubator (Binder) with a gas mixture comprised of 93% N_2, 5% CO_2, and 2% O_2. To note, apart from untreated cells, cells treated with the same volume of DMSO in all concentrations (0.4% v/v) were considered as solvent control.

Viability assessment of cells

Resazurin assay is a rapid and sensitive measurement of the viability of mammalian cells. The base of this colorimetric assay is an irreversible reduction of purple resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) to pink resorufin (7-hydroxy-3H-phenoxazin-3-one) by aerobic respiration of cells with active metabolism (21). For viability assessment, 20 µl resazurin/well (0.1 mg/ ml, Sigma) was used and cells were incubated for 2 h at 37°C. Eventually, the optical density (OD) of wells was measured at 600 nm using a microplate reader (Epoch), and the following formula was used to calculate cell viability (%): $(100-(OD_T-OD_U/OD_B-OD_U))\times100$, in which OD_T, OD_U and OD_B were OD of treated cells, untreated cells and blank control, respectively.

Statistical analysis

Results were statistically analyzed by GraphPad Prism software using a one-way analysis of variance (ANOVA) test. All experiments were carried out at least in triplicates and three times, and results are expressed as mean ± standard deviation (SD). p values less than 0.05, 0.01, 0.001 and 0.0001 are exerted by *, **, *** and ****, respectively.

Results

Upon treatment of cells with GBA, AUR and UMB, results of the resazurin assay demonstrated that each agent induced its toxic effect in a distinct manner. As presented in Figure 2, the viability of MT-2 cells was not significantly changed after treatment with GBA in normoxia. Nevertheless, administration of 10 and 20 µM GBA in hypoxia significantly ($p < 0.0001$) reduced viability after 72 and 96 h.

Figure 3 presents the results of the viability assay after AUR treatment. As shown, 10 and 20 µM AUR had no significant toxicity in normoxia and hypoxia, thus MT-2 cells were further treated with 40 µM AUR at three-time points. Results revealed that the highest concentration of AUR significantly ($p < 0.05$) decreased viability after 72 and 96 h in normoxia and hypoxia.

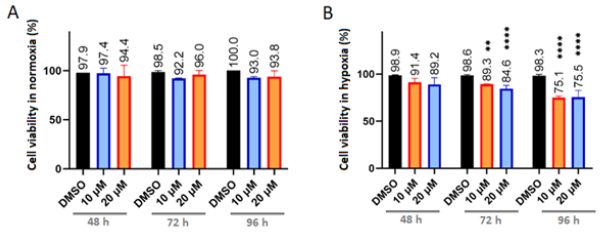

Figure 2. Viability of MT-2 cells upon GBA treatment. Cell viability was assessed after treatment with 10 and 20 µM GBA for 48, 72 and 96 h in normoxia (A) and hypoxia (B). Resazurin assay was carried out at least three times and results are presented as mean ± SD.

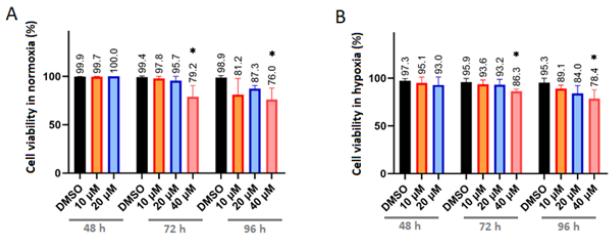

Figure 3. Viability of MT-2 cells upon AUR treatment. Cell viability was assessed after treatment with 10, 20 and 40 µM AUR for 48, 72 and 96 h in normoxia (A) and hypoxia (B). Resazurin assay was carried out at least three times and results are presented as mean ± SD.

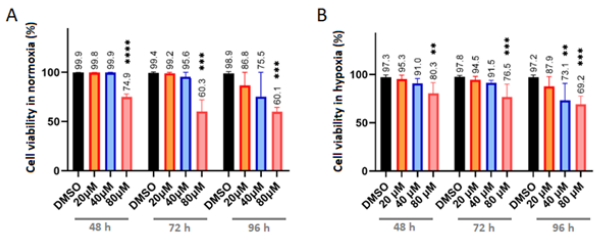

Figure 4. Viability of MT-2 cells upon UMB treatment. Cell viability was assessed after treatment with 20, 40 and 80 µM UMB for 48, 72 and 96 h in normoxia (A) and hypoxia (B). Resazurin assay was carried out at least three times and results are presented as mean ± SD.

Obtained findings demonstrated that UMB had the lowest cytotoxicity among all agents. As shown in Figure 4, the viability of cells did not considerably change upon treatment with 20 and 40 µM UMB in normoxia. Nevertheless, 80 µM UMB induced significant ($p < 0.0001$ and $p < 0.001$) cytotoxicity after 48, 72 and 96 h. Similarly in hypoxia, only 80 µM UMB significantly ($p < 0.01$ and $p < 0.001$) reduced cell viability during three consecutive days.

Discussion

Natural coumarins are a large class of phenolic substances that consist of benzene and α-pyrone rings. Production of these agents has been reported in approximately 150 different species belonging to about 30 different families, such as Rutaceae, Umbelliferae and Apiaceae (22). Coumarins possess valuable pharmacological activities, including anti-inflammatory, antibacterial, antifungal and antiviral effects. In addition, coumarins induce cancer chemopreventive and anticancer effects and also have the potential to improve the efficacy of radiotherapy and chemotherapy (23). GBA, AUR and UMB are valuable coumarin derivatives from *Ferula* species that were studied for their cytotoxic effects against ATL cells in the present study. Since T lymphocytes are commonly faced with hypoxia in a carcinoma state (14), we evaluated the cytotoxicity of these agents in different O_2 contents.

Time- and dose-dependent toxicity of GBA has been reported in human ovarian, lung and prostate carcinoma cells, and upregulation of caspase 9, downregulation of antiapoptotic protein BCL-xL and inhibition of androgen receptor signaling pathway have been introduced as mechanisms of its anticancer action (24-26). In addition, it has been demonstrated that GBA has the potential to improve the accumulation and efficacy of arsenic trioxide in ATL cells (27). The current study is the first report indicating low doses of GBA (10 and 20 µM) induced significant toxicity on ATL cells in hypoxia. Accordingly, GBA could be considered as a potent coumarin to design novel chemotherapeutic regimens for ATL treatment.

Antiproliferative and apoptosis-inducing effects of AUR have been shown on human gastric, colon and renal carcinoma cells (28-30), as well as T-cell leukemia and lymphoma cells (31, 32). It has been suggested that AUR induced its anticancer effects through stimulation of the caspase cascade and suppression of mitochondrial respiration and mTOR pathway (32, 33). In the current study, we reported that AUR induced similar toxicity on ATL cells in normoxia and hypoxia, although it has less cytotoxicity when compared with GBA.

Previous studies indicated that UMB exerts anticancer effects on human leukemia, lymphoma and melanoma cells, and also gastric, breast and lung carcinoma cells through cell cycle arrest and modulation of WNT, NF-κB and TGFβ signaling pathways (34-40). Present results revealed that UMB was the only coumarin that induced toxic effects on ATL cells at all time points in normoxia and hypoxia, although its concentration was higher than that for GBA and AUR.

In conclusion, obtained results demonstrated, for the first time, that GBA, AUR and UMB are potent anticancer agents against ATL cells, although they act in different manners; GBA was the most toxic coumarin in hypoxia, AUR induced similar effects in normoxia and hypoxia, and low toxicity of UMB was stable during the time and different O_2 contents. Nevertheless, future studies on other ATL cell lines are recommended to better evaluate the toxic effects of GBA, AUR and UMB *in vitro*.

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