Cytotoxicity of Vincristine on the 5637 Cell Line Is Enhanced by Combination with Conferone

Vajiheh Neshati[a], Maryam M. Matin[a,b,*], Mehrdad Iranshahi[c], Ahmad R. Bahrami[a,b], Javad Behravan[c], Samaneh Mollazadeh[a], and Fatemeh Behnam Rassouli[a]

[a] Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran. Fax: +98 (0) 51 18 79 64 16. E-mail: Matin@um.ac.ir
[b] Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[c] Department of Pharmacognosy and Biotechnology, Biotechnology Research Center, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

* Author for correspondence and reprint requests

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Bladder cancer is one of the most common cancers worldwide, with the highest incidence in industrialized countries. There are three major histological subtypes of bladder cancer: transitional cell carcinoma (TCC) (>90%), squamous cell carcinoma (<10%) and adenocarcinoma (1–2%). The present study was carried out to assess the effects of conferone, a sesquiterpene coumarin isolated from *Ferula badrakema*, on a TCC subline, 5637 cells. In order to test the effects of conferone, 5637 cells were treated with different concentrations (16, 32, 64, 128 μg/ml) of conferone. The results indicated that conferone did not have any significant cytotoxic effect on these neoplastic cells. To determine the combining effects, the cells were cultured in the presence of different concentrations of conferone (16, 32, 64, 128 μg/ml) and vincristine (30, 40, 50 μg/ml) in combination. The morphological changes were then observed and cytotoxicity effects were studied using the MTT assay 24, 48 and 72 h following drug administration. The cells were more rounded and granulated after treatments with both drugs in comparison to vincristine only. The results of the MTT assay confirmed the morphological observations. After 48 h of combined treatment with 40 μg/ml vincristine and 16 μg/ml conferone, the cytotoxicity of vincristine was increased by 23.6%.

Key words: Conferone, Vincristine, 5637 Cell Line

Introduction

Bladder carcinoma is the most prevalent malignant tumour of the urinary system. In addition to the bladder, other urogenital sites may also be at risk for developing cancer, including the urethra, ureters and renal pelvis. Although surgery and the use of radiotherapy control and cure the majority of cases, nearly 30% of patients would eventually die as a result of the progressive form of the disease. Although progress has been made in identifying anticancer drugs with activity in metastatic bladder cancer, these tumours are relatively resistant to most forms of chemotherapy (Niell *et al.*, 1985). Transitional cell carcinoma (TCC) is the most common type of bladder cancer (Lee *et al.*, 2003). A major problem in the management of TCC is the low sensitivity to chemotherapy and the high recurrence after transurethral resection, which occupies a large proportion (approx. 40%) among bladder cancer patients. Therefore drug resistance is a major and difficult problem which has to be resolved in TCC chemotherapy. Vincristine (Fig. 1) is a cell cycle-specific alkaloid which arrests cells at the metaphase stage. TCC cells show resistance to *Vinca* alkaloids such as vincristine (Yu *et al.*, 1998). Plants are an excellent source of pharmaceuticals, and drugs based on compounds isolated from plants form a major part of the pharmaceutical armamentarium against cancer (Newman *et al.*, 2003). The genus *Ferula* belongs to the family Umbelliferae and comprises 130 species distributed from the Mediterranean region to Central Asia. This genus is a good source of biologically active compounds such as coumarins (Iranshahi *et al.*, 2004a, b), terpene alcohols, and sesquiterpene derivatives (Iranshahi *et al.*, 2003). *Ferula badrakema* (Rechinger *et al.*, 1994), like other species of the genus *Ferula*, is a rich source of sesquiterpene coumarins (Bukreeva

and Pimenov, 1991). Several studies have shown that some drimane-type sesquiterpene coumarins such as conferone, mogoltacin and diportlandian (Fig. 1) can increase the drug accumulation and effectiveness by P-glycoprotein inhibition (Barthomeuf *et al.*, 2006; Behnam Rassouli *et al.*, 2009; Munoz-Martinez *et al.*, 2004).

In the present study we assessed the effects of conferone, a sesquiterpene coumarin, on vincristine cytotoxicity in 5637 cells, which is a TCC subline.

Material and Methods

Conferone preparation

Fruits of *Ferula badrakema* were collected from Hezarmasjed Mountains, northeast of Iran, in August 2005, and identified at Ferdowsi University of Mashhad Herbarium (FUMH), Iran. The air-dried fruits (500 g) were ground to a powder, defatted with petroleum ether and extracted exhaustively by maceration with dichloromethane at room temperature. After filtration, the extract was concentrated under reduced pressure to yield 20 g of a brown residue. Part of the extract (15 g) was subjected to column chromatography on silica gel (5 × 50 cm) using petroleum ether with increasing volumes of acetone (petroleum ether/acetone 20:1, 15:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1 and 0:1). The fractions were compared by TLC (silica gel using petroleum ether/acetone as solvent), and those giving similar spots were combined. Five fractions were finally obtained. Fraction 1 which was named conferone, was selected to identify its effect on anticancer drug activity. Fraction 1 contained 15 mg of conferone; its structure was confirmed by 1D- and 2D-NMR (nuclear magnetic resonance) spectra as well as melting point. These spectroscopic data were in agreement with those previously described in the literature (Abd El-Razek *et al.*, 2003).

Cell culture

5637 cells were obtained from Pasteur Institute (Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Scottland) at 37 °C in a humidified atmosphere containing 90% air and 10% CO_2. In order to subculture 5637 cells, they were washed with phosphate buffered saline (PBS) and incubated with 0.25% trypsin and 1 mM EDTA for 3–5 min. Then detached cells were resuspended in fresh serum-containing medium to inactivate the trypsin and transferred to new labeled flasks.

Preparation of solutions with different concentrations of conferone

In order to prepare solutions with different concentrations of conferone, 2 mg conferone were dissolved in 1 ml dimethyl sulfoxide (DMSO, Merck, Germany), sterilized by 0.2 μm filters (Millipore, Millex-GV) and used as stock solution. Different concentrations (16, 32, 64, 128 μg/ml) were prepared by dilution of different volumes of stock solution in different amounts of culture medium.

MTT-based cytotoxicity assay

The assessment of cell viability was carried out by the MTT assay (Mosmann, 1983) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Deisenhofen, Ger-

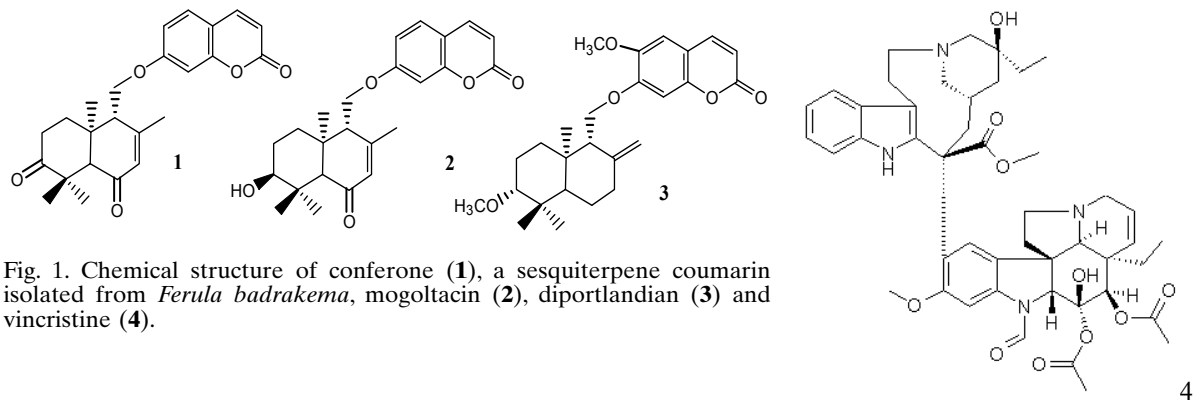

Fig. 1. Chemical structure of conferone (**1**), a sesquiterpene coumarin isolated from *Ferula badrakema*, mogoltacin (**2**), diportlandian (**3**) and vincristine (**4**).

many). This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzymes of viable cells into an insoluble coloured formazan product, which can be determined spectrophotometrically after dissolving in DMSO. Briefly, the cells were cultured in T_{25} and T_{75} flasks. When cells reached 90% confluency, they were removed from the culture dishes by trypsinization and suspended in 10 ml culture medium. Cells were then counted and $8 \cdot 10^3$ exponentially growing cells were seeded per well in 96-well plates. The total volume of each well reached 200 μl by adding culture medium. The cells were left to grow for 24 to 48 h. After this time, first the effects of vincristine and conferone on 5637 cells were assessed separately. Then cells were exposed to combinations of these compounds. The MTT assay was performed after 24, 48, and 72 h of treatment for all stages of the experiment.

Because the solvent of conferone was DMSO, all combined concentrations of DMSO and vincristine, equivalent to conferone and vincristine, were prepared as control groups. To perform the MTT assay, 5 mg/ml fresh and sterilized MTT dye in PBS was prepared, then 20 μl MTT solution were added to each well and the plates were incubated at 37 °C for 4 h. During this period the living cells produced blue, insoluble formazan from the yellow, soluble MTT. The remaining MTT solution was removed and 200 μl of DMSO were added to each well to dissolve the formazan crystals. Absorbance for each well was measured at 570 nm (single wavelength) using an ELISA plate reader. All experiments were performed in triplicate. The percentage of living cells against the control was calculated using the following formula: living cells (%) = (absorbance of treated cells in each well/mean absorbance of control cells) · 100. The dose-response curves were calculated at the above-mentioned concentrations of conferone and vincristine combinations and expressed as the mean percentage fraction of control ± standard error of means (SEM). IC_{50} values were determined by calculating the concentration of the drugs at which 50% of the cells could survive (Durmaz *et al.,* 1999) using Minitab software program.

Morphological alterations

Cells were treated with various concentrations of conferone (16, 32, 64 and 128 μg/ml) and vincristine (30, 40 and 50 μg/ml) in combination and observed under a light-inverted microscope for morphological alterations for 24 to 72 h.

Statistical analysis

Statistical procedures were performed with SPSS, JMP4 and MSTAT softwares. The significant level was ascertained by one way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. Results were expressed as the mean ± SEM. p *V*alue of <0.05 in the Tukey test and <0.001 in the LSD test were considered significant.

Results and Discussion

Effects of conferone on 5637 cells

Treatment of cells with solutions of different concentrations of conferone (16, 32, 64 and 128 μg/ml) showed that these doses had not any cytotoxic effect on 5637 cells.

IC_{50} value of vincristine on 5637 cells

The cells were treated with solutions of different concentrations (25, 50, 100 and 250 μg/ml) of vincristine for 24, 48 and 72 h and cell survival was measured by the MTT assay. The results indicated that the IC_{50} value of vincristine was 49 μg/ml in 5637 cells.

Effects of vincristine and conferone combination on 5637 cells

For the determination of the effect of conferone on the cytotoxicity of vincristine, 24 combinations of different concentrations of conferone (16, 32, 64 and 128 μg/ml) and vincristine (30, 40 and 50 μg/ml), close to its IC_{50} value, were used. Evaluation of the viability of the cells during vincristine + conferone treatment and comparison with that of the cells during vincristine + DMSO treatment (control) showed that the viability of the cells was greatly decreased during vincristine + conferone treatment. By using one way ANOVA and Tukey test, it was shown that 24 and 48 h after drug administration, there were significant differences in the cell viabilities between most of the conferone + vincristine combinations and their DMSO equivalents.

After calculating the increase percentage of vincristine cytotoxicity caused by different concentrations of conferone, final statistical analysis (LSD test) showed that there was a significant

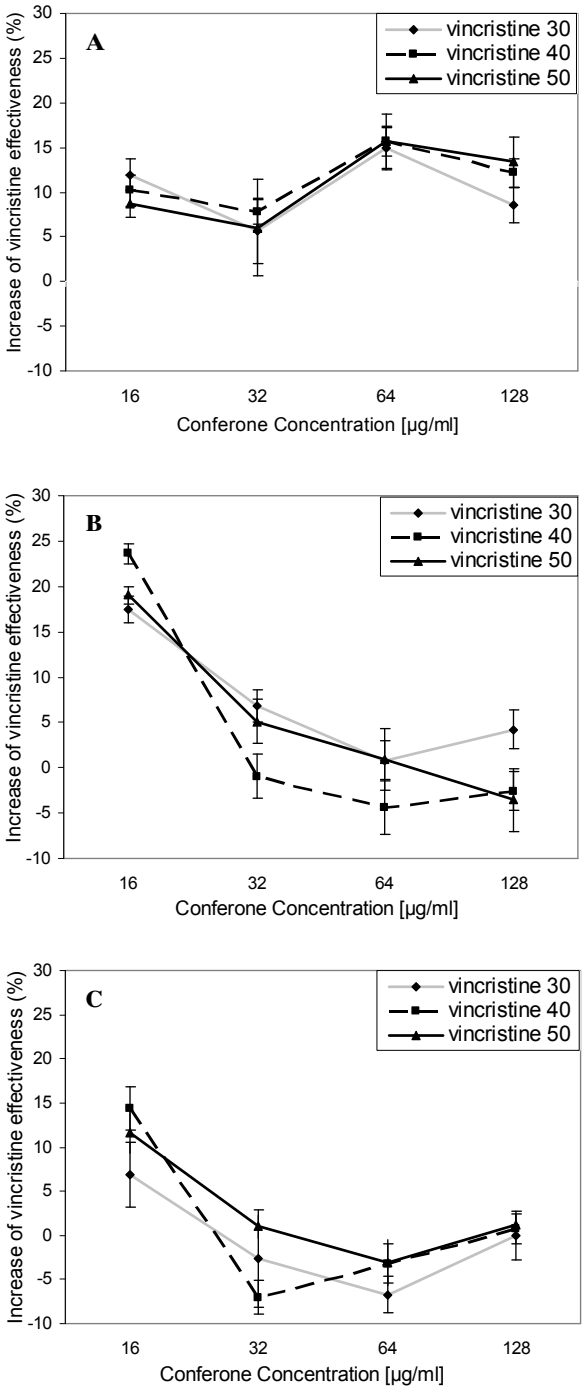

Fig. 2. Dose-response curve of different concentrations of conferone and vincristine: (A) 24, (B) 48, and (C) 72 h after drug administration.

difference between the effect of 16 μg/ml conferone on the cytotoxicity of 40 μg/ml vincristine after 48 h (23.6%) and that of other combinations of concentrations during three consecutive days (Fig. 2).

Morphological alterations

The effects of 24 different combinations, including four conferone concentrations (16, 32, 64 and 128 μg/ml) and four DMSO contents (0.8%, 1.6%, 3.2% and 6.4% DMSO) with three vincristine concentrations (30, 40 and 50 μg/ml), were assessed over three consecutive days. The most obvious morphological changes were observed with the combination of 16 μg/ml of conferone and 30 μg/ml of vincristine after 24 h. The morphology of the cells was changed to spherical forms with granulated cytoplasm as seen in Fig. 3. At higher concentrations of conferone and vincristine, the cytotoxic effects were not more prominent.

Development of drug resistance in tumour cells represents a significant barrier to successful chemotherapy. This cellular resistance is known as multidrug resistance (MDR) (Zhai *et al.*, 2006). Overexpression of P-glycoprotein (P-gp) is the main mechanism of MDR (Wu *et al.*, 2007), which is associated with the development of vincristine resistance (Ozgen *et al.*, 2000). In 1981, it was discovered that drug resistance could be reversed by the addition of P-gp inhibitors such as verapamil which has a widely clinical use (Tsuruo *et al.*, 1981). It was also demonstrated that cnidiadin, a furanocoumarin, inhibited the P-gp transport and reversed the resistance of multi-resistant cells overexpressing P-gp to *Vinca* alkaloids (Miski and Ulubelen, 1985). It has also been shown that TCC cells are resistant to *Vinca* alkaloids (Yu *et al.*, 1998) by *MDR1* gene overexpression (Wu *et al.*, 2006).

In the present study the effects of conferone, a sesquiterpene coumarin from *Ferula badrakema*, on the cytotoxicity of vincristine was investigated in 5637 cells, and it was shown that conferone enhances the cytotoxicity of vincristine. The most significant changes in morphology and cell viability occurred 48 h after drug administration, and in this period, 16 μg/ml of conferone increased the cytotoxicity of vincristine by 23.6%. Increasing the concentration of conferone to 32, 64, and 128 μg/ml did not increase the cytotoxicity of vincristine any more. This is probably caused by

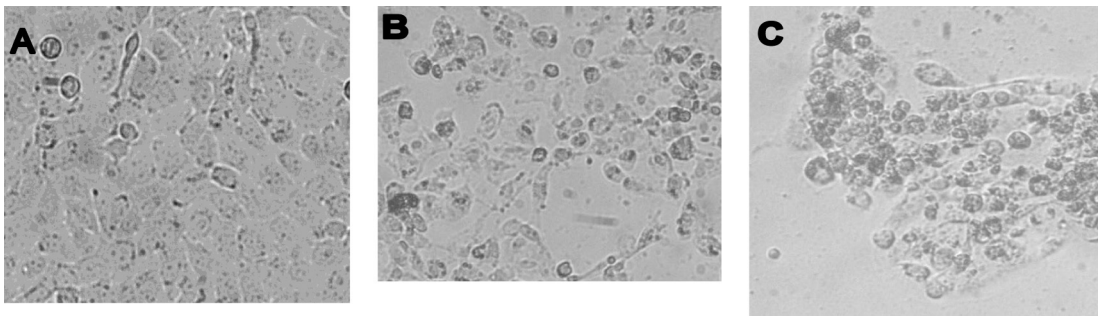

Fig. 3. 5637 cells cultured for 48 h: (A) without any treatment, (B) treated with 0.8% DMSO and 40 μg/ml vincristine, and (C) treated with 16 μg/ml conferone and 40 μg/ml vincristine (×40).

the increase of the DMSO concentration which has prevented the conferone effects observed. Since the single use of conferone at 16, 32, 64 and 128 μg/ml did not have any toxic effects on 5637 cells, conferone is a compound effective at nontoxic concentrations. These results can be explained by the fact that conferone can probably block P-gps by attachment to these pumps, which results in accumulation of vincristine inside the cells. It has been shown that conferone, isolated from *Ferula schtschurowskiana,* enhances the cytotoxicity of vinblastine by competitively blocking the P-gp transport, which was shown by decreasing the P-gp photolabeling efficiently. Conferone, the first identified natural sesquiterpene coumarin from *Ferula*, is a promising drug candidate for reversion of MDR encoded by the *MDR1* gene (Barthomeuf *et al.*, 2006). The capacity of conferone to bind P-gp with high affinity is in agreement with the observation that a binding site for sesquiterpenes exists within the transmembrane domain of P-gp (Munoz-Martinez *et al.*, 2004). In summary, as the main cause of failure of cancer therapy is related to inherent or acquired overexpression of efflux pumps by tumour cells, the potency of sesquiterpenes, as blockers of P-gp-mediated drug transport activity, would make these components suitable for entry into clinical studies.

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