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Original Article

Cytotoxic effects of some 1-[(benzofuran-2-yl)-phenylmethyl]-imidazoles on MCF-7 and Hela cell lines

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Abstract

Aromatase inhibitors have been used as a second line therapy after tamoxifen, in estrogen-receptor positive (ER+) women to reduce estrogen concentration in plasma and breast tumors. It has been shown that benzofuran(phenylmethyl)imidazoles are potent aromatase inhibitors at nano-molar concentrations, about 1000 times more potent than aminoglutetimide. In this study, cytotoxic activity of some of these potent aromatase inhibitors (compounds 1-6) on MCF-7 and Hela cell lines were evaluated. MCF-7 and Hela cells were cultured on RPMI medium and the cells obtained after the third generation were used in this study. The cytotoxic activity of the compounds was first screened at 20 and 100 μM. The IC_{50} values were then determined, using MTT method. Briefly, after 24 h incubation of the cells, the compounds, doxorubicine and the medium were added to the cells and incubated for further 48 h (37 ^{o}C, 5% CO_2). The MTT solution was then added and the absorbance was measured by ELISA plate reader at 540 nm after 4 h incubation. The results indicated that the IC_{50} values for compounds 1-6 were around 35-80 μM for MCF-7 and 47-85 μM for Hela cells. Compound 5, the 4-chloro derivative, showed almost the highest toxicity on both cell lines. i.e. IC_{50}s were 35 μM and 55 μM for MCF-7 and Hela cells, respectively. The cytotoxic activity could be in part due to the aromatase inhibition but other mechanisms like DNA degradation may also be involved.

Keywords: Cytotoxicity; Benzofuran(phenylmethyl)imidazoles; Aromatase inhibitor; MCF-7; Hela

INTRODUCTION

Aromatase is responsible for the formation of estrogens from their androgen precursors (1). It has been shown that the aromatization of androgens is more pronounced in malignant than normal breast tissues (2) and a locally increased estrogen production may stimulate proliferation of estrogen responsive breast cancer cells. Aromatase inhibitors have been used as second line therapy after tamoxifen in postmenopausal estrogen-receptor positive (ER+) women to reduce plasma and breast levels of estrogens and therefore suppress the stimulus on growth of estrogen-dependent metastases (3,4).

1-[(Benzofuran-2-yl)-phenylmethyl]-imidazoles have been introduced as potential antimycotic agents (5,6). It was then shown that these imidazole derivatives were potent inhibitors of placental microsomal aromatase, being about 1000 times more potent than aminoglutethimide (7). We have found that the resolved enantiomers of two of these compounds unexpectedly resulted in reduced stereoselectivity in that both

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enantiomers of the 4-chloro derivative (compound 5, R_1 = 4-Cl, R_2 = H) had identical potencies (IC_{50} = 8.4 nM) and the (+) and (-) forms of the 4-fluoro derivative (compound 4, R_1 = 4-F, R_2 = H) had IC_{50} values of 5.3 and 65 nm, respectively (8). The finding was then confirmed by docking of the enantiomers into the active site model of aromatase (9), both of which well accommodated into the model (8). However, replacement the phenyl ring with aliphatic substituents caused about 100 times reduction in aromatase inhibitory activity (10).

The combination therapy is well established for breast cancer treatment in which tumors are attacked by different drugs with diverse mechanisms. For instance, the combination of letrozole, a non-steroidal aromatase inhibitor with fulvestrant an antiestrogen was clearly more effective than either agent when used alone (11). The effect of paclitaxel on cell growth of human tumor cells expressing aromatase was enhanced by co-administration with exemestane, seemingly due to the reduction of the paclitaxel dose required, which resulted in reduced toxicity (12). Therefore, if an aromatse inhibitor could also act as a cytotoxic agent, this may potenciate its activity as an anticancer agent.

In this study, cytotoxicity of 1-[(benzofuran-2-yl)-phenylmethyl]imidazoles (Fig. 1) on MCF-7 as aromatase positive (13) and Hela as aromatase negative cell lines (14) were investigated to find whether these aromatase inhibitors are able to reduce the viability of these cells. A similar cytotoxicity on both cell lines may indicate some other mechanisms involved for their activities rather than aromatase inhibition.

MATERIALS AND METHODS

Materials

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), tripan blue and dimethylsulfoxide (DMSO) were purchased from Merck, Germany. Roswell Park Memorial Institute (RPMI)-1640 culture medium, foetal bovine/calve serum (FBS), or FCS, sodium pyrovate, penicillin/streptomycin and trypsin-EDTA were purchased from Gibco, Scotland. The absorbance was measured with an ELISA plate reader (Starfinn 2100, USA). Doxorubicine, as positive control, was purchased from Farmitalia. MCF-7 (sterogen-dependent human breast cancer) (15) and Hela (16) (human, black, cervix, carcinoma, epithelioid) cells were purchased from Pasteure Institute, Tehran, Iran. 1-[(Benzofuran-2-yl)-phenylmethyl] imidazoles (Fig. 1) was prepared previously (17) according to the method of Pestillini et al. (6).

Sample and culture medium preparations

Both cell lines were grown in RPMI 1640 medium. Each 500 ml of the medium consisted of 5.2 g RPMI powder, 1 g of sodium bicarbonate, 5 ml of L-glutamine (2 mM), 5 ml of sodium pyruvate (1 mM), 2.5 ml of penicillin (10000 IU/ml) and 2.5 ml of streptomycin (10 mg/ml) supplemented with 50 ml heat-inactivated fetal calf serum (FCS) in deionised water (18). Completed medium was sterilized by filtering through 0.22 µm microbiological filters. The pH of the medium was then adjusted between 7.3 and 7.6 using concentrated HCl or NaOH and kept at 4 °C before use. Cell lines were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

The stock solutions of compounds 1-6 (200- 1000 µM) and doxorubicin, (200 µg/ml or 368 µM) were prepared by dissolving the compounds in the minimum volume of DMSO and phosphate buffered saline (PBS) was then added to reach to the appropriate volume. 20 µl of each stock solution was added to the microplate containing 180 µl of the cell suspensions. The final concentrations of the compounds

1-6 and doxorubicin were 20-100 µM and 36.8 µM (20 µg/ml), respectively.

In-vitro cytotoxicity assay

The cytotoxic effects of compounds 1-6 against the MCF-7 and Hela cells were determined by a rapid colorimetric assay using MTT. The results were compared with untreated control (19). In this assay, mitochondrial succinic dehydrogenase enzyme of live cells would metabolically reduce the yellow soluble MTT salt into a blue insoluble formazan product. The blue solid could be dissolved in DMSO and measured spectrophotometrically (20).

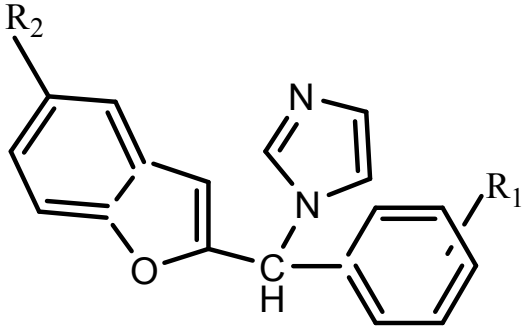
The cytotoxic activity of the compounds was first screened at 20 and 100 µM. Briefly, after 2-3 subcultures, 180 µl of the cells (5×10^4 cells/ ml of media) was seeded in 96 well microplates and incubated for 24 h (37 °C, air humidified 5% CO_2). 20 µl of different concentrations of compounds 1-6 were then added and the microplates were further incubated for 48 h in the same condition. Doxorubicin was used as a positive control and the first column of the microplate containing 180 µl of the cell suspension and 20 µl RPMI was regarded as negative control. The blank wells were consisted of 200 µl of the RPMI medium. To evaluate cell survival, each well was then incubated with 20 µl of MTT solution (5 mg/ml in PBS) for 3 h. Afterwards, the media in each well was gently replaced with 200 µl DMSO and pipetted up and down to dissolve the formazan crystals. The absorbance of each well was measured at 540 nm using an ELISA plate reader. Each experiment was carried out in triplicate and repeated three times.

Standard curves (absorbance against number of cells) for each cell line were constructed and used for the calculation of percent cell survival. In the negative control, percent cell survival was taken as 100%. The percentage of cell viability was calculated using the following formula:

$$\%\ \text{Cell Survival} = \frac{(\text{Mean absorbance of the test compound} - \text{Mean absorbance of the blank})}{(\text{Mean absorbance of the negative control} - \text{Mean absorbance of the blank})} \times 100$$

Table 1. Comparison of the aromatase inhibitory activity (data adopted from reference 7) and cytotoxicity of compounds 1-6 on MCF-7 and Hela cells.

Compound	Aromatase inhibition (7) IC_{50} nM	Effects on MCF-7 IC_{50} µM	Effects on Hela IC_{50} µM
1	43.2	80	80
2	7.9	61	65
3	19.7	50	85
4	7.3	39	74
5	7.7	35	55
6	73.5	55	47



1 R_1 = H, R_2 = H
2 R_1 = 2-CH_3, R_2 = H
3 R_1 = 2-OCH_3, R_2 = H
4 R_1 = 4-F, R_2 = H
5 R_1 = 4-Cl, R_2 = H
6 R_1 = H, R_2 = Br

Fig. 1. Chemical structure of 1-[(benzofuran-2-yl)-phenylmethyl]imidazols.

Statistical Analysis

The results are the mean of three triplicate experiments. SIGMASTAT® (Systat Software Inc. USA) was used for statistical analysis. Analysis of variance (ANOVA) followed by Tukey test was used to see the differences amongst various groups. The significance level was set at $P<0.05$.

RESULTS

The results indicated that the test compounds could reduce the viability of both cell lines at 20 µM to only about 80%. We consider compounds as cytotoxic when they reduce the viability of cells to less than 50%. By assuming this, all compounds were cytotoxic to both cell lines at 100 µM concentrations and viability was reduced to less than 10%. The cytotoxic activity of different concentrations of compounds 1-3 and 4-6 are shown in Fig. 2 and Fig. 3, respectively. The IC_{50} values are around 35-80 µM for MCF-7 and 47-85 µM for Hela cells. Compound 5, the 4-chloro derivative showed almost the highest cytotoxicity on both cell lines. i.e. IC_{50} were 35 µM and 55 µM for MCF-7 and Hela cells, respectively. A comparison of the IC_{50} values for their cytotoxic effects and their placental aromatase inhibitory activity are shown in Table 1.

DISCUSSION

1-[(Benzofuran-2-yl)-phenylmethyl]-imidazoles are highly potent aromatase inhibitors being about 1000 times more potent than aminoglutetimide. In the present study, cytotoxicity of the compounds 1-6 was first determined at 20 µM and 100 µM on MCF-7 "an aromatase positive" and Hela "an aromatase negative" cell lines. The cytotoxicity of different concentrations (between 20-100 µM) of these compounds was subsequently tested against both cell lines to estimate

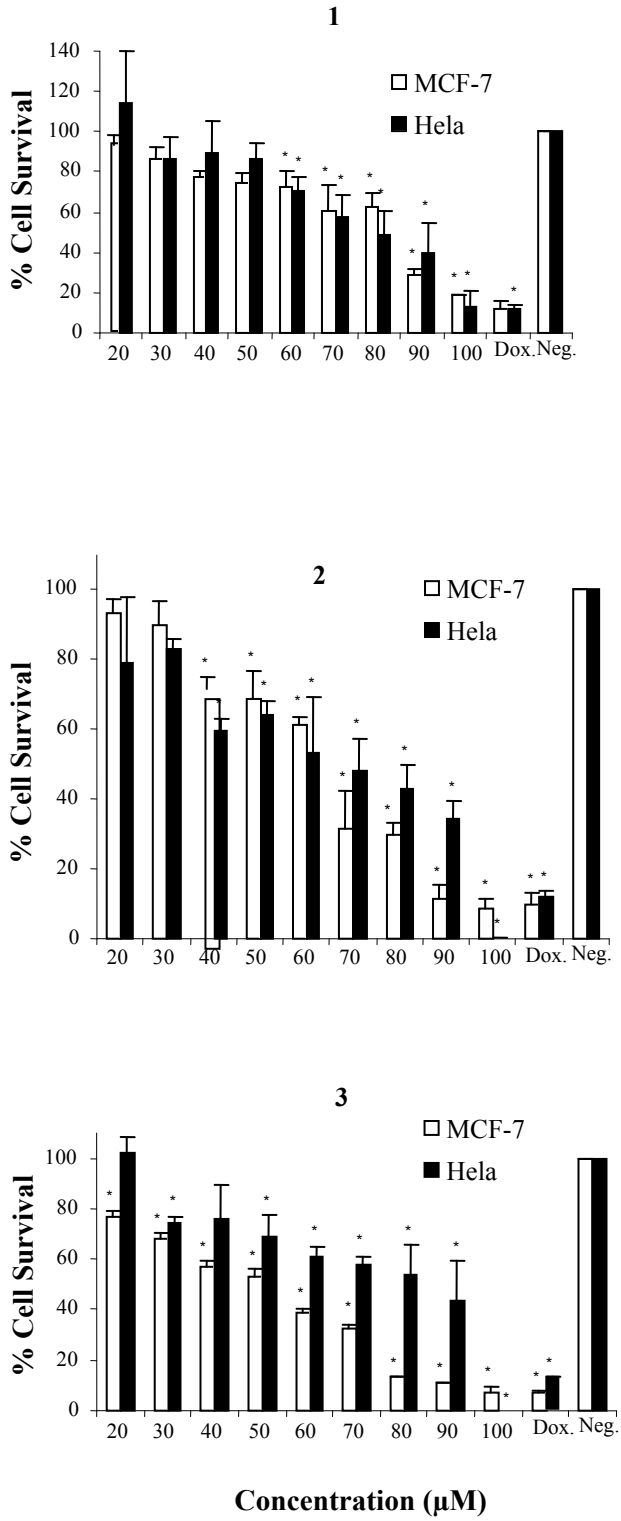


Fig. 2. Cytotoxic effects of compounds 1-3 on MCF-7 and Hela cells. Following exposure to different concentrations of compounds 1-3, cell viability was assessed using the MTT method. Data are presented as mean ± SD, *$P<0.05$, n = 9, Dox. = Doxorubicine 36.8 µM, Neg. = Negative control.

their IC_{50} values. Although these results are mostly in agreement with their aromatase inhibitory activity, the IC_{50} values of their cytotoxic effects are about 1000 times higher than their placental aromatase inhibitory activity as shown in Table 1. On the other hand, remarkable difference was not observed between the cytotoxicity of these compounds on MCF-7, the aromatase positive cells, and Hela, the aromatase negative cells. However, it seems that compounds 1-6 are slightly more cytotoxic on MCF-7 than Hela cells.

Auvray et al. demonstrated that MR 20492 and MR 20494, two indolizinone derivatives, and fadrozole (CGS16949A) were strongly inhibited the human aromatase, however, neither were cytotoxic to Hela cells at a concentration of 10 µM (21).

On the other hand, Yano et al studied the cytotoxicity of fadrozole on MCF-7 cells. Their results indicated that testosterone-induced growth of MCF-7 was inhibited in a dose dependent manner and a complete growth inhibition was observed at fadrozole concentrations of as low as 10 nM, which is corresponding to the concentration required for inhibition of human aromatase in placental microsoms; responsible for the production of estrogens from testosterone or androstendione precursors (22), while, as it is expected, cell growth stimulated by estradiol was not affected by fadrozole at the concentrations of less than 10 µM. Their results were lead to the suggestion that the inhibition of intracellular estrogen production participates in the anti-tumor activity of fadrozole on breast cancer cells.

Recently some 1-[benzofuran-2-yl-(4-alkyl/aryl-phenyl)methyl]-1H-triazoles, the close analogues of compounds 1-6, were prepared by Pautus et al. and their CYP26A1 inhibitory activity were evaluated using a MCF-7 cell-based assay. The IC_{50} values for these triazole analogues were reported to be 7-100 µM using MCF-7 assays (23). One of these

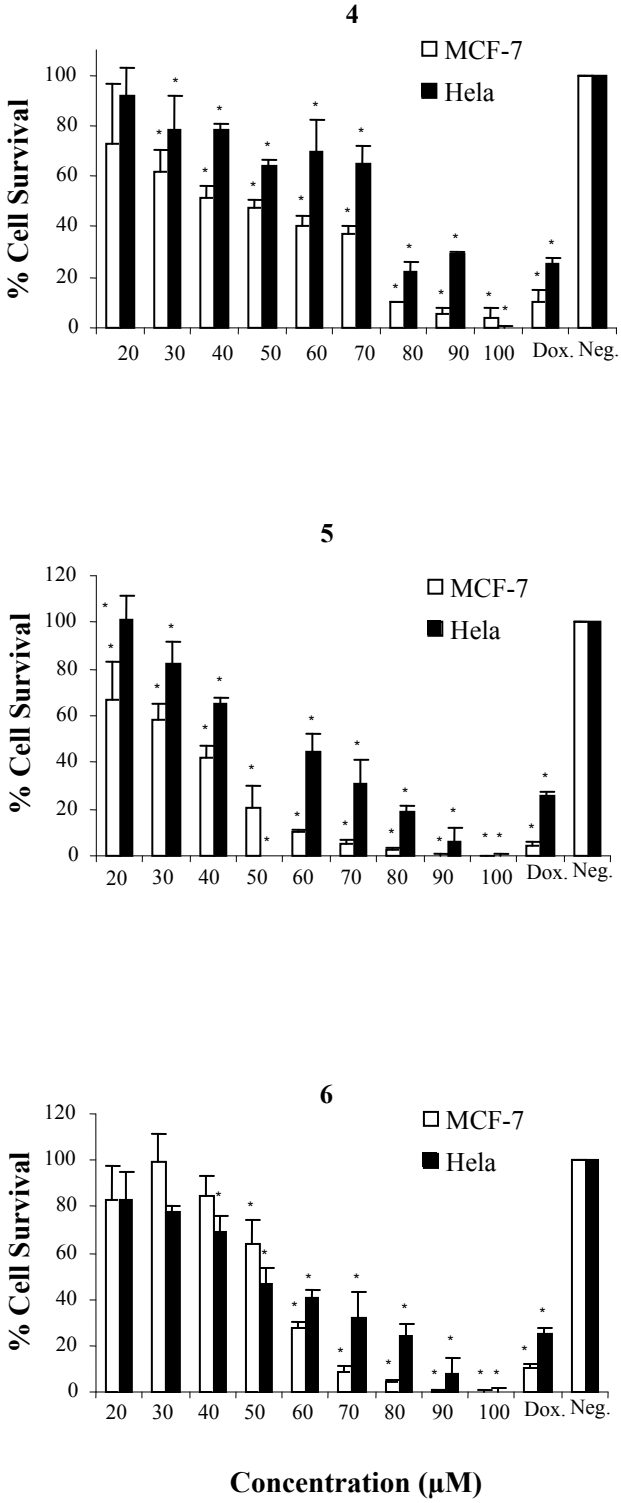


Fig. 3. Cytotoxic effects of compounds 4-6 on MCF-7 and Hela cells. Following exposure to different concentrations of compounds 4-6**,** cell viability was assessed using the MTT method. Data are presented as mean ± SD, **P*<0.05, n = 9, Dox. = Doxorubicine 36.8 µM, Neg.= Negative control.

compounds was also evaluated as an aromatase inhibitor. The IC_{50} values for aromatase inhibitory activity and MCF-7 based assay were 590 nM and >40 μM, respectively (24).

In conclusion, although compounds 1-6 are highly potent inhibitors of human aromatase in placental microsoms, it is still unclear that these compounds can also inhibit the aromatase activity in the breast cancer cell cultures at the same extent and/or act as cytotoxic agent with elaborating other mechanisms. On the other hand, the moderate cytotoxicity of these compounds on both MCF-7 and Hela cell lines may lead to this assumption that their cytotoxicity can be at least in part due to the aromatase inhibition, though other mechanisms like DNA degradation may also responsible. A genotoxicity study is currently underway to investigate the possible mechanisms of the cytotoxicity of these compounds.

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