Original Article

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Cytotoxic Evaluation of some new and Potent Azole Derivatives as Antimicrobial Agents

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Abstract

In recent years use of antifungal drugs in human medicine has increased, especially with the advent of AIDS epidemy. Despite the growing list of azoles, their clinical value has been limited by their relatively high risk of toxicity and the emergence of drug resistance. Efforts have focused on the development of new, less toxic and more efficacious antifungal agents. We previously described synthesis of some new azole derivatives. We also evaluated all the synthesized compounds for their antifungal activity. Most of our compounds showed desirable activity against different species of microorganisms. Here we choose thirteen of these compounds, 5 benzotriazole derivatives (**1a-5a**), 5 imidazole derivatives (**1b-5b**) and 3 triazole derivatives (**1c-3c**) to evaluate their cytotoxic activities against a human cancer cell line (MCF-7) using colorimetric MTT cytotoxic assay. Their cytotoxic activities were compared to clotrimazole as a positive control. Our results collectively showed that most of our synthesized compounds had less cytotoxicity against MCF-7 compared to clotrimazole.

Keywords: Azole, Antifungal, MTT assay, MCF-7 cell line.

1. Introduction

Through the 1980s azole compounds have been introduced as orally active compounds in medicine to conflict fungal diseases. They represent a successful strategy for the development of antifungal drugs with wide spectrum activity (1). Azole antifungal agents, exhibit a good profile of tolerance in the dose ranges recommended in invasive candidiasis (2). Ergosterol biosynthesis pathway is the target of azole derivatives (3). Azoles inhibit 14α-sterol demethylase, the key enzyme in sterol biosynthesis causing depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols. Since ergosterol maintains the integrity of cell membrane, preventing its production would lead to membrane damage, change in its permeability, and hence growth inhibition of fungal cells (2). Although various azole antifungals are currently available as therapeutic agents, due to their related hepatotoxicity, the discovery of new active drugs in infectious processes has been of great importance (4). Each azole antifungal has relative potential to induce acute liver injury, in more serious cases, hepatic dysfunction or acute liver failure (5). However, these antifungal agents still have other problems, such as drug resistance and low bioavailability (6). This situation has put forth an urgent need to develop more effective antifungal agents with novel chemical structures which are

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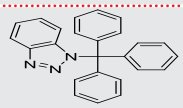
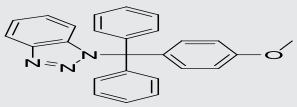
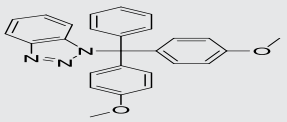
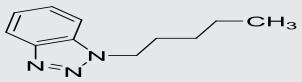
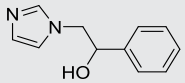
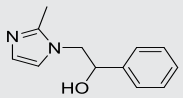
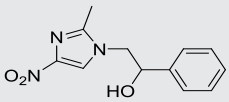
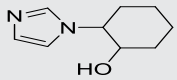
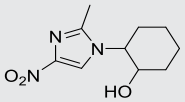
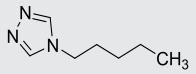
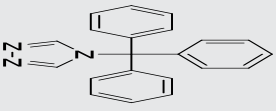
helpful for overcoming drug-resistance and improving antifungal potency (7). In this regard we synthetized a variety of new compounds of imidazole, triazole and benzotriazole derivatives via a structural modification on available azole drugs introduced in our previous work (8-10). As most of our new compounds showed desirable antifungal activity here we are going to evaluate their toxicity in comparison to a standard drug.

2. Material and methods

All chemicals and solvents were purchased from Merck (Germany). Clotrimazole powder was purchased from Darou Pakhsh Pharma Chem Co., Iran. Newly synthesized compounds were obtained from Department of Medicinal Chemistry, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran (Table 1). The purity of the compounds was checked by TLC, their melting point and IR spectroscopy.

Table 1. Chemical name, structure and other properties of the tested compounds.

Entry	Chemical Name	Chemical Structure	Code	M.p. (˚C)	Log p
1	1-trityl-1H-1,2,3-benzotriazole		**1a**	215	6.51
2	1-[(4-methoxyphenyl) (diphenyl) methyl]-1H-1,2,3-benzotriazole		**2a**	220	6.38
3	1-[bis(4-methoxyphenyl) (phenyl) methyl]-1-1H-1,2,3- benzotriazole		**3a**	222	6.26
4	1-pentyl-1H-1,2,3-benzotriazole,		**4a**	271.5	3.11
5	l-octyl-1H-1,2,3-benzotriazole		**5a**	309.7	4.36
6	2-(1H-1-imidazolyl)-1-phenyl-1-ethanol		**1b**	145	0.77
7	2-(2-methyl-1H-1-imidazolyl)-1-phenyl-1-ethanol		**2b**	113	1.44
8	2-(2-methyl-4-nitro-1H-1-imidazolyl)-1-phenyl-1-ethanol		**3b**	138	ND*
9	2-(1H-1-imidazolyl)-1-cyclohexanol		**4b**	174	0.16
10	2-(2-methyl-4-nitro-1H-1-imidazolyl)-1-cyclohexanol		**5b**	124	ND*
11	4-pentyl-4H-1,2,4-triazole		**1c**	Liquid	0.98
12	4-octyl-4H-1,2,4-triazole.		**2c**	Liquid	2.24
13	1-trityl-1H-1,2,3-triazole		**3c**	204	4.38

*Not Defined.

2.1. Cell line and cell culture

A human cancer cell line, MCF-7 (breast carcinoma) was purchased from the national cell bank of Pasteur Institute of Iran (Tehran, Iran). Using aseptic techniques, the cells were cultured in sterile T25 flasks in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera, UK), containing 10% Fetal Bovine Serum (FBS) (Biosera, UK), 100 units/ml penicillin and 100 µg/ml streptomycin (Biosera, UK), and incubated at 37 °C in a humidified atmosphere and 5% CO_2. When the cells covered approximately 80% of the flask surface, they were sub-cultured. To sub-culture, the media was first poured off from the flask and the cells were washed with 1× PBS to remove the residual culture media and were then treated with 1ml 25% trypsin-EDTA (Biosera, UK). To inactivate the trypsin, as soon as the cells detached, a culture media containing 10% FBS was added to the flask. The cells were then washed two times with complete medium for 10 min at 300 g and were then counted and prepared for MTT assay.

2.2. Cell counting

The cells were gently mixed to ensure that they were well distributed. A 10 µl cell suspension was taken out and mixed well with 10 µl of Trypan Blue solution. 10 µl of this cell suspension was then applied to the hemocytometer. Using a microscope, the live unstained cells were counted (Trypan Blue can incorporate into dead cells due their damaged membrane). The final number of viable cells per 1 milliliter was measured using the following formula:

Number of viable cells=Counted unstained cells×10000×2(as dilution factor)×Total volume of cell suspension.

2.3. Determination of optimal cell number for MTT assay

To determine the optimal cell number for MTT assay, cells were trypsinized and released from the culture flask and counted as explained above. The cells were then re-suspended at 5×10^6 cells/ml in complete culture medium and serially diluted from 50×10^3 to 1×10^3 cells/ml. Different concentrations of the cells were then plated at 100 µl per well in sextuplicate, in 96-well cell culture microplate. The plate was also included 3 control wells containing only cell culture medium (the blanks). The cells were then incubated for 48 hours. After incubation, 10 µl MTT reagent (0.5 mg/ml; Sigma, Germany) was added to each well and the plate was incubated for more than 3 hours at 37 °C. Using an inverted microscope, the cells were periodically checked for the appearance of clearly visible purple precipitate. 100 µl of DMSO was added to all wells, including controls. The plate was covered and left in the dark at room temperature for 1 hour and then the absorbance of all wells including the blanks, were measured at 570 nm. The average values from sextuplicate readings were determined and subtracted from the average value for the blank (blanks should have OD values of 0 to 0.1). The absorbance plot was depicted versus cell number/ml. The optimal cell number is the number that falls within the linear portion of the curve and has an absorbance between 0.75 and 1.25. For MCF7 cell line, the optimal cell number was obtained approximately 10×10^3 cells.

2.4. MTT assay for the tested compounds

The cells were plated at the 10×10^3 concentration in 100 µl per well in a sextuplicate manner for each compound. Three wells for cell-based negative controls (without treatment), and three wells of cell culture medium alone (as blanks) was also included. The cells were incubated for 24 hours to recover and reattach and were then treated with different concentrations of each chemical (1-2000 µM). After 48 hours, the culture medium was completely removed and 100µl of MTT reagent (0.5 mg/ml) was added to each well including controls. Different concentrations of clotrimazole (1-100 µM), were also used as positive control. Each experiment was separately repeated three times.

2.5. Data analysis

Excel 2013 was used for calculation. For each compound an Inhibition Concentration 50 (IC_{50}), indicating the 50% growth inhibition of the cells, was calculated and reported using CurveExpert 1.5 software. One-way ANOVA was then used to compare the IC_{50} of each compound with IC_{50}

of clotrimazole

3. Resuls

In the present study, we determined the cytotoxic effects of a variety of synthetic compounds of benzotriazole, imidazole and triazole using colorimetric MTT cytotoxic assay. The IC_{50} of each compound as well as clotrimazole, as the standard drug, are summarized in Table 2. As shown in this table, tritylbenzotriazoles derivatives (**1a-3a**) displayed less cytotoxicity. In this series, compound **3a** with two methoxy groups had more effect than **2a** with just one methoxy group; both of them had more activities than **1a** which does not have any methoxy group in its structure. This could imply that the methoxy group enhances the cytotoxic effect of trirtylbenzotriazole compounds. But in the case of alkyl benzotriazole derivatives, both compounds (**4a-5a**) had IC_{50} less than 50 µM, however, compound **4a** (IC_{50}=47.75) showed a little more cytotoxic effect than **5a** (IC_{50}=49.38).

Regarding Phenyl-ethanol imidazole derivatives (**1b-3b**) we observed that all had IC_{50} more than clotrimazole (IC_{50}>50 µM). Generally, this decreased cytotoxicity could be attributed to phenyl-ethanol substitution instead of chlorotrityl moiety in clotrimazole. Compound **2b** with a methyl group at position 2 of the imidazole ring, showed more cytotoxic effect than **1b**. On the other hand, compound **3b**, with both methyl and nitro group on its imidazole ring, demonstrated more activity than **2b**. It seems that the presence of a hydroxyl group together with less hindrance on imidazole ring in the structure of these compounds, decreases penetration into the cells and results in less cytotoxic effects. In the case of compounds with cyclohexanol moiety (**4b-5b**) with IC_{50}>200 µM, it seems that having both methyl and nitro groups in imidazole ring of compound **5b**, decreases the activity of this compound. However, in comparison to other compounds in this group, phenylethanol substitution on imidazole ring generally promotes its cytotoxicity.

Among alkyltriazole compounds, as shown in Table 2, **1c** and **2c** had IC_{50} equal or more than clotrimazole. It might denote that increase in the number of alkyl chains from 5 to 8 carbons can increase cytotoxicity. In this group, compound **3c** showed more cytotoxic effect (IC_{50}=25 µM) than clotrimazole. The presence of 1, 2, 4 triazole instead of imidazole ring significantly increases the activity of the compound. In addition, comparing **1c-2c** with **3c** indicated that trityl moiety could also elevate the cytotoxicity of the molecule rather than linear substitution in contrast to the first group (**1a-5a**).

Our results indicated that all compounds show significant differences in their IC_{50} in comparison to clotrimazole except for **4a**, **5a** and **1c**. Statistical analysis showed that despite compounds **2c** and **3c** which have higher cytotoxic effects than clotrimazole, the IC_{50} of others are remarkably higher than clotrimazole (P<0.0001) (Figure 1). Among all investigated compounds, **3c** showed the most cytotoxic effect which has the most similarity in structure to clotrimazole. It seems that re-

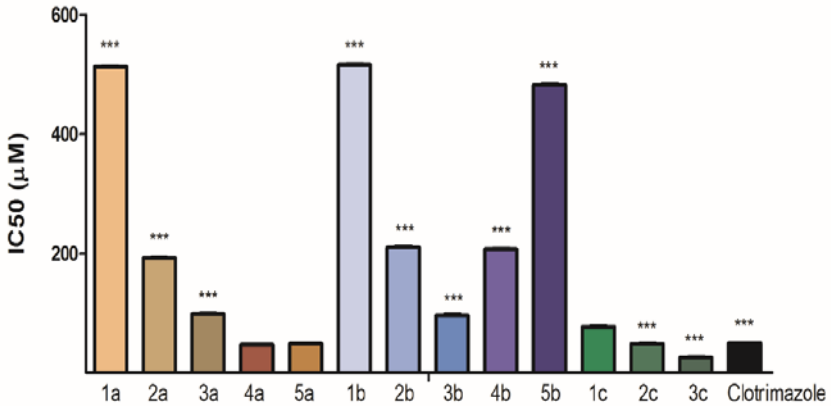


Figure 1. The comparison of IC_{50} of compounds with that of clotrimazole's. The data is presented as mean±SD. *** Differences are significant at P<0.001 (2-tailed).

Table 2. Cytotoxic effects of azole tested compounds on MCF-7 cell.

Compound	IC_{50} (µM)	SD
1a	512.71	1.79
2a	192.57	2.22
3a	98.81	2.42
4a	47.75	1.21
5a	49.38	1.50
1b	515.89	2.65
2b	210.44	3.19
3b	96.67	2.72
4b	207.55	2.10
5b	482.25	2.68
1c	77.75	1.88
2c	48.98	1.95
3c	26.35	1.97
Clotrimazole	50.41	1.17

placement of imidazole ring with triazole moiety remarkably enhances the cytotoxic activity of the compound.

4. Discussion

We previously synthesized some azole derivatives and tested them against different species of microorganisms (11). In comparison to clotrimazole or fluconazole, our compounds showed desirable antifungal activity. Here we selected thirteen of them and compared their cytotoxicity with clotrimazole. It seems that substitution of benzotriazole ring instead of imidazole ring in the structure of clotrimazole might lead to significant reduction of cytotoxic effects of these compounds (**1a-5a**). Compound **1a** had shown higher potency against *E. flocosum* than clotrimazole in previous studies (11) and here showed less toxicity. It could also be concluded that increasing the length of alkyl chain in benzotriazole compounds causes less cytotoxicity. Furthermore, as could be observed, alkylbenzotriazole had more cytotoxic activity than tritylbenzotriazole. We suggested that steric hindrance of trityl group compared to linear group in benzotriazole derivatives, may result in this difference. Compounds **4b-5b** had previously shown desirable antibacterial activity against gram positive and gram negative bacteria (12) and here they showed less toxicity.

5. Conclusion

In conclusion, some of our tested compounds while having already shown desirable antifungal or antibacterial activity in previous studies, exhibited less cytotoxic behaviour in comparison to clotrimazole in this study. Accordingly they could be good applicants for further studies in the point of *in vivo* revisions, mechanism discovery, dose determination, rout of administration *etc*.

Acknowledgment

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Conflict of Interest

None declared.

6. References

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