Cytotoxicity of some 1-(2,4-dihydroxyphenyl)-3-(4-phenylpiperidin-1-yl) prop-2-en-1-one derivatives using MTT assay

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1. Introduction

Cancer, as a broad group of various diseases, is normally expressed by unregulated cell growth in many types of cancer. Uncontrollable cell division could result in the formation of malignant tumors which attack the adjacent parts of the body. In 2007, cancer caused about 13% of all human deaths around the world (7.9 million) (1). There are over 200 different known human cancers (1). Ovarian cancer is a common neoplasm in western countries and the leading cause of death in women with gynecological malignancies. The heterogeneity of ovarian cancer forces the researcher to find new strategies and targets to individualize the therapy for this common disease (1). Burkitt lymphoma is a high-grade, rapidly-growing and aggressive B-cell non-Hodgkin's lymphoma, with three recognized forms: those endemic to Africa, sporadic forms, and those associated with immunodeficiency states (2). Human foreskin fibroblast (HFFF2) is a normal fibroblast like stem cell obtained from human foreskin fibroblast and is used as a predictive model. The most usual treatments for cancer are based on chemotherapy, radiation therapy and surgery. Radiotherapy consists of a vital element in the treatment of breast cancer but has limiting factors for a successful treatment such as relative side effects and different radioactive responses (3).

Chemotherapy is, therefore, a noninvasive method wherein cytotoxic agents are used in the treatment of the disease. Doxorubicin as a successful example of chemical agents was used to treat cancer since 1960s, and its clinical pharmacology was well established (Figure 1) (4). It is approved worldwide for the treatment of leukemia, bone cancer, multiple myeloma and ovarian carcinoma and is considered as the most effective drug in the treatment of breast cancer (5,6). Due to multidrug resistance for chemotherapy agents, demand for newer agents has been a great challenge in pharmaceutical sciences (7). Different derivatives bearing piperidine substructure were previously reported as anticancer agents (8,9). In a recent study, insertion of piperidine ring into benzofuran scaffold has led to a dramatic increase in the activity of the synthesized compounds as inhibitors of mTOR (mammalian target of rapamycin) signaling, a newly discovered target in oncology (Figure 2) (10). In order to extend a reasonable structure activity relationship

Abstract

Some N-substituted piperidine structures were synthesized and evaluated for cytotoxicity. The N-substituted piperidine with propene substructure could be considered as a lead structure for further studies of structure activity relationship to develop more potent compounds in future. The compounds were evaluated against four different cell lines using the standard MTT assay method and doxorubicin was used as the reference drug. The IC₅₀ values were determined by constructing dose-response curves and revealed that three of the synthesized compounds were active against breast cancer cell lines. Compound 6a bearing hydroxyl at para position of piperidine ring was the most active compound within this series.

Keywords: Piperidine, Cytotoxicity, Breast cancer.
pattern for the piperidine structures and to find out a proper lead structure for further studies, some piperidine structures were synthesized and evaluated for anticancer activity in this study. The selection of analogues for evaluation was in such a way that a whole overview of the chemical space could be obtained with the least time and material consumption. The cytotoxicity of the compounds was determined using MTT assay (11).

2. Materials and methods

2.1. Synthesis of the target compounds (6a-d)

Synthesis and purification of the target compounds, 3-(Piperidin-1-yl)-1-(4-substituted phenyl)prop-2-en-1-one derivatives (6a-d), have been carried out according to a previously described procedure (Scheme 1) (12). As depicted in Scheme 1, annulation of chromanone ring preceded by primary acylation of resorcinol yielded compound 3. Subsequently, the hydroxyl moiety was alkylated and a further monobromination reaction was conducted. Then, the compound was reacted with piperidine to afford the final compounds according to the previously described rearrangement (12).

2.2. Cytotoxicity activity

2.2.1. Materials

Doxorubicin, RPMI-1460 and L-glutamine were purchased from Sigma, and Antibiotic/Antimicotic Solution and fetal bovine serum were purchased from Gibco (Grand Island, NY). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Roche (Applied Science, Indianapolis, IN).

2.2.2. Cell lines

Ovarian cancer (OVCAR3), Human Burkitt's Lymphoma (CA46), Breast cancer (T47d) cell lines and human foreskin fibroblast (HFFF2) were purchased from Pasteur Institute of Iran. The cells were cultured in polystyrene plates enriched with Dulbecco’s Modified Eagle Medium (DMEM) and RPMI 1640 medium (Gibco BRL), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma), and incubated at 37 °C in a humid atmosphere with 5% CO₂. All lineages were maintained in the logarithmic phase. The media were changed twice weekly and regular examinations were performed. Evaluation of the cytotoxic effect against all lineages were inoculated at 10,000 cells per well. The plates were preincubated for 24 h at 37 °C to allow adaptation of cells prior to the addition of the test compounds. Freshly prepared solutions of the different compounds were tested at 10 µM. Subsequently, the plates were inoculated for 24 h in an atmosphere of 5% CO₂ and 95% relative humidity. Control groups included those treated with 0.1% DMSO (negative control) and 10 µM of doxorubicin (positive control). All cells were monitored under optical microscopy (400×) before addition of MTT solution to evaluate the morphological changes in vitro after treatment with different concentrations of each compound. Cell viability was estimated by measuring the rate of mitochondrial reduction of MTT. All substances were dissolved in dimethyl sulfoxide (DMSO) prior to dilution. Compounds that inhibited the proliferation more than 50% were selected for determination of the half maximal inhibitory concentration (IC₅₀). IC₅₀ values were determined over a range of concentrations (10-100 µM). All compounds were tested in triplicate, as three independent experiments (13).

2.3. In vitro cell viability assay (MTT assay)

The MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells, only. Briefly, 4 h after the end of incubation of cells with different compounds, 20 µL of MTT solution (2.5 mg.mL⁻¹ in phosphate-buffered saline) was added to each well; the supernatant was removed and 200 µL of 0.04 M HCl in isopropyl alcohol was added to dissolve the formazan crystals. The optical densities (OD) were evaluated at 570 nm using a Rayleigh spectrophotometer. Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as control (blank) and was treated in the same way as the drug-containing media. Results were expressed as the percentage of cell proliferation, comparing with 0.1% DMSO control and were calculated as follows: viability (%)=(mean OD treated - mean OD background)/(mean OD untreated cultured, i.e. 0.1% DMSO - mean OD blank wells)×100. Interactions of compounds and media were estimated on the basis of the variations between drug-containing medium and drug-free medium to escape from false-positive or false-negative (13).
2.4 Statistical analyses
All experiments were performed in at least three replicates per compound and results shown are the average of three independent experiments. Data are represented as the range of mean±SEM and calculated significance was tested by the Student’s t-test (14).

2.5 Docking simulation study
To perform docking simulation, DNA pdb code 1Z3F was retrieved from protein data bank. Water molecules together with the complexed co-crystal ligand were removed from DNA structure. Compound 6a was saved as mol2 and converted to pdbqt using MGLtools 1.5.6. Autodock vina was used for docking purpose and the grid dimensions were set as two times the length of compound 6a (20 Å)(15). The center of box was defined as the center of the co-crystal structure (center_x=2.641, center_y=13.273 center_z=28.985). The exhaustiveness parameter in vina was set to 1000. All visualizations of DNA-ligand interactions were done using VMD software (16).

3. Results and discussion
3.1. Cytotoxicity
The IC_{50} values of the piperidine derivatives against ovarian cancer (OVCAR3), Human Burkitt’s Lymphoma, Human fibroblast foreskin (HFF), and Breast cancer (T47d) cell lines are summarized in Table 1. In general, it was shown that T47d cells were more vulnerable to all compounds than the other cell lines excepted in the case of compound 6d lacking a hydroxyl moiety at the four position of piperidine ring. Interestingly, compound 6a was even more active than doxorubicin, used as the reference drug, against T47d and CA46 cells. The compound 6d was poorly active against all cell lines, with IC_{50} values higher than 100 µM, and in comparison with doxorubicin as positive control. In general, compound 6a revealed the most cytotoxicity against all cell lines except HFF. The presence of hydroxyl group at orto position to carbonyl moiety resulted in increased cytotoxicity as in compounds 6a and 6b as well as a broader activity spectrum. This finding could be related to the planarity of the ring in compounds bearing OH moiety which was previously described through NMR spectra of this series of compounds (12). On the contrary, presence of methoxy at the mentioned position could destroy the planarity of the conjugated system attached to propene linker and result in compounds with narrow spectrum (6c and 6d). Despite a narrow spectrum for compound 6c, it was more active than doxorubicin against T47d cell line. Cytotoxicity of the studied
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compounds was also evaluated in normal cells. The human HFF cell line was used as the predictive model. For this purpose, therapeutic index (TI) was calculated as TI=IC_{50} (HFF)/IC_{50} (tumor cell). According to Table 1, although compound 6a presented the highest cytotoxicity against the investigated tumor cell lines, it could be considered as the best antitumor drug candidate due to its higher therapeutic indices. The TI values for compound 6a were 1.8, 2.95 and 3.17 for the cell lines OVCAR3, CA46 and T47d, respectively. In contrast, the obtained TI value for doxorubicin indicates its low toxicity for normal cells in case of OVCAR3 cells (TI=3.23) while compound 6a showed lower toxicity to normal cells in case of T47d (TI=3.17) and CA46 (TI=2.95) cell lines. The morphological changes in all cell lines especially adherent cells such as HFF, OVCAR3 and T47d were clear in low concentrations (25µM) for all the synthesized structures. This might indicate the chemical contamination for lineage cells, and could alter the normal biological behavior in vitro. Ultrastructure, gene expression profiles and in vivo study of these compounds might be required to show other features of their effects on live normal and tumor cell lines in order to describe cell and molecular mechanisms of their functions. The mechanism of activity for the flat anticytotoxic compounds could be through intercalation with DNA (17). Since these compounds are unsaturated carbonyl structures conjugated with phenyl ring, it was inferred that the plausible mechanism for their activity is DNA intercalation. To deal with this hypothesis, the most active compound, 6a, was subjected to docking simulation study with DNA. The best docking pose in terms of ΔG is depicted in Figure 3. It seems that the flat part of the molecule is well fitted between C-G base pairs of DNA with a negative binding energy (-6.8 kcal) suggesting an intercalating mechanism for this compound.

4. Conclusion

Four N-substituted piperidine structures were synthesized and evaluated for anticancer activity against four cell lines. The experiments were designed so that some minor but principal modifications of the substituents could be studied with the few synthesized structures. It was shown that all compounds except 6d lacking hydroxyl at para position of piperidine ring were active agents against T47d with the order of 6a>6c>6b based on their IC_{50} values. An important issue with the structure activity relationship of this series of compounds was the presence of OH at para position of piperidine ring. Other outstanding modification was orto modification in the phenyl group attached to propene linker at N moiety of the ring. This modification was able to devastate the planarity of the conjugated system. Based on the TI values, compound 6a could

Figure 3. Intercalation of compound 6a with DNA at C-G base pair site using docking simulation.
be proposed as a convenient lead compound for further studies of structure activity relationship against breast cancer cell lines.

Conflict of Interest:
None declared.

5. References

Table 1. Cytotoxicity (IC50 values) of compounds 6a-d against different cell lines

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µM) 95%Confidence interval in parenthesis</th>
</tr>
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<tbody>
<tr>
<td>OVCAR3</td>
<td>CA46</td>
</tr>
<tr>
<td>6a</td>
<td>R=H Y=OH</td>
</tr>
<tr>
<td>6b</td>
<td>R=H Y=X=OH</td>
</tr>
<tr>
<td>6c</td>
<td>R=CH3 Y=X=OH</td>
</tr>
<tr>
<td>6d</td>
<td>R=CH3 Y=X=OH</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>28.5845 (16.66-73.83)</td>
</tr>
</tbody>
</table>

* P-value <0.05 in comparison with the positive control (Doxorubicin); ** TI represents Therapeutic Index.