In-vitro cytotoxicity evaluation of novel N-substituted bis-benzimidazole derivatives for anti-lung and anti-breast cancer activity

Prakash S. Sukhramani 1*, Poonam S. Sukhramani 1, Sarav A. Desai 2 and Maulik P. Suthar 3

1Veerayatan Institute of Pharmacy, Jakhania, Mandvi, Kutch, Gujarat, India
2Pioneer College of Pharmacy, Sayajipura, Vadodara, Gujarat, India
3S. K. Patel College of Pharmaceutical Education & Research, Ganpat University, Kherva, Mehsana (N.G.), India

ABSTRACT

Benzimidazoles of both natural and synthetic sources are the key components of many bioactive compounds. Several reports have shown antifungal, antiviral, H2 receptor blocker and antitumor activities for benzimidazoles and their derivatives. In-vitro cell-based cytotoxicity assay is an easy and cost effective tool for hit ranking and lead optimization at the early stage of drug discovery. In the present study, novel bis-benzimidazole derivatives were screened for cytotoxicity against HEK-293T (Human erythrocyte kidney cell line), MDA-MB453 (Human Breast carcinoma cell line), MDA-MB468 (Human Breast carcinoma cell line), NCI-H522 (Human Lung cancer cell line) and NCI-H23 (Human Lung cancer cell line) with use of short term cytotoxicity MTT assay protocol. Compound 9c, 9g, 9i have exhibited significant cytotoxic activity after 48 hrs comparable with standard drug doxorubicin and were considered to be the best candidate of the series that could be a good starting point to develop new lead compounds in the fight against cancer.

Keywords: Bis-benzimidazole derivative; Cytotoxicity; Lung and Breast cancer; MTT assay.

INTRODUCTION

Cancer is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. [1, 2] Benzimidazoles of both natural and synthetic sources are the key components of many bioactive compounds. The usual synthesis involves
condensation of o-phenylenediamine with formic acid, or the equivalent trimethyl orthoformate. By altering the carboxylic acid used, this method is generally able to afford substituted benzimidazoles. [5] Biologically active benzimidazoles have been known for a long time and they can act as antifungal, anti-leishmanial, antiviral, H2 receptor blocker and antitumor activities. In recent years, benzimidazole derivatives have attracted particular interest due to their anticancer activity or as potential in-vitro anti-cancer and anti-HIV agents. [6]

Some of the complications occur during in-vivo cytotoxic screening that is intravenous administration of chemotherapeutic drugs cause significant individual differences in biotransformation and biodistribution. To overcome this problem, in-vitro cytotoxic screenings are used in which the effect of chemotherapeutic drug is being studied on the tumor cells in culture outside the body. There are two basic types of in-vitro cancer screening method - (a) chemo-sensitivity and (b) chemo-resistance. [7, 8] Human breast carcinoma cell lines (MCF-7, MDA-MB453), Human Non-Small Cell Lung cancer cell line (NCI-H23, NCI-H522) and HEK-293T (Human Embryonic Kidney 293 normal cells) are used for the in-vitro screening of newly synthesized compounds.

Common basic steps of in-vitro cytotoxic screening include: (a) isolation of cells, (b) incubation of cells with drugs, (c) assessment of cell survival and (d) interpretation of the result. The trypan blue dye exclusion assay is the most commonly accepted method for the measurement of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability. [7] It is now well-documented that apoptosis or programmed cell death is the key mechanism by which Chemotherapeutic agents exert their cytotoxicity. [4] Colorimetric assay (MTT) is mainly useful in determination of cellular proliferation, viability and activation. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts MTT are especially useful for assaying the quantification of viable cells. MTT works by being converted to a formazan dye only by metabolic active cells. [3, 7] Formazan dyes were solubilized and are directly quantified using an ELISA reader with their respective reference wavelengths.

MATERIALS AND METHODS

Compounds:
A series of nine synthesized and characterized compounds (9a-9i) were procured from Pharmaceutical Chemistry Department, S. K. Patel College of Pharmaceutical Education & Research, Ganpat University, N. Gujarat, India as shown in Table 1. These compounds were screened for anti-lung and anti-breast cancer activity.

Media
Leibovitz L-15 Medium with L-Glutamine (Biological Industries, Lot No: 928726), FBS (Fetal Bovine Serum, South American origin) (Quaditive, Lot No: 103128), SFM HEK-293 (Serum Free Media, Hyclone, Lot no: ARF26635), Thioglycollate medium (TGM) (Himedia, Lot No: YHI25), Tryptone soya broth (TSB) (Himedia, Lot No: YH031) and Cell proliferation kit (MTT) 1000 tests (Biotium, Inc., Cat. No: 30006).
<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Name of the Compound</th>
<th>Structure of the Compound</th>
<th>Mol. Formula / Mol. Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9a</td>
<td>(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl) phenylmethanone</td>
<td><img src="image" alt="Structure of 9a" /></td>
<td>C_{26}H_{24}N_{4}O_{4} 408.49</td>
</tr>
<tr>
<td>2</td>
<td>9b</td>
<td>1-(4-fluorophenyl)-3-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)propan-1-one</td>
<td><img src="image" alt="Structure of 9b" /></td>
<td>C_{28}H_{27}FN_{4}O_{4} 454.54</td>
</tr>
<tr>
<td>3</td>
<td>9c</td>
<td>2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)-N-phenylacetamide</td>
<td><img src="image" alt="Structure of 9c" /></td>
<td>C_{27}H_{27}N_{5}O 437.54</td>
</tr>
<tr>
<td>4</td>
<td>9d</td>
<td>2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)-N-p-tolylacetamide</td>
<td><img src="image" alt="Structure of 9d" /></td>
<td>C_{28}H_{29}N_{5}O 451.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>( \text{C}<em>{27}\text{H}</em>{26}\text{N}<em>{6}\text{O}</em>{3} ) 482.53</td>
<td></td>
</tr>
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</tr>
<tr>
<td>5</td>
<td>9e</td>
<td>2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)-N-(4-nitrophenyl)acetamide</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>9f</td>
<td>N-(4-methoxyphenyl)-2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)acetamide</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>( \text{C}<em>{28}\text{H}</em>{29}\text{N}<em>{5}\text{O}</em>{2} ) 467.56</td>
</tr>
<tr>
<td>7</td>
<td>9g</td>
<td>2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)-N-o-tolylacetamide</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>( \text{C}<em>{28}\text{H}</em>{29}\text{N}_{5}\text{O} ) 451.56</td>
</tr>
<tr>
<td>8</td>
<td>9h</td>
<td>2-(4-methyl-6-(1-methyl-1H-benzo[d]imidazol-2-yl)-2-propyl-1H-benzo[d]imidazol-1-yl)-N-(2-nitrophenyl)acetamide</td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td>( \text{C}<em>{27}\text{H}</em>{26}\text{N}<em>{6}\text{O}</em>{3} ) 482.53</td>
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Cell lines
HEK-293T (Human embryonic kidney normal cell line), NCI-H23 (Human Non-Small Cell Lung cancer cell line), NCI-H522 (Human Non-Small Cell Lung cancer cell line), MDA-MB453 (Human breast adenocarcinoma cell line) and MCF-7 (Human breast cancer cell line) were purchased from NCCS, Pune.

Microbial and fungal culture
Candida albicans, Bacillus subtilis, Candida sporogenes, Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Subculture of adherent cell lines (HEK 293T, MDA-MB453, NCI-H522) [7, 8]
Cultures were observed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without Ca\(^{2+}\)/Mg\(^{2+}\) using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1 ml per 25 cm\(^2\) of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the incubator and left for 2-10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing HEK-293 medium. 100-200 µl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed HEK-293 medium and incubated as appropriate for the cell line.

Determination of bacteria and fungi in normal and carcinoma cell lines [7]
Cell line was cultured in the absence of antibiotics at NCCS, Pune. Cell suspension was prepared by scraping attached cells with the use of a cell scraper and maintained the pH 7.5-8.0. In 1.5 mL cell suspension, 2 mL thioglycollate medium (TGM) and 2 mL tryptone soya broth (TSB) were added and inoculated with two different strains; Candida albicans (0.1 mL) Bacillus subtilis (0.1 mL). Then in 1.5 mL cell suspension, 1 mL TGM was added and inoculated with 0.1 mL Candida sporogenes and 2 mL (TGM), 2 mL (TSB) were left uninoculated as negative controls. Broths were incubated at 32 °C. Test and Control broths were examined for turbidity after 14 days.

Anti-cancer Activity
MTT Assay: [7]
The cells were preincubated at a concentration of 1 x 10\(^6\) cells/ml in culture medium for 3 hrs at 37 °C and 6.5 % CO\(_2\). Then, the cells were seeded at a concentration of 5 x 10\(^4\) cells/well
in 100 µl culture medium and at various concentrations (0.005-100 µM/ml) of standard doxorubicin and synthesized compounds (dissolved in 2 % DMSO (dimethylsulphoxide) solution) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 hrs at 37 °C and 6.5 % CO₂. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37 °C and 6.5 % CO₂. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product in between 550 and 600 nm according to the filters available for the ELISA reader was used. The reference wavelength should be more than 650 nm.

IC₅₀, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of compound) vs % cell inhibition. A line drawn from 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC₅₀ value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

\[
\frac{(At - Ab)}{(Ac - Ab)} \times 100 \%
\]

Where, \( At = \) Absorbance of Test,
\( Ab = \) Absorbance of Blank (Media),
\( Ac = \) Absorbance of control (cells)

% cell inhibition = 100 – % cell survival

RESULTS AND DISCUSSION

Total bacterial and fungal count
The examination of the test and control broths after 14 days incubation confirmed the absence of turbidity. Absence of turbidity in the test broth means that there was no evidence of bacterial, fungal and cross contamination.

Cytotoxicity Assay
The effect of novel compound aliquots (test) and doxorubicin (standard) on the growth of MCF-7, MDA-MB453, NCI-H522 and NCI-H23 cell lines were examined by the MTT assay. Dose response curves constructed between the range 0.005 – 100 µg/ml and 0.005 – 100 µM for compound aliquots and doxorubicin (control) respectively, express decreasing number of viable cells with increasing concentration of compounds aliquots as well as doxorubicin. Calculation of IC₅₀ value was done using GraphPad Prism Software (Ver. 5.01) (Figure 1, 2, 3 and 4). The susceptibility of cells to the compound aliquots and doxorubicin was characterized by IC₅₀ and R² values (Table 2). Results indicate that the cytotoxic effect steadily strengthens with increase in the concentration.
Figure 1: Activity of Compound (9c) against various cell lines by MTT assay (48 hrs)

Figure 2: Activity of Compound (9g) against various cell lines by MTT assay (48 hrs)

Figure 3: Activity of Compound (9i) against various cell lines by MTT assay (48 hrs)
Comparable cytotoxicity of Compound 9g, 9i was found against lung cancer cell line and Compound 9e exhibited activity in breast cancer cell line. For Compound 9g, IC₅₀ was found to be 50.48 µg/ml in NCI-H522 cell line and 51.45 µg/ml in NCI-H23 cell line and for Compound 9i, IC₅₀ were 47.41 µg/ml in NCI-H522 and 45.22 µg/ml in NCI-H23 but no activity found against normal cell line. Whereas, Compound 9c was found to be active only on breast carcinoma cell line with IC₅₀ of 55.89 µg/ml in MDA-MB453 cell line and 52.09 µg/ml in MCF-7 cell line. However, all compounds were found to be devoid of any activity against HEK-293T (normal) cell line but doxorubicin (standard) was found active against same lung cancer and breast cancer cell lines with lower IC₅₀.

CONCLUSION

A series of new N-substituted Bis-Benzimidazole derivatives were synthesized as well as characterized for their structure elucidation and screened for anti-cancer activity at various concentrations (0.005-100 µM/ml) using Doxorubicin as standard by MTT assay. Data indicates that among the synthesized compounds; 9c, 9g and 9i Compound displayed greater cytotoxicity with comparable IC₅₀. The results described indicate that these compounds could
serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

Acknowledgement
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REFERENCES