MOLECULAR DOCKING AND BIOLOGICAL ACTIVITY OF N-(4-METHOXY)-BENZOYL-N’-PHENYLTHIOUREA AND N-(4-TRIFLUOROMETHYL)-BENZOYL-N’-PHENYLTHIOUREA AS ANTI-BREAST CANCER CANDIDATES

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ABSTRACT
Current breast cancer therapy does not always work optimally, and cases of resistance have been reported. Therefore, it is imperative to develop new effective drugs with minimal side effects through predictions of the epidermal growth factor receptor (EGFR) signalling pathway inhibition. Among chemicals with the potential as anticancer candidates for breast cancer are phenylthiourea derivatives. In this study, two phenylthiourea derivatives were synthesized: N-(4-methoxy)-benzoyl-N’-phenylthiourea and N-(4-trifluoromethyl)-benzoyl-N’-phenylthiourea. These compounds were docked with the EGFR receptor (code: 1M17.pdb) to predict their cytotoxic activity in silico using AutoDock tools. Furthermore, the microculture tetrazolium technique (MTT) was used to investigate in vitro cytotoxicity against MCF-7 cells. The in silico test revealed that the compounds N-(4-trifluoromethyl)-benzoyl-N’-phenylthiourea and N-(4-methoxy)-benzoyl-N’-phenylthiourea had a binding score of -8.2 and -7.3 kcal/mol, respectively, and the in vitro cytotoxic activity testing showed IC₅₀ values of 0.37 mM and 0.38 mM, demonstrating significant EGFR inhibitory activity in MCF-7 cells. Therefore, it can be concluded that N-(4-trifluoromethyl)-benzoyl-N’-phenylthiourea has better cytotoxic activity than N-(4-methoxy)-benzoyl-N’-phenylthiourea.

Keywords: Molecular Docking, Cytotoxic Activity, Breast Cancer, Phenylthiourea.


INTRODUCTION
Breast cancer is the most prevalent cancer in the world, which, together with lung cancer and colorectal cancer, is among the top five leading causes of cancer-related mortality.¹ The increasing number of new breast cancer cases is a health problem that needs immediate attention. Breast cancer treatment is generally a combination of radiotherapy, surgery, and chemotherapy. Therapy options depend on the cancer stage: radiotherapy and surgery for early-stage cancers and chemotherapy for advanced cancers. However, because drug options and undesirable side effects are known limiting factors of chemotherapy,²,³ it is thereby necessary to develop novel chemotherapy drugs with higher anticancer activity and minimal side effects. The development of anticancer drugs is currently towards growth factor receptor (GFR)-targeted therapy. Epidermal growth factor receptor (EGFR)/HER1 plays an essential role in delivering signals needed in cell proliferation. Changes in the EGFR regulatory system and a mutation in the EGFR gene can cause overexpression of EGFR, resulting in uncontrolled cell growth, as seen in several types of cancer, including breast cancer.⁴,⁵ EGFR overexpression in approximately 40% of breast cancer patients correlates with a poor clinical prognosis.⁶ Chemotherapeutic agents that have been developed for breast cancer therapy include thiourea and phenylthiourea derivatives. After synthesizing several phenylthiourea derivatives, Li discovered that N-(5-Chloro-2-hydroxybenzyl)-N-(4-hydroxybenzyl)-N’-phenylthiourea inhibits the enzymatic activity of EGFR (IC₅₀ = 0.08 mM) and HER-2 (IC₅₀ =0.35 mM). In addition, these compounds...
can inhibit the proliferation of MCF-7 cells.\(^7\) In a different study, Huang synthesized thiourea-derived compounds and observed their cytotoxic activity against the NCI-H460, A549, HepG3, and SKOV3 cell lines. The results indicated that thiourea-derived compounds have greater anticancer activity than 5-fluorouracil.\(^8\) In this study, two phenylthiourea derivatives were synthesized, namely N-(4-methoxy)-benzoyl-N'-phenylthiourea (4-OCH\(_3\)-BPTU) and N-(4-trifluoromethyl)-benzoyl-N'-phenylthiourea (4-CF\(_3\)-BPTU). Before the synthesis, their cytotoxic activity was predicted \textit{in silico} (molecular docking) using the AutoDock Vina 1.2.0 program. The phenylthiourea derivatives were docked with EGFR (PDB: 1M17) with the erlotinib as the original ligand.\(^9\) A smaller Binding Score (BS) obtained from the molecular docking indicates lower binding energy of the docked compounds, which means that the ligand-receptor bonds are more stable and produce greater activity.\(^10\) Infrared (IR), \(^1\)H-NMR, \(^13\)C-NMR, and mass spectrometers were used to identify the two synthesized compounds.\(^11,12,13\) To determine the \textit{in vitro} cytotoxic activity of the two compounds against MCF-7 cells, the microculture tetrazolium technique (MTT) was used.\(^14,15\) The IC\(_{50}\) values derived from this process were then compared with those of hydroxyurea (HU, the simplest compound first synthesized from urea) and erlotinib. Here, erlotinib was selected as a comparison compound because it has been used clinically to stop the growth of cancer cells. In addition, the selectivity of the synthesized compounds for cancer cells was observed using normal cells, namely Vero cells. This study was designed to obtain new compounds derived from phenylthiourea as anticancer candidates for breast cancer by predicting whether or not and to what extent they inhibited EGFR signalling.

**Experimental**

**Molecular Docking**

MarvinSketch v. 19.17.0 was used to draw the chemical structures of 4-OCH\(_3\)-BPTU and 4-CF\(_3\)-BPTU. Afterward, Avogadro v. 1.2.0 was used to convert the derived 2D sketches into 3D form for docking. Finally, the geometric structures of both compounds were optimized using the Merck molecular force field (MMFF94) and then stored in mol2 file format.

**Prediction of \textit{In Silico} Cytotoxic Activity**

The compounds, 4-OCH\(_3\)-BPTU and 4-CF\(_3\)-BPTU, were docked with EGFR receptor (PDB: 1M17) in the AutoDock Vina 1.2.0 program to predict the \textit{in silico} cytotoxicity activity. Furthermore, the binding scores generated by the docking were observed for any similarity with those of HU and erlotinib for comparison.

**Synthesis**

N-phenylthiourea was reacted with triethylamine (TEA) and 4-methoxy or 4-trifluorobenzoyl chloride in tetrahydrofuran (THF). In a water bath, the mixture was refluxed, then the sample was applied on thin-layer chromatography (TLC) plate. TLC was performed every hour until a spot appeared on the plate. This process was followed by THF evaporation and then recrystallization.\(^16\) IR, \(^1\)H-NMR, \(^13\)C-NMR, and HRMS spectroscopy techniques were used to identify the structure of the synthesized compound.\(^11,12\)

**Cytotoxic Activity against MCF-7 Cells**

Cultures of MCF-7 breast cancer cells and normal cells, i.e., Vero cells, were grown on 96-well plates for a 24-hour incubation in a CO\(_2\) incubator. Afterward, various concentrations of the test compounds, HU and erlotinib were added to the 96-well plates. A culture medium that did not contain MCF-7 and Vero cells was used as a control. The microplate to which the test compounds, HU and erlotinib were added was then re-incubated for 24 hours. The next step was inverting the microplate at 180°C to remove the media in the wells and washing each well with 100 \(\mu\)L of PBS; in this process, the PBS was discarded. Then, the microplate was added with the MTT reagent (100 \(\mu\)L, 0.5 mg/mL) and incubated for 4 hours. Following the incubation was the addition of 100 \(\mu\)L of 10% SDS in 0.01 n HCl into each well to dissolve the formed formazan crystals, stopping the MTT reaction. After wrapping the microplate in a paper, it was incubated at 37°C for 24 hours; finally, the absorbance was measured and read at 595 nm using an ELISA reader and the surviving fraction was calculated.\(^17,18\) Probit analysis was used to determine the IC\(_{50}\) values of the two synthesized compounds, HU and erlotinib for MCF-7 cells and Vero cells.\(^14\) The selectivity of the synthesized compounds for cancer cells was calculated as a ratio of the IC\(_{50}\) for normal cells to the IC\(_{50}\) for cancer cells.
RESULTS AND DISCUSSION

In Silico Cytotoxic Activity

Table-1 shows the in silico test outcome of the compounds 4-OCH$_3$-BPTU, 4-CF$_3$-BPTU, HU, and erlotinib. The method validation obtained a root-mean-square deviation (RMSD) value of 1.02 Å, which meets the validation requirements$^{21}$. As seen in Table-1, 4-OCH$_3$-BPTU (-7.3 kcal/mol) and 4-CF$_3$-BPTU (-8.2 kcal/mol) had a lower binding score (BS) than HU (-3.8 Kcal/mol). Compared with erlotinib (-7.5 kcal/mol), 4-CF$_3$-BPTU had a lower BS, while 4-OCH$_3$-BPTU had a slightly higher BS. The -OCH$_3$ group at the para position has low lipophilic and electronic properties but can substantially boost electrons; thus, adding the -OCH$_3$ group results in BS that is only slightly higher than or almost similar to erlotinib but smaller than HU. On the contrary, the –CF$_3$ group at the para position has high lipophilic and electronic properties and high electron-withdrawing capabilities. Therefore, with the addition of the –CF$_3$ group, the resultant in silico cytotoxic activity was proven greater than the compound added with the -OCH$_3$ group.

Table-1: In Silico Test Results: Binding Scores and Interaction Binding of 4-OCH$_3$-BPTU, 4-CF$_3$-BPTU, HU, and Erlotinib

<table>
<thead>
<tr>
<th>Substituents</th>
<th>Binding Scores (kcal/mol)</th>
<th>Interaction Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M17 (Ligand: AQ4)</td>
<td>-7.5</td>
<td></td>
</tr>
<tr>
<td>Erlotinib</td>
<td>-7.5</td>
<td></td>
</tr>
<tr>
<td>Hydroxyurea (HU)</td>
<td>-3.8</td>
<td></td>
</tr>
<tr>
<td>4-CF$_3$-BPTU</td>
<td>-8.2</td>
<td></td>
</tr>
<tr>
<td>4-OCH$_3$-BPTU</td>
<td>-7.3</td>
<td></td>
</tr>
</tbody>
</table>

Synthesis

The compounds 4-OCH$_3$-BPTU and 4-CF$_3$-BPTU were synthesized from 4-methoxy and 4-trifluorobenzoyl chloride with N-phenylthiourea, producing shiny white, water-insoluble crystals. Figure-1 shows the structure of the synthesized compounds, with the details presented in Table-2.

In vitro Cytotoxic Activity

As presented in Table3, the compounds 4-OCH$_3$-BPTU and 4-CF$_3$-BPTU had an IC$_{50}$ value of 0.38 and 0.37 mM, respectively, indicating higher cytotoxic activities than HU (IC$_{50}$ = 9.76 mM) and erlotinib (IC$_{50}$ = 0.92 mM). The table also shows that 4-CF$_3$-BPTU had a slightly lower IC$_{50}$ value than 4-OCH$_3$-BPTU, which means that the former has a higher cytotoxic activity than the latter. This result also corresponds to
the *in silico* activity prediction in which 4-CF$_3$-BPTU was found to have a smaller BS than 4-OCH$_3$-BPTU. Based on the physicochemical quality, the –CF$_3$ group at the para position has high lipophilic and electronic properties, allowing it to penetrate the cancer cell membrane. For these reasons, it is predicted to inhibit the phosphorylation of EGFR in the intracellular domain, resulting in the inhibition of intracellular signalling, which is involved in cell proliferation.

![Structures of 4-OCH$_3$-BPTU (a) and 4-CF$_3$-BPTU (b)](image)

**Table-2:** Characterization of N-(4-methoxy)-benzoyl-N’-phenylthiourea (4-OCH$_3$-BPTU) and N-(4-trifluoromethyl)-benzoyl-N’-phenylthiourea (4-CF$_3$-BPTU)

<table>
<thead>
<tr>
<th>Characteristics and Methods</th>
<th>N-(4-methoxy)-benzoyl-N’-phenylthiourea (4-OCH$_3$-BPTU)</th>
<th>N-(4-trifluoromethyl)-benzoyl-N’-phenylthiourea (4-CF$_3$-BPTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>White crystal</td>
<td>White crystal</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>70</td>
<td>67</td>
</tr>
<tr>
<td>m.p. (°C)</td>
<td>108–109</td>
<td>125–126</td>
</tr>
<tr>
<td>$^1$H-NMR (DMSO-d$_6$, 500 MHz)</td>
<td>δ 3.90 (s, 3H, OCH$_3$); δ 7.02 (d, J=8.9 Hz, 2H, Ar-H); δ 7.29 (t, J=7.6 Hz, 1H, Ar-H); δ 7.43 (t, J=7.6 Hz, 2H, Ar-H); δ 7.72 (d, J=7.6 Hz, 2H, Ar-H); δ 7.88 (d, J=8.9 Hz, 2H, Ar-H); δ 9.07 (s, 1H, O=C-NH-C=S); δ 12.68 (s, 1H, S=C-NH-Ar).</td>
<td>δ 7.31 (t, J=7.8 Hz, 2H, Ar-H); δ 7.43 (t, J=7.8 Hz, 1H, Ar-H); δ 7.70 (d, J=8.0 Hz, 2H, Ar-H); δ 7.81 (d, J=7.8 Hz, 2H, Ar-H); δ 8.02 (d, J=8.0 Hz, 2H, Ar-H); δ 9.19 (s, 1H, O=C-NH-C=S); δ 12.45 (s, 1H, S=C-NH-Ar).</td>
</tr>
<tr>
<td>$^{13}$C-NMR (DMSO-d$_6$, 125 MHz)</td>
<td>δ 55.8 (1C, OCH$_3$); δ 114.6 (2C, Ar); δ 123.6 (2C, Ar); δ 124.3 (1C, Ar); δ 127.0 (2C, Ar); δ 129.0 (1C, Ar); δ 129.8 (2C, Ar); δ 137.8 (1C, Ar); δ 164.2 (1C, Ar); δ 166.5 (1C, C=O); δ 178.7 (1C, C=S).</td>
<td>δ 124.4 (1C, CF$_3$); δ 126.4 (2C, Ar); δ 126.4 (2C, Ar); δ 127.3 (2C, Ar); δ 128.2 (1C, Ar); δ 129.1 (2C, Ar); δ 135.1 (1C, Ar); δ 135.4 (1C, Ar); δ 137.5 (1C, Ar); δ 165.8 (1C, C=O); δ 178.1 (1C, C=S).</td>
</tr>
<tr>
<td>IR (KBr), $v_{max}$ (cm$^{-1}$)</td>
<td>1661 (C=O amide); 1593 and 1505 (C=C aromatic); 3300 and 1593 (NH stretch secondary amides); 1075 and 805 (C=S).</td>
<td>1671 (C=O amide); 1600 and 1497 (C=C aromatic); 3269 and 1600 (NH stretch secondary amides); 1082 and 838 (C=S).</td>
</tr>
<tr>
<td>HRMS (m/z)</td>
<td>C$<em>{15}$H$</em>{13}$N$_2$O$_2$S: (M-H)$^-$ = 285.0692</td>
<td>C$<em>{15}$H$</em>{18}$N$_2$OSF$_3$: (M-H)$^-$ = 323.0472</td>
</tr>
</tbody>
</table>

**Table-3:** IC$_{50}$ Values of the Test and Comparison Compounds for MCF-7 and Vero Cells and Their Selectivity Index Values

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (mM) MCF-7 Cells</th>
<th>IC$_{50}$ (mM) Vero Cells</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OCH$_3$-BPTU</td>
<td>0.38</td>
<td>22.59</td>
<td>59.45</td>
</tr>
<tr>
<td>4-CF$_3$-BPTU</td>
<td>0.37</td>
<td>37.28</td>
<td>100.76</td>
</tr>
<tr>
<td>HU</td>
<td>9.76</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>0.92</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
In addition to cytotoxic activity, the test compounds were examined on their selectivity for MCF-7 cancer cells using Vero cells (normal cells). A Selectivity Index (SI) is a selectivity parameter that measures the safety of a drug. SI was calculated using the formula below:

\[
Selectivity\ Index\ (SI) = \frac{IC_{50}\ normal\ cells}{IC_{50}\ cancer\ cells}
\]

Based on Table-3, the compounds 4-OCH$_3$-BPTU and 4-CF$_3$-BPTU each had an SI value of 59.45 and 100.76, indicating high selectivity for MCF-7 cancer cells (SI > 2).\textsuperscript{20,21} Also, 4-CF$_3$-BPTU had a higher SI value or, thus, better selectivity than 4-OCH$_3$-BPTU. Nevertheless, according to the SI values, the two test compounds can kill cancer cells with little or no impact on normal cells.

**CONCLUSION**

The test compounds 4-CF$_3$-BPTU and 4-OCH$_3$-BPTU generally exhibit higher \textit{in silico} and \textit{in vitro} cytotoxic activity than hydroxyurea (HU) and erlotinib, except 4-OCH$_3$-BPTU which has lower \textit{in silico} cytotoxic activity than erlotinib. Overall, 4-CF$_3$-BPTU has a higher cytotoxic activity than 4-OCH$_3$-BPTU both \textit{in silico} and \textit{in vitro}.

**ACKNOWLEDGEMENT**

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