New N-allylthiourea derivatives: Synthesis, molecular docking and in vitro cytotoxicity studies

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INTRODUCTION

Chemotherapy is one method of cancer treatment that works by inhibiting the growth of cancer cells or killing them. It is known that chemotherapy has many adverse side effects, such as hair loss, diarrhea, dry mouth, nausea, vomiting, and fatigue [1]. Therefore, it is important to evolve newer, more effective and more selective anticancer agents [2,3]. In the present study, thiourea derivatives were developed as potential anticancer agents by modifying the structure of N-allylthiourea (ATU) [3-8]. The choice of N-allylthiourea was due to...
its thiol group, a pharmacophore which is responsible for its biological activity. In addition, N-allylthiourea possesses a primary amine group (-NH$_2$). These groups allow for possible modifications that may lead to the development of ATU as an anticancer agent.

The chemical modification of the structure of N-allylthiourea was effected by reacting it with derivatives of benzoyl chlorides, resulting in generation of N-benzoyl-3-allythiourea (A – D, Figure 1) using the Schotten-Baumann reaction [7]. In the first step, the amine group of N-allylthiourea reacted with the C-carbonyl of benzoyl chloride forming an amide, with the release of a proton and a chloride ion. The addition of the weak base, triethylamine (TEA) was necessary to neutralize the acidic proton formed, otherwise, the reaction would not have proceeded. Moreover, the addition of TEA blocked the protonation of the amine, a reaction which would have made it impossible for the base to react as a nucleophile [7].

![Figure 1](image-url)  
**Figure 1:** (i) Steps in the synthetic process according to the Schotten-Baumann procedure. (ii) Chemical structures of the four derivatives of N-allylthiourea: A. 3, 4-dichlorobenzoyl-3-allythiourea; B. 4, 4-dichlorobenzyol-3-allythiourea; C. 4-fluorobenzoyl-3-allythiourea; D. 4-methylbenzoyl-3-allythiourea

Molecular modeling is usually conducted to examine the affinity of a ligand to its docking site through evaluation of the energy of drug-receptor binding. It has been shown that the evaluation of the interaction between a molecule and its docking protein involved in signal transduction may aid in identifying potential biological activity, as well as possible mechanisms of action [8,10,11]. Studies indicate that the 1M17, 1XKK, and 3POZ receptors are used as models of the epidermal growth factor receptor, EGFR [1,12]. The standard ligands for these receptors are erlotinib, lapatinib, and TAK-285, respectively (TAK-285 is a selective EGFR inhibitor) [12]. In addition, these receptors resemble thiourea derivatives (EGFR/HER-2 inhibitors) which act by inhibiting the tyrosine kinase receptor (RTKs) in the intracellular region [13-15]. As inhibitors of EGFR, thiourea derivatives exhibit anti-proliferation activities against tumor cells, including human breast cancer cells [3,4,14].

One important parameter in molecular docking is the Rerank Score (RS). It is the energy of the ligand-receptor interaction, and a measure of bond energy between ligand and receptor [7,8,10]. The smaller the RS value, the more stable the bond between ligand and receptor. Therefore, a strong interaction implies a high biological activity [8].

In the present study, the cytotoxicities of N-allylthiourea and its derivatives against MCF-7 breast cancer cell line were investigated using MTT assay [6,16,18].

**EXPERIMENTAL**

**Chemicals and reagents**

N-allylthiourea was purchased from Merck; 3, 4-dichlorobenzoyl chloride; 2, 4-dichlorobenzoyl chloride; 4-fluorobenzoyl chloride, and 4-methylbenzoyl chloride were products of Sigma-Aldrich. The reactions were monitored by Thin Layer Chromatography (TLC) carried out on silica gel 60 F254 plates (Merck). Spot detection was performed at UV 254 nm [19]. Purification of the products was carried out by recrystallization [7,19].

Infrared spectra were measured using a Perkin-Elmer Spectrum One spectrophotometer and major absorptions were listed in cm$^{-1}$. $^1$H-NMR (600 MHz) and $^{13}$C-NMR (150 MHz) spectra were measured on JEOL JNM-ECS 600 instrument with DMSO-d$_6$ as solvent and chemical shifts reported in ppm on the δ-scale. Positive ion HR-ESI-MS was accomplished by an Applied Biosystems QSTAR XL Nanospray™ system. In silico studies were carried out using Molegro® Virtual Docker version 5.5; 1M17, 1XKK, and 3POZ receptors were obtained from Protein Data Bank (www.pdb.org). The 2-D and 3-D structures of N-allylthiourea derivatives and the ligand were measured using ChemBioOffice Ultra 11.0 and MMFF94 for minimization of the energy [8,11].

**Cell culture**

MCF-7 cell lines were obtained from the Laboratory of Gene Regulation at Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Japan. The cells were grown on high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Nacalai.
Tres, Japan) containing 10% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin-streptomycin (Sigma, USA). The cells were harvested from culture dish using Trypsin-EDTA 0.25 % (Sigma, USA). The MTT reagent (5 mg/ml) was prepared by dissolving MTT (Nacalai, Japan) in phosphate-buffered saline (PBS).

Docking

Receptor and ligand were downloaded from Protein Data Bank. The ligand-receptor complexes were measured in the cavity. The 3-D structures of derivatives of N-allythiourea were replaced to the fixed cavity. The ligands were docked towards the 1M17, 1XKK, and 3POZ receptors [12]. In the next step, Docking Score, Rerank Score, RMSD value, and the state of the environment of each compound derivative, such as hydrogen bonds, hydrophobic interactions, and electronic bonds, were determined [11,16,20].

Synthesis of N-allythiourea derivatives

N-Allythiourea (0.025 moles) was dissolved in 20 mL of tetrahydrofuran. Benzoil chloride derivatives (1 eq) and triethylamine (7 mL) were added to the solution, stirred for 30 min at room temperature, and refluxed for 3-4 h. Thereafter, the mixture was evaporated and washed with saturated aqueous sodium bicarbonate solution. The crude product was purified using column chromatography, with hexane: acetone (3:2); chloroform: hexane (5:2) and chloroform: hexane: ethyl acetate (1:6:2) as mobile phases at the indicated volume ratios. Recrystallization was done using ethanol: water at a volume ratio of 1:1 [7].

EVALUATION OF CYTOTOXICITY

The cytotoxic effects of the derivatives against MCF-7 cells were evaluated using MTT assay. The cells were seeded into 96-well plates at a density of 8 x 10^3 cells/well and incubated at 37 °C for 24 h in an atmosphere containing 5% CO_2. The synthesized compounds were applied to the cells separately at different concentrations. After a 24-h incubation, MTT (5 mg/mL in PBS) was added to each well, and incubation was continued for 4 h at 37 °C to allow for formation of formazan crystals. Then, the reaction in each well was stopped by addition of the stopper reagent, a solution of 10% sodium dodecyl sulphate (SDS, Merck) in 0.01 N HCl (Merck), which also served to solubilize the formazan crystals. The plates were kept overnight in the dark at room temperature, after which the absorbance of the formazan solution in each well was read at 570 nm in an ELISA plate reader [2,17,18].

RESULTS

Derivatives synthesized from N-allythiourea

The desired derivatives were synthesized through nucleophilic substitution of N-allythiourea with benzoyl chloride derivatives i.e. 3, 4-dichlorobenzoyl chloride; 2, 4-dichlorobenzoyl chloride; 4-fluorobenzoyl chloride, and 4-methylbenzoyl chloride, yielding compounds A, B, C and D, respectively (Figure 1). The detailed physicochemical and spectral data of the compounds are described below:

Compound (A) was obtained as a white crystal (49% yield) with the following spectral characteristics: IR (cm⁻¹) = 1672; 3467; 1548; and 1447; ¹H-NMR δ_H (ppm, J in Hz) = 4.30 (t, J = 5.2, 2H), 5.20 (d, J = 12, 1H), 5.25 (d, J = 3, 1H), 6.00 - 5.93 (m, 1H), 7.88 (d, J = 6, 2H), 7.89 (d, J = 6, 2H), 8.21 (s, 1H), 10.83 (s, 1H), and 11.61 (s, 1H); ¹³C-NMR δ_C (ppm) = 46.86, 116.69, 128.81, 130.57, 130.63, 131.18, 132.71, 132.96, 135.64, 165.80, and 180.09; HR-ESI-MS m/z = 288.9222 [M + H]^+ (calculated value for C₉H₁₂ON₂Cl₂S = 288.9262). These spectral data agreed with the structure of 3,4-dichlorobenzoyl-3-allythiourea (A).

The synthesized compound (B) was a white crystal obtained at a yield of 75%. Its spectral data were: IR (cm⁻¹) = 1684, 3467, 1585 and 1425; ¹H-NMR δ_H (ppm, J in Hz) = 11.81 (s, 1H), 10.64 (s, 1H), 7.75 (d, J = 2, 1H), 7.62 (d, J = 8.3, 1H), 7.54 (d, J = 5.1, 1H), 6.02-5.92 (m, 1H), 5.25 (d, J = 10.3, 1H), 5.20 (d, J = 1.6, 1H), and 4.29 (t, J = 3.6, 2H); ¹³C-NMR δ_C (ppm) = 180.24, 167.09, 136.17, 133.88, 133.45, 131.66, 131.03, 129.54, 127.80, 117.33 and 47.31; HR-ESI-MS m/z = 288.9966 [M + H]^+ (calculated value for C₁₁H₁₂ON₂Cl₂S = 288.9969). These spectral data agreed with the structure of 2,4-dichlorobenzoyl-3-allythiourea (B).

Compound (C) was a white crystalline salt (48% yield) with the following spectral data: IR (cm⁻¹) = 1674; 3429; 1568 and 1449; ¹H-NMR δ_H (ppm, J in Hz) = 4.30 (t, J = 12, 2H), 5.20 (d, J = 12, 1H), 5.26 (d, J = 18, 1H), 6.00-5.94 (m, 1H), 7.36 (t, J = 18Hz, 2H), 8.03 (t, J = 12, 2H), 10.93 (s, 1H), and 11.43 (s, 1H); ¹³C-NMR δ_C (ppm) = 46.83, 115.42, 116.65, 128.73 and 131.55 (2C); 13C-05, 164.00, 166.99, 180.30; HR-ESI-MS m/z = 239.0650 [M + H]^+ (calculated value for C₁₁H₁₂ON₂FS = 239.0654). These spectral data
agreed with the structure of 4-fluorobenzoyl-3-allythiourea (C).

The derivative compound (D) was a white crystal (35% yield). Its spectral features were: IR (cm⁻¹) = 1663, 3467, 1609 and 1448; ¹H-NMR δₘ (ppm, J in Hz) = 11.26 (s, 1H), 11.00 (s, 1H), 7.87 (d, J = 8.2, 2H), 7.33 (d, J = 7.9, 2H), 6.02-5.93 (m, 1H), 5.28 (d, J = 42, 1H), 5.20 (d, J = 12, 1H), 4.30 (t, J = 3.6, 2H), and 2.39 (s, 3H); ¹³C-NMR δₐ (ppm) = 180.176, 166.847, 144.736, 131.941, 129.929, 128.861 (2C), 127.574 (2C), 117.801, 82.160, 21.769. HR-ESI-MS m/z = 235.0905 [M + Na]+ (calculated value for C₁₂H₁₅ON₂S = 235.0905). These were consistent with the structure of 4-methylbenzoyl-3-allythiourea (D).

**Docking results**

![Image](image-url)

**Figure 2:** The amino acids involved in the interaction process between ligands and epidermal growth factor receptor. (1) 2-D interaction of compound A with 1M17; (2) H-bond interaction with 1XKK in 3-D illustration; and (3) 2-D interaction of ligand standard TAK-285 with 3POZ.

The interaction between the synthesized compounds (ligands) and amino acid residues are shown in Table 1 and illustrated in 2D in Figure 2.

**Cytotoxicity of compounds A - D**

The cytotoxic activities of the four derivatives were assessed using MTT assay, and the results are shown in Table 2. The viabilities of the cells obtained from the measurement of the absorbance of formazan formed after treatment with MTT reagent, and representative morphologies of MCF-7 cell line after treatment are shown in Figure 3.

**DISCUSSION**

In drug development and discovery, it is important to ensure that the drug-like physicochemical properties are maintained as described by Lipinsky’s rule [9]. Most orally-administered drugs are relatively small and moderately lipophilic molecules.

**Table 1:** Interactions between the synthesized compounds (ligands) and amino acid residues in the EGFR receptor

<table>
<thead>
<tr>
<th>Code</th>
<th>1M17</th>
<th>3POZ</th>
<th>1XKK</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Met769, Thr766, Leu820, Leu768, Ile765, Gln767, Ala719</td>
<td>Met793, Thr854</td>
<td>Met793, Gly796, Gln791, Thr790, Val726, Ile744, Ala743, Ile789, Leu788</td>
</tr>
<tr>
<td>B</td>
<td>Met769, Thr766, Leu820, Leu768, Ile765, Gln767, Ala719</td>
<td>Met793, Thr854</td>
<td>Met793, Gly796, Gln791, Thr790, Val726, Ile744, Ala743, Ile789, Leu788</td>
</tr>
<tr>
<td>C</td>
<td>Met769, Thr766, Leu820, Lau764, Leu768, Ile765, Gln767, Ala719</td>
<td>Met793, Thr854</td>
<td>Met793, Gly796, Gln791, Thr790, Ala743, Ile789, Leu788</td>
</tr>
<tr>
<td>D</td>
<td>Met769, Thr766, Leu820, Lau764, Leu768, Ile765, Gln767, Ala719</td>
<td>Met793, Thr854</td>
<td>Met793, Gly796, Gln791, Thr790, Ala743, Ile789, Leu788</td>
</tr>
<tr>
<td>ATU</td>
<td>Met769, Leu768, Gln767, Leu820</td>
<td>Met793, Thr790, Gln791, Ala743, Ile789, Leu844</td>
<td>Met793, Thr790, Gln791, Ala743, Ile789, Leu844</td>
</tr>
<tr>
<td>HU</td>
<td>Met769, Leu768, Gln767</td>
<td>Met793, Thr790, Gln791, Ala743, Ile789, Leu844</td>
<td>Met793, Thr790, Gln791, Ala743, Ile789, Leu844</td>
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<tr>
<td>SL</td>
<td>Met769, Gln767, Gly772, Pro770, Asp831</td>
<td>Met769, Arg841</td>
<td>Phe856, Cys775</td>
</tr>
</tbody>
</table>

*Note: ATU = Allylthiourea; HU = hydroxyurea; SL = standard ligand*
Table 2: Docking (RS) and IC_{50} data

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent</th>
<th>Docking Score (kcal/mol)*</th>
<th>IC_{50} (mM) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3,4-Cl_{2}</td>
<td>-94.7885</td>
<td>0.213±1.61</td>
</tr>
<tr>
<td>B</td>
<td>2,4-Cl_{2}</td>
<td>-94.1892</td>
<td>0.231±1.34</td>
</tr>
<tr>
<td>C</td>
<td>4-F</td>
<td>-91.2717</td>
<td>0.261±1.92</td>
</tr>
<tr>
<td>D</td>
<td>4-CH_{3}</td>
<td>-68.5424</td>
<td>0.230±1.05</td>
</tr>
<tr>
<td>ATU</td>
<td>-</td>
<td>-42.2023</td>
<td>2.793±1.66</td>
</tr>
<tr>
<td>HU</td>
<td>-</td>
<td>-44.2384</td>
<td>5.219±1.81</td>
</tr>
</tbody>
</table>

*Rerank Score is the value of energy after re-ranking by considering RMSD factor and chemical bonds involved [15].

Chemdraw® is a helpful tool for predicting the lipophilicity of organic compounds [8]. The log P values of N-benzoyl-3-allythiourea, N-allylthiourea and hydroxyurea are 2.0450, 0.0799, and -1.8000, respectively [7]. The higher the log P value, the more lipophilic the compound. Based on this parameter, the derivatives of N-allylthiourea are more lipophilic than N-allylthiourea and hydroxyurea. Thus, theoretically, the derivatives of N-allylthiourea can penetrate the cell membrane more easily, and so possess higher biological activities than the parent compound. The infrared spectra of the synthesized compounds A-D showed peaks above 1663 - 1684 cm^{-1}, indicating the presence of C=O carbonyl groups in their structures. Furthermore, the chemical structures of compounds A-D were confirmed through several parameters such as ratio of protons as seen from integrations and splitting patterns of the peaks in ¹H-NMR spectra, number of peaks in ¹³C-NMR spectra, and the exact mass of molecular ion measured by HR-MS.

Based on in silico studies on the interactions between the ligands and EGF receptor, compound A interacted with amino acid residues such as Met769, Met793, and Thr854 through sterical bonds and H-bonds. The interactions of other compounds (B, C and D) followed a similar pattern. The synthesized compounds exhibited the same ATP binding site for the H-bond binding of the amino acid residues Met793 and Met769 to the EGFR. However, N-allylthiourea and hydroxyurea bound amino acid residues only through sterical bonds: H-bonds were not involved.

The presence of the lipophilic benzoyl group in N-benzoyl-3-allythiourea resulted in enhanced biological activity due to stabilization of the ligand-receptor bond. This is consistent with the Rerank Score values of the N-allylthiourea derivatives (compounds A - D) which were lower than those of N-allylthiourea (lead compound) and hydroxyurea (clinically-used anticancer drug). The addition of substituents with certain lipophilic, electronic and steric properties improves the stability of ligand-receptor bonds.
The variation of substituents on N-benzoyl-3-allyltiourea caused differences in the number of interaction with amino acids. However, it did not bring about significant differences in Rerank Score values. From the results of in silico studies, some derivatives of N-allyltiourea were predicted to have greater anticancer activities than hydroxyurea. The results of in vitro cytotoxicity performed on MCF-7 cell lines using the MTT method revealed that the IC50 of compounds A - D (0.21 – 0.38 mM) were less than that of N-allyltiourea (IC50 = 5.22 mM). These results indicate that the four derivatives of N-allyltiourea were more cytotoxic than the lead compound, N-allyltiourea. In addition, cytotoxicity studies revealed that all derivatives of N-allyltiourea were more cytotoxic against MCF-7 cell lines when compared to the commercial anticancer drug, hydroxyurea (IC50 = 2.79 mM).

CONCLUSION

Molecular docking data have predicted that N-allyltiourea derivatives are epidermal growth factor inhibitor receptors. This is supported by results from cytotoxicity studies using MCF-7 cancer cell lines. Therefore, N-allyltiourea derivatives possess potential anticancer properties that can be harnessed for the development of new anticancer drugs.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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