ORIGINAL ARTICLE

Anticancer evaluation of *N*-benzoyl-3-allylthiourea as potential antibreast cancer agent through enhances HER-2 expression

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ABSTRACT

Breast cancer with HER-2 overexpression is sensitive to drugs which target the receptor or its kinase activity. Although the anti-HER-2 therapies commonly used have improved patient outcome, resistance usually occurs. In this present study, we investigated a modification of the chemical structure of allylthiourea derivatives in order to enhance the cytotoxicity effect on breast cancer cells with HER-2 overexpression. The aim of this research was to predict the absorption, distribution, metabolism, excretion, and toxicity by *in silico* study and to explore the effect *N*-benzoyl-3-allylthiourea (BATU) on MCF-7 cell line with overexpressing of HER-2 using MTT assay and western blot. The result showed that the cytotoxicity effects of BATU on MCF-7/HER-2 cell line (IC_{50} value 0.64 mM) were higher than on MCF-7 cell lines (IC_{50} value 1.47 mM). In addition, the cytotoxic effects of BATU on MCF-7 and MCF-7/HER-2 were higher than allylthiourea as a lead compound (IC_{50} value 5.22 and 3.17 mM). The results also confirmed that the BATU compound has the ability to effectively enhance its cytotoxicity against MCF-7/HER-2 through enhanced HER-2 expression and inhibition of nuclear factor kappa B (NF-kB) activation. Above all, the BATU compound is effective in increasing HER-2 expression and inactivating NF-kB transcription factors, thereby resulting in inhibition of protein expression which works a significant part in cell proliferation. Therefore, the BATU compound has the potential to be developed as a complementary drug in breast cancer therapy with HER-2 positive.

Key words: Allylthiourea, cytotoxicity, HER-2, MCF-7, N-benzoyl-3-allylthiourea, nuclear factor kappa B

INTRODUCTION

Based on the World Health Organization in 2018, a total of 627,000 deaths are caused by breast cancer, the highest contributor to death from all types of cancer in women. In cases of breast cancer, 30% of occurrences are due to the overexpression of HER-2 with a poor prognosis.[1,2] A large number of HER-2 receptors are able to influence the continuous proliferation of tumor cells[3] and induce

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spontaneous dimerization and autophosphorylation, trigger the activation of focal adhesion kinase, and induce migration processes and cancer cell metastasis.[4]

The most common treatment used for breast cancer cases with HER-2 is the lapatinib chemotherapy agent. According to Johnston *et al.* (2006), lapatinib plays as a tyrosine kinase inhibitor. However, the use of lapatinib has been reported to be resistant to HER-2 positive breast cancer cases.[5] Other anticancer group such as the thiourea derivative, which acts as an inhibitor of epidermal growth factor receptor (EGFR) kinase, causes high antiproliferation activity against tumor cells. In addition, these compounds play a significant role in inhibiting the protein tyrosine kinase and NADH oxidase, which both contribute to the growth of tumor cells.[6,7]

In recent years, several studies were conducted on the development of thiourea derivatives as anticancer agents. The results showed that the compound developed had a pharmacophore group thiourea (-HN-C (=S)-NH-).[8-12] The synthesis and relationship of the structural activity of N-benzyl-N-(X-2-hydroxybenzyl)-*N*-phenylurea and thiourea derivatives as anticancer showed that the derivatives of these compounds work as potential inhibitors of EGFR and HER-2 and have a high antiproliferation activity against MCF-7. Furthermore, recent studies on allylthiourea derivatives showed the activity of these compounds against T47D and MCF-7 line breast cancer cells.[13,14] In this study, the activity of allylthiourea derivatives, namely N-benzoyl-3-allylthiourea (BATU) [Figure 1], will be tested for the anticancer activity using MCF-7 cells with HER2 overexpression.

MATERIALS AND METHODS

Absorption, distribution, metabolism, excretion, and toxicity prediction

The prediction of pharmacokinetic properties including absorption, distribution, metabolism, excretion, and toxicity (ADMET) of BATU compounds was carried out using the online pkCSM tool program.[15-17] These BATU and comparative compounds were drawn in 2D and 3D molecular structures with ChemBioOffice Ultra 13.0 programs, which were subsequently stored as * .sdf files. Then, the structures of the BATU compound were translated into the SMILES format using the Online SMILES Translator program. In the SMILES format, these compounds were processed using the online pkCSM tool to predict ADMET of the compounds.[17]

Molecular docking

The *silico* test involved the use of human EGFR with ID code 3PP0 downloaded from Protein Data Bank (PDB). This protein contains the standard ligand SYR127063. The work procedure of the *in silic*o test was conducted in several stages, starting with the preparation of the test ligand used to make a 2D and 3D structure of the compound through the ChemBioOffice Ultra 13.0 programs. The next step was energy minimization with MMFF94. Then, the structures were stored with the extension * mol2/SYBYL2. Then, the Molegro Virtual Docker 5.5 program is used to carry out the molecular docking process.[17-19]

Cell culture

The test subjects used were MCF-7/HER-2 and MCF-7 cells, collected from the Nara Institute of Science and Technology, Japan. The cell cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) media containing FBS 10% (v/v) from Sigma and 1% (v/v) penstrep antibiotics from Nacalai Tesque. Then, 0.25% trypsin-EDTA from Nacalai Tesque was used for cell harvesting.

MTT assay

The MCF-7/HER-2 and MCF-7 cells were distributed into 96 well plates and incubated in a CO_2 incubator for 24 h. After which, the test solution was added in various concentration series and re-incubated for another 24 h. After that, each plate was added 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS from Sigma. The incubation continued for 4 h at 37°C until formazan was formed. The MTT reaction was stopped with 10% SDS from Nacalai Tesque in 0.01 N HCl from Merck, after which the incubation was allowed to continue overnight at room temperature. The uptake was read by ELISA reader at a wavelength of 570 nm and the absorbance results read were converted into the percentage of life.[14,20]

Western blot

In this method, 8×10^5 cells were planting in a 6 well plate and incubated for 24 h then treated with the sample for 24 h. After which, the cells were lysed using lysis buffer consisting of 1% NP40, 5 mM EDTA in Tris buffer, for 30 min. The cell

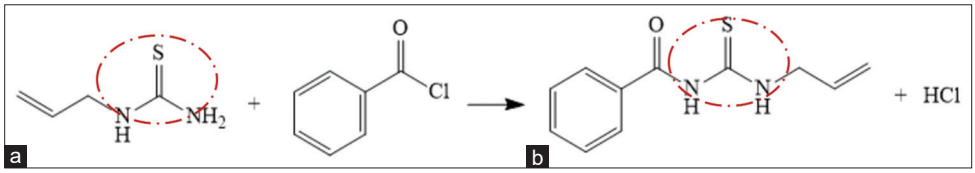

Figure 1: The route structure of N-benzoyl-3-allylthiourea. Allylthiourea (a) reacted with benzoyl chloride in an alkaline state will produce the target compound N-benzoyl-3-allylthiourea (b)

suspension was centrifuged at 15,000 rpm for 20 min. The SDS-polysaccharide gel was then electrophoresed with a current of 30–40 mA for approximately 1 h. Then, the protein was transferred to the Polyvinylidene difluoride (PVDF) membrane using a blotting machine for 1–2 h. Next, the PVDF membrane was blocked with the use of blocking buffer for 1 h, after which it was incubated with first antibodies which was anti-HER-2 at 1:50 dilution and anti-p65 at 1:300 dilution. The protein at that point formed hybridization with the first antibodies and then incubated with second antibodies known as antimouse IgG, at 1: 2000 dilution. The protein in the membrane was detected with the use of Chemilumi-one plus.

RESULTS

Absorption, distribution, metabolism, excretion and toxicity prediction

The predicted results of the pharmacokinetic properties of ADMET from the BATU and allylthiourea (ATU) compounds as well as the comparative hydroxyurea (HU) and lapatinib compounds are shown in Table 1.

Docking

A description of the interaction of amino acid residues from the BATU compound at the pdb code HER-2 receptor 3PP0 and the acquisition of the docking value (Rerank Score [RS]) is shown in Figure 2.

Based on the docking result, it can be known that BATU has binding energy with receptors which is reflected by the RS is lower than ATU. The BATU derivative also shows a lower RS than hydroxyurea.

Cytotoxicity assay

IC_{50} values of BATU compounds against MCF-7 and MCF7/HER2 cells by MTT method and amino acid residues that interact with BATU compounds are shown in Table 2. The results of cytotoxic tests on MCF-7 and MCF7/HER-2 breast cancer cells are shown in Figure 3.

The result of cytotoxicity showed that the treatment of BATU compounds caused changes in morphology and cell density of MCF-7/HER-2 along with an increase in test concentration. MCF-7/HER-2 breast cancer cells in control are wide in shape with clear cytosol, colonized, and attached to the base of the tissue culture dish. After the treatment of BATU compounds, some cells appear to be smaller and detached from TCD (a). The morphological changes of MCF-7/HER-2 cells are in line with the increase in the concentration of the test compound (b).

HER-2 protein expression and p-65 localization

The expression of HER-2 due to the treatment of BATU and ATU compounds at various concentrations of MCF-7/HER-2 cells and localization of p-65 transcription factor (nuclear factor kappa B [NF-kB]) by western blot method are shown in Figure 4.

The results of western blot analysis showed an increase in HER-2 protein expression along with an increase in

Table 1: Predictions of absorption, distribution, metabolism, excretion, and toxicity

Code	Absorption		Distribution		Metabolism		Excretion		Toxicity	
	Intestinal absorption (%)	Skin permeability (log Kp, cm/h)	VDss (logL/kg)	BBB permeability (logBB)	CYP2D6 substrate	CYP2D6 inhibitor	Tot clearance (logml/min/kg)	Renal OCT2 substrate	Ames toxicity (mol/kg)	Hepatotoxic
ATU	93.196	−3.026	−0.060	−0.063	No	No	−0.064	No	No	No
BATU	89.841	−3.123	−0.112	−0.263	No	No	0.095	No	No	No
HU	73.127	−4.319	−0.495	−0.545	No	No	0.659	No	No	No
LP	95.160	−2.735	−0.293	−0.737	No	No	0.557	No	No	No

BBB: Blood-brain barrier, BATU: N-benzoyl-3-allylthiourea, ATU: Allylthiourea, HU: Hydroxyurea, LP: Lapatinib, OCT2: Organic cation transporter 2, CYP2D6: Cytochrome P2D6 isoform, VDss: Steady state of volume distribution

Table 2: IC_{50} values of Rerank scores and amino acids interacting

Code	IC_{50} (mM)		RS (kcal/mol)	Amino acid residues interaction	
	MCF7	MCF7/HER-2		Steric-Bond	Hydrogen-Bond
BATU	1.47	0.64	−93.3816	Met801; Gly804; Thr798; Thr862; Gln799; Ala751; Ile752; Val797; Asp863; Asn850	Thr862; Met801
ATU	5.22	3.17	−53.0235	Thr862; Asp863; Lys753	Thr862
HU	2.79	2.00	−35.5542	Asp863; Asn850	-
LP	0.16	0.08	−154.6920	Ala751; Asp863; Asp808; Asn850; Thr862; Met801; Leu726; Lys753; Thr789; Ile752; Gly804; Val853; Leu852; Val851; Cys805	Asp863; Thr862; Met801
LS	-	-	−143.222	Ala751; Asp863; Asn850; Thr862; Met801	Asp863; Thr862; Met801

LS: Ligand standard SYR127063, BATU: *N*-benzoyl-3-allylthiourea, ATU: Allylthiourea, HU: Hydroxyurea, LP: Lapatinib, RS: Rerank score

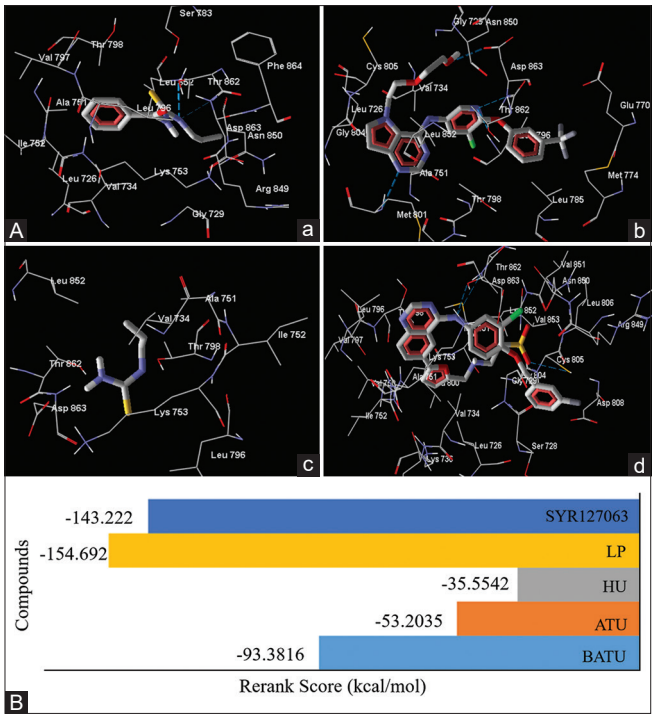

Figure 2: (A) Interactions between compounds (a) N-benzoyl-3-allylthiourea, (b) SYR127063, (c) allylthiourea and (d) lapatinib with amino acids at HER-2 receptors with using MVD programs are shown in 3D. In the picture, the blue lines show interactions in hydrogen bonds. Tests carried out on human epidermal growth factor-2, pdb codes 3PP0 by calculating the RMSD factor. (B) The graphs of *in silico* test results indicated by the Rerank score parameter values, from N-benzoyl-3-allylthiourea, allylthiourea, hydroxyurea, and LP, which are compared with the standard ligand SYR127063. The more negative value indicates the more stable interaction energy

the concentration of BATU compounds. At the lowest concentration (100 μM) did not show a different expression intensity compared to the control, and the intensity of expression increases with increasing concentration of BATU compounds.

DISCUSSION

The prediction results of the pharmacokinetic properties of ADMET [Table 1] show that the BATU compound has good skin permeability (log Kp <-2.5).[15] Similarly, for the prediction of distribution volume, the BATU compound has a log value of steady state of volume distribution (VDss) >-0.15, hence it has a fairly good distribution volume. However, the comparative compound has a low distribution volume since it has a log value of VD <-0.15. Additionally, the ability of BATU compounds to penetrate the blood–brain barrier (BBB) is quite low because it has a log value of BBB>-1. Therefore, there is a need for the consideration of the drugs' ability to penetrate the BBB in order to increase the efficacy of drugs whose pharmacological activities are in the brain. In addition, the ability of the compound to inhibit cytochrome P450 was shown by cytochrome P2D6 isoform (CYP2D6). Hence, from Table 1, it is shown that the BATU compound does not affect cytochrome P450. Furthermore, organic cation transporter 2 (OCT2) is a transporter in the kidneys which works an important part in the disposition and clearance of drugs as well as endogenous compounds. It is seen that all BATU do not affect the OCT2 substrate. Then, the toxicity of compounds was determined by the Ames toxicity test. This test assesses the mutagenic potential of a

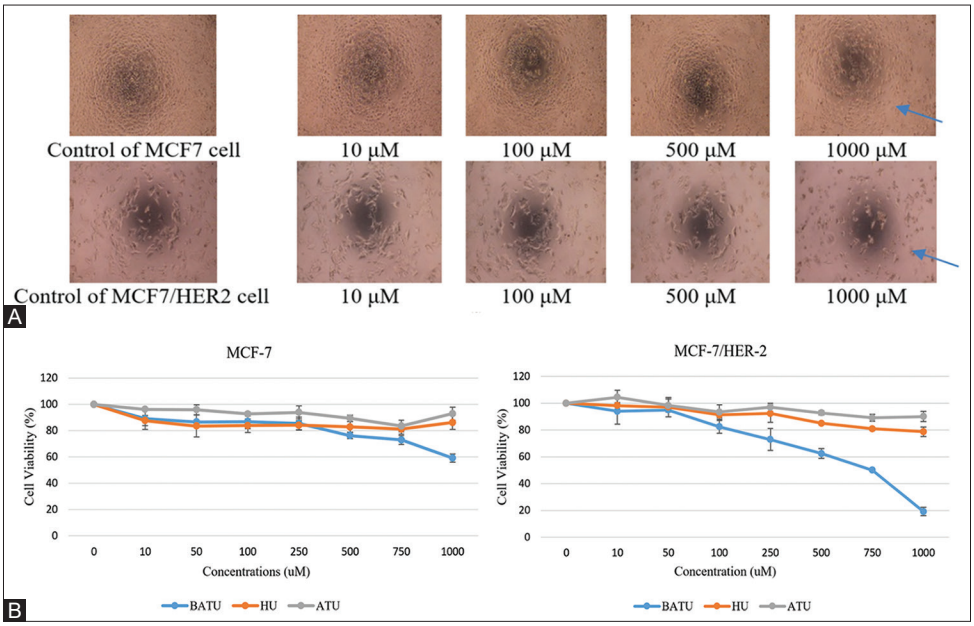

Figure 3: (A) Effect of sample treatment of N-benzoyl-3-allylthiourea compound on the morphology of MCF7 and MCF-7/HER-2 cells after incubation for 24 h. Observation of cell morphology at 24 h was carried out with an inverted microscope at a magnification of ×100. Blue lines indicate dead cells. (B) Percentage of living cells (cell viability) curve after treatment of test compounds in different concentrations of (a) MCF-7 and (b) MCF-7/HER-2 cells of N-benzoyl-3-allylthiourea, allylthiourea, and hydroxyurea compounds

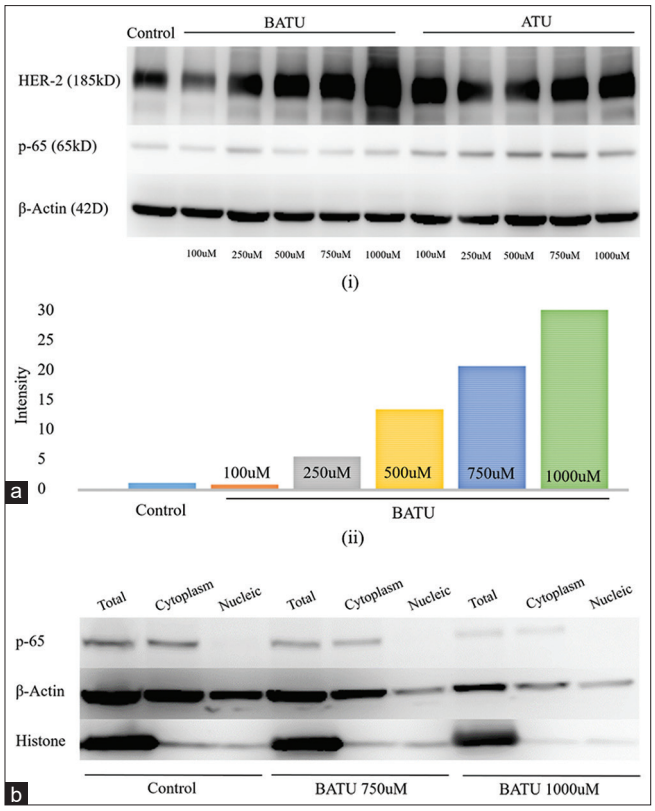

Figure 4: (a) (i) HER-2 expression of test compounds at various concentrations to MCF-7/HER-2 cells. Cells without treatment (control) and cells with treatment (N-benzoyl-3-allylthiourea and allylthiourea) with concentrations of 100, 250, 500, 750, and 1000 uM for 24 h, analyzed by western blot method. (ii) Analysis of the results was carried out using ImageJ® software to measure the intensity of the HER-2 expression band on the MCF-7/HER-2 cell line compared to the control. (b) Expression and localization of p65 (nuclear factor kappa B) due to the treatment of N-benzoyl-3-allylthiourea samples on MCF-7/HER-2 cells for 24 h by western blot method at N-benzoyl-3-allylthiourea concentrations of 750 uM and 1000 uM

compound with the use of bacteria. From Table 1, it can be determined that BATU compounds and comparators do not have mutagenic and nonhepatotoxic potential.

Furthermore, the BATU compound has a binding energy with the receptor as shown in Table 2, reflected by a lower RS compared to ATU. The BATU compounds also show a lower RS compared with HU, considering the fact that BATU derivatives have advantages over ATU and HU, and with the ability to bind with the amino acids Met801 and Thr 862 through hydrogen bonds, and amino acids Gly804, Thr798, Gln799, Ala751, Ile752, Val797, Leu796 through steric bonds as shown in Figure 2. Furthermore, BATU compounds interact with amino acid residues at the same ATP binding site as standard ligands, namely Thr862 and Met801 through hydrogen bonds. The results of this molecular modeling study showed that the BATU compound has the potential to be developed with the prediction of having higher biological activity compared with ATU and HU.

The results also show the IC_{50} value of BATU compound was smaller than ATU on MCF-7 and MCF-7/HER-2 cell line. This is an indication that the BATU compound has a higher cytotoxic effect on breast cancer cell compared with the ATU. Besides, the cytotoxic test also shows that the BATU compound has a higher activity against the MCF-7/HER-2 than on the MCF-7 cell line. Hence, it is suspected that the compound works more selectively against MCF-7 cells which are overexpressed by HER-2. The results of this cytotoxic test were strengthened by an analysis of HER-2 protein expression using the western blot method. The results of this western blot analysis show that the BATU compound exhibits expression activity on HER-2 protein.

It is also clear from the results from this study that the higher the concentration of the BATU compound, the higher the expression of HER-2 protein in MCF-7/HER-2 cell line. This study shows that the localization of p65 in the cytoplasm and inactivation of p65 in the cell nucleus were overexpressed by BATU compounds, with concentrations around IC_{50} values or higher concentrations of about 1000 μM. Therefore, this has proven the fact that BATU compound has the ability to effectively inhibit the activation of NF-kB.

Based on these results, there is need to do further research on BATU compound developed as a potential therapy drug and for the reduction of the occurrence of resistance to anticancer drugs for breast cancer with HER-2 expression. Moreover, the flow cytometry test can be proposed as a suggestion to be done in the next research to check if BATU could engage cell cycle profile.

CONCLUSION

Based on this study, it can be concluded that the BATU compound is effective in increasing HER-2 expression and inactivating NF-kB transcription factors, thereby resulting in inhibition of protein expression which works a significant part in cell proliferation. Therefore, the BATU compound has the potential to be developed as a complementary drug in breast cancer therapy with HER-2 positive.

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Conflicts of interest

There are no conflicts of interest.

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