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Original Article

Synthesis, molecular docking and antitumor activity of *N,N'*-Carbonylbis(*N*-Ethylbenzamide)

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ABSTRACT

We have designed and synthesized *N,N'*-carbonylbis(*N*-ethylbenzamide) to find new urea derivative with antitumor activity. The compound was synthesized from reaction of *N,N*'-diethylurea and benzoyl chloride in tetrahydrofuran. The designed molecule was docked into binding site of p38α MAP Kinase (pdb. 3HEG) to predict its binding affinity. The antitumor activity was tested *in vitro* on human breast cancer cell line (MCF-7 and T47D) by MTT assay and compared with hydroxyurea as a reference compound. The synthesis yield 30% and confirmed as *N,N'*-carbonylbis(*N*-ethylbenzamide) by spectroscpopic data. The compound showed higher *in vitro* antitumor activity than hydroxyurea either against MCF-7 or T47D and displayed better *in silico* binding property. It concluded that the synthesized compound is recommended to be developed further antitumor agents for human breast cancer.

Keywords: *N,N'*-carbonylbis(*N*-ethylbenzamide), synthesis, 3HEG, antitumor activity.

INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer-related deaths in 2012 [1]. The number of new cases is expected to rise by about 70% over the next 2 decades. More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths. Among women the 5 most common sites diagnosed were breast, colorectum, lung, cervix, and stomach cancer. Meanwhile, anticancer drugs that have long been used gradually becomes less effective and the cancer cells becomes resistant to anticancer drugs [2, 3, 4]. Therefore, there is an urgent need for development of new anticancer agents with divergent and unique structure which is different from the existing antitumor agents.

It has been well established that urea derivatives have got a significant place in modern medicinal chemistry. Urea derivatives have been reported in the literature as insecticide [5], anticonvulsant [6], antibacterial and antifungal agents [7], anticancer agent [8], hypoglycemic agent [9]. Furthermore, a large variety of urea derivatives were reported to possess potent inhibiting effects on receptor tyrosine kinases (RTKs) [10], raf-1 kinases [11], p38α MAP kinases [12]. The p38 mitogen-activated protein kinase (p38 MAPK) is a crucial component for the regulation of many cellular processes, including cell proliferation, differentiation, survival and migration [13]. Recent evidence has shown that p38α may also play a causative role in cancer.

Hydroxyurea (HU) is a classical anticancer drugs which is still usefull to treat several types of cancer, but not including breast cancer [14, 15, 16]. Nevertheless, it has been reported that HU has antitumor activity on breast cancer cell line with IC_{50} of 307 μM [17]. Another urea derivative, sorafenib, is an inhibitor of several tyrosine kinase and Raf kinase [18]. Nambodiri et al. (2010) reported that sorafenib could interacted with p38α MAPK [19]. *In silico* study indicated that N-CO-N in urea's structures are pharmacophores that capable to form hydrogen bonding with amino acid in binding site of the target enzymes [20].

A serie of ring-substituted benzoylurea derivatives had reported were more active than hydroxyurea according to Brine Shrimp Lethality Test. Quantitative Structure Activity Relationship and *in*

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silico study revealed that binding affinity of benzoylurea derivatives to the enzyme were better than hydroxyurea due to steric (MR) and hydrophobic (log P) factors and the presence of aromatic ring in the structure of urea [21]. In subsequent study, benzoylurea has been modified into *N,N'*-dibenzoyl-*N,N'*-dimethylurea which exhibits antitumor activity against human breast cancer cell line, MCF-7 [22]. Methyl substitution on both the N urea and one additional aromatic ring on the other N increase the activity of benzoylurea. Antiproliferative activity test using MTT assay was reported by many researcher [23, 24]. El-Shawy et al. (2011) and Lu et al. (2013) reported that aromatic ring and urea group in benzylurea is the pharmacophore responsible to tumor growth inhibition activity of some benzylurea derivatives [25, 26].

In the present study, we attached two ethyl gropups and two aromatic rings on both N atoms of urea to obtain *N,N'*-carbonylbis(*N*-ethylbenzamide). The synthesis procedure was based on Schotten-Baumann acylation method [27], with modification in solvent. Antitumor activity were tested using MTT assay against two human breast cancer cell line, T47D and MCF-7, and compared with two anticancer drugs, hydroxyurea which containing same pharmacophore and 5-fluorouracil which has been clinically used in the treatment of breast cancer. *In-silico* activity were performed by Molegro Virtual Docker 5.5 using 3HEG as target molecule.

MATERIALS AND METHODS

General: All reagents and solvents were purchased from standard commercial suppliers. *N,N'*-carbonylbis(*N*-ethylbenzamide) **(Figure 1)** were synthesized from the reaction of starting material *N,N'*-diethylurea with benzoyl chlorides in the organic solvent, tetrahydrofuran (THF), and using triethylamine as catalyst. The structure of synthesized compound were identified by IR, ^{1}H-NMR, ^{13}C-NMR, and MS spectroscopy; whereas their purity were determined by melting point and TLC tests.

Melting point was measured with an Electrothermal melting point apparatus without correction. IR spectrum was recorded in KBr on Jasco FT-IR 5300 and major absorption was listed in cm^{-1}. ^{1}H-NMR and ^{13}C-NMR spectrum was taken at JEOL ECS-400 spectrometer (400MHz), and chemical shifts were reported in ppm on the δ-scale from internal standard Me_4Si. Mass spectrum was measured with Agilent 6890N spectrometer using EI method. TLC was carried out on aluminium plates coated with silica gel F_{254} (E Merck) using UV lamp 254 nm to spot detection. *In silico* molecular docking was running out using Molegro Virtual Docker (MVD) ver 5.0 (CLC Bio). CS ChemBioDraw Ultra ver 11.0 (Cambridge Soft) was employed to built 3D structure of compounds. Antitumor activity was tested *in vitro* against MCF-7 and T47D cell line by MTT method and expressed in IC_{50}, concentration of the compounds inducing a 50% inhibition of cell growth of treated cells compared to the growth of control cells. Hydroxyurea (HU) and 5-fluorouracil (5-FU) were used as the reference drugs. The absobance at 595 nm was recorded using ELISA Microplate Reader (Benchmark).The IC_{50} values were calculated by probit analysis using SPSS 20.

Synthesis of *N,N'*-carbonylbis (*N*-ethylbenzamide): *N,N'*-diethylurea 0,0125 mol dissolved in 20 ml THF was mixed with 4 ml triethylamine in a 200 ml conical flask. The solution of benzoyl chloride 0,0250 mol in 20 ml THF then added drop wise to the mixture at 0–5 °C ice bath while stirring. Then the temperature was raised to 70 °C and the mixture was refluxed for 3 hours. Reflux was continued at room temperature for next 20 hours and the mixture was concentrated by evaporating the solvent. The product was washed with cold water and saturated sodium bicarbonate respectively, and the solid residu was crystallized from ethanol–water (1:1).

***In silico* Molecular Docking:** The structure of p38α MAP kinase (pdb. 3HEG at 2.2 Å resolution) was obtained from the Protein Data Bank (www.rcsb.org) [28]. Compound structures were built with ChemBioDraw Ultra 11.0 and their geometry optimization were performed using the MMFF94 method in the program and saved as Sybyl Mol2 format. In the molecular docking, the test compounds were placed into binding site of 3HEG (cavity-1) by align method to the reference ligand (BAX). BAX is sorafenib molecule, an urea anticancer drug which capable to complex with the enzyme [19]. The binding affinity between ligand and enzyme (docking score) was predicted using MolDock Score and post analysed as Rerank Score (RS). The highest-scoring pose (lowest energy) should represent the best-found binding mode. Evaluation of the interaction was based on the their RS which is the sum of ligand-protein interaction energy and internal ligand energy, including hydrogen bonds between ligands and protein. The validation of docking was carried out by redocking the BAX into binding site (cavity-1) of 3HEG. A more negative binding affinity indicates stronger binding. The best docked structures have to follow these criteria : i) they must occupy the same cavity in the enzyme similar to BAX, (ii) they have the highest-scoring pose (lowest binding energy);

which can be observed visually by comparing the structure of docked molecule with crystal structure of BAX inside the binding site. The ligand-protein complexes of the top-scoring poses were used for further visual inspection.

***In vitro* Antitumor activity:** In vitro antitumor activity was tested by MTT assay and expressed in IC_{50}, concentration of the compounds inducing a 50% inhibition of cell growth of treated cells compared to the growth of untreated cells [24]. Hydroxyurea and 5-Fluorouracil used as reference drugs. Cells were seeded at a density of $8x10^4$ cells/well into 96- well microplates, followed by treatment with tested compounds at concentration ranging between 100 – 1000 µg/mL and incubated in 5 % CO_2 incubator at 37 ^{0}C. After 24 hours, each wells were added by 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (100 µL of 0.5 mg/mL MTT in PBS). Then 100 µL 10 % SDS in 0.1 N HCl was added after 3 hours and plates were incubated in the dark at 37 ^{0}C overnight. Cell viability was determined from the absorbance which was observed at 595 nm on ELISA-reader and exppressed in percentages with respect to untreated cells. The IC_{50} values was calculated with probit analysis. Each experiment was performed at least three times.

RESULT AND DISCUSSION

Synthesis product, *N,N'*-carbonylbis (*N*-ethylbenzamide): *N,N'*-carbonylbis(*N*-ethylbenzamide) **(Figure 1)** were synthesized from *N,N'*-diethylurea in one steps. The compounds is white solids and water insoluble substances. Structure of the compound were identified by IR, ^{1}H-NMR,^{13}C-NMR, and MS spectroscopy as follows : Yield 30% as white crystal, m.p. 147 ^{0}C. ^{1}H-NMR ($CDCl_3$, 400 MHz): 1,1 (6H, t, *J*=7.2 Hz, CH_3); 3,2 (4H, m, CH_2); 7,3 (10H, m). ^{13}C-NMR ($CDCl_3$, 400 MHz) : 13,47 (C_8); 42,99 (C_7); 127,65 (=C_4); 128,67 (=C_5); 132,21 (=C_6); 136,03 (=C_3); 159,03 (C_1=O); 170,96 (–C_2=O). IR (KBr), ν_{maks} (cm^{-1}) : 3087 (Csp_2–H), 2977 (C–H), 1717 (C=O_{ar}), 1676 (-C=O(–N–)), 1580 (C=C), 858 (C–H_{ar}). MS (EI): m/z 324 (M^+), calculated Mass $C_{19}H_{20}N_2O_3$: 324.15.

The synthesis procedure used was capable to produce the designed compound. The main feature for the formation of the compound is the absence of hydrogen on both N of diethylurea and the presence of 2 benzoyl grooup. This is proved by ^{1}H-NMR spectrum showing an additional H peak in the benzene region (δ= 7,3 ppm). The C=O of benzoyl is proved by ^{13}C-NMR chemical shift at 170 ppm, supported by the IR spectrum showing absorption band at 1717 cm^{-1} and 1676 cm^{-1}.

The *N,N'*-carbonylbis(*N*-ethylbenzamide) was produced by nucleophilic acylation [27] between benzoyl chlorides and *N,N'*-diethylurea. The structural change of *N,N'*-diethylurea to *N,N'*-carbonylbis(*N*-ethylbenzamide) was characterized by the conversion of –NH– moeity of diethylurea to –N–C=O(ar). As there are two symmetric -NH-moeity, the new compounds bear two symmetric benzoyl group, each attach on their N atom.

Antitumor Activity: The synthesized compound was subjected to *in vitro* antitumor activity test on human breast carcinoma cell line, compared with HU and 5-FU. The results were listed in **Table 1**. Table 1 show that *N,N'*-carbonylbis (*N*-ethylbenzamide) have IC_{50} lower than hydroxyurea and 5-FU. This indicated that the compound had antitumor activity against MCF-7 and T47D cells higher than HU. Its activity on MCF-7 also higher than 5-FU, an anticancer drug that is clinically used in the treatment of human breast carcinoma. It seems that benzoyl and ethyl group on the structure of urea may increase antitumor activity.

Molecular Docking: The capability of tested compound to interact with target molecule 3HEG was investigated by docking study, and the results were compared to HU. Human breast cancer were known to overexpress in MAP kinase, represented by 3HEG in this study. It was reported that HU could interacted with ERK, one type of this enzyme [29]. *N,N'*-carbonylbis(*N*-ethylbenzamide) display better score (RS= -74.55) than HU (RS= -37.12) which indicate high affinity of the new compound to 3HEG. Further visual inspection denoted that the carbonyl (C=O) group and two N atom of *N,N'*-carbonylbis(*N*-ethylbenzamide) capable to form hydrogen bonds with amino acid residue Asp 168 in binding site of 3HEG (cavity-1). There is no hydrogen bond between HU and 3HEG. These interaction denoted the importance of N–CO–N moeity of urea for binding and the subsequent inhibitory capacity. Besides hydrogen bonds, there are van der Waals intermolecular forces (hydrophobic interactions) performed by compound with amino acid residues Asp 168, Tyr 35, Glu 71, Leu 167, and Phe 169. Such interactions supported the better score (RS) for the compound compare with the HU. Molecular docking of *N,N'*-carbonylbis(*N*-ethylbenzamide) to 3HEG is shown in **Figure 2** and **Figure 3**. The comparative docking study of the *N,N'*-carbonylbis(*N*-ethylbenzamide) with anticancer drug hydroxyurea supports the molecular design of this new compound.

CONCLUSION

of *N,N'*-carbonylbis(*N*-ethylbenzamide) has been designed and successfully synthesized from *N,N'*-

diethylurea and benzoyl chloride. The compound showed higher activity against human breast cancer cell lines (MCF-7 and T47D) than hydroxyurea. The compound was also more active against MCF-7 compared with 5-fluorouracil, an anticancer drug which has been clinically used in the treatment of human breast cancer. It is recommended to develop the compound as antitumor agents for human breast cancer.

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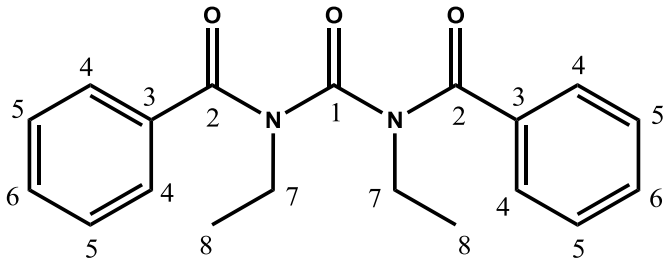

Figure 1: Structure of *N,N'*-carbonylbis(*N*-ethylbenzamide)

Table 1. IC_{50} growth inhibition activity of tested compounds against human breast cancer cell lines

Compound	IC_{50} (mM)	
	MCF-7	T47D
N,N'-carbonylbis(*N*-ethylbenzamide)	0.64	0.79
Hydroxyurea	31.32	5.60
5-Fluorouracil	15.37	*

* = not determined

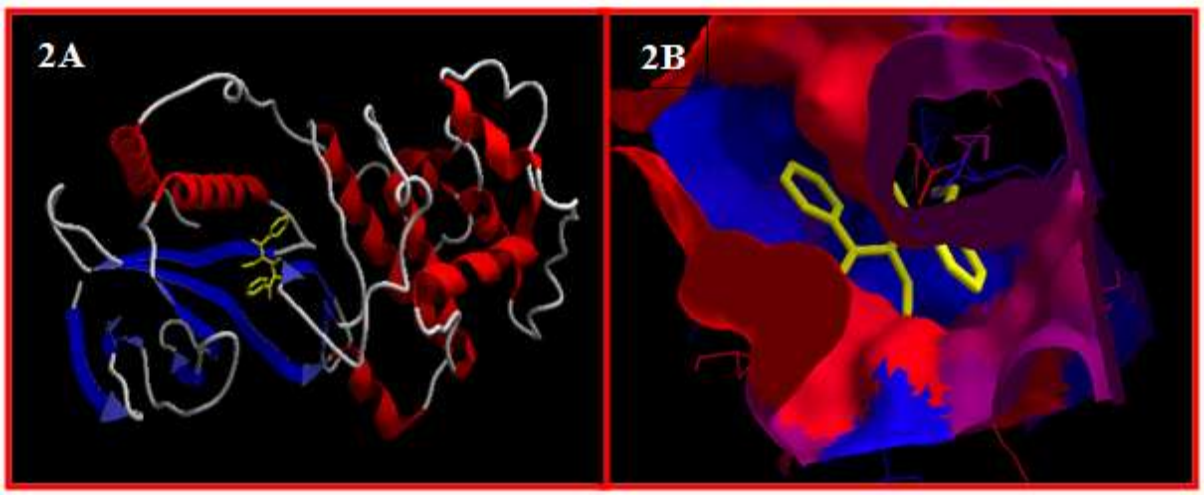

Figure 2 : docked conformation of *N,N'*-carbonylbis(*N*-ethylbenzamide) molecule in secondary structure of p38α MAPK (2A), and in hydrophobic environment of enzyme's binding site, cavity-1 (2B)

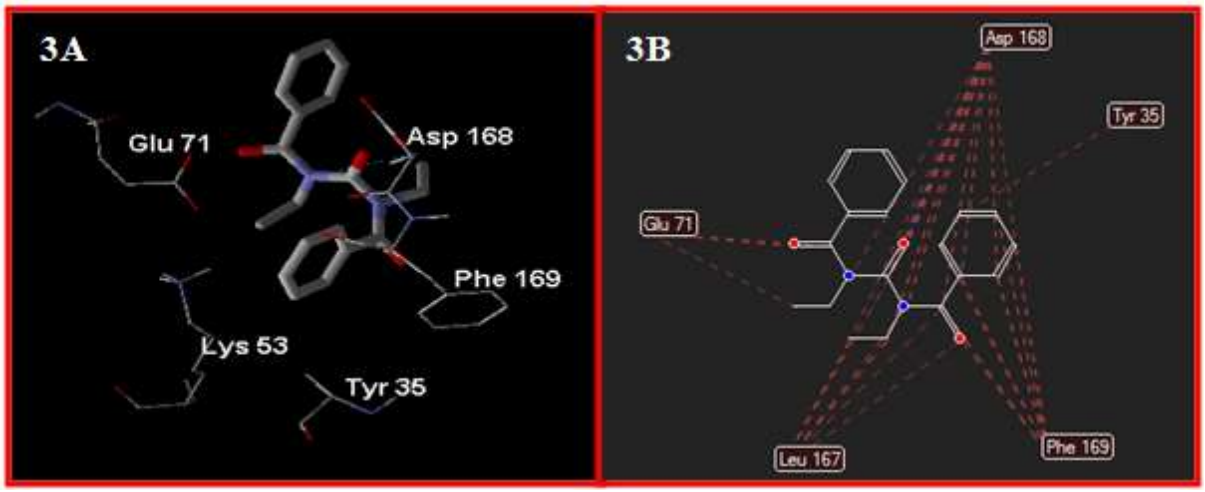

Figure 3 : docked conformation showing hydrogen bonds between *N,N'*-carbonylbis(*N*-ethylbenzamide) molecule and amino acid residue Asp 168 (3A), and 2D-picture showing hydrophobic interaction performed by compound with amino acid residues Asp 168, Tyr 35, Glu 71, Leu 167, and Phe 169 (3B).

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