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Antimicrobial Activity and Molecular Docking of Benzoyl-*N,N'*-dialkylurea against Target Proteins in Microbial Cells

Aktivitas Antimikroba dan Docking Molekul Benzoil N,N'-dialkilurea terhadap Target Protein-protein dalam Sel Mikroba

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ABSTRACT

This research aims to evaluate the antimicrobial activity of benzoyl dialkylurea derivatives to meet the global need for new antibiotic lead compounds. Four compounds (BDMU, BEU1, BEU2, BEU3) were tested *in vitro* against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Candida albicans* by broth dilution method using ciprofloxacin and nystatin as standards. Docking simulations were performed to target essential proteins including DNA gyrase, FabH, RNA polymerase in bacterial cells and DHFR in *C. albicans*. The BDMU, BEU1, BEU2, BEU3 exhibited antibacterial activity while BEU1 and BEU2 showed weak antifungal activity against *C. albicans*. The BDMU, BEU2, BEU3 considered promising growth inhibition against *P. aeruginosa*. *In silico* molecular docking on DNA gyrase from *P. aeruginosa* (PDB. 6M1S) was proposed as a model for mechanism of action in bacterial cells. The monobenzoyl of dialkylurea containing urea functionality with chloro-substituent at position of 2 and 4 on aromatic rings were potential as lead compound to generate new antibacterial agent.

Keywords: Benzoyl-*N,N'*-dialkylurea, antimicrobial activity, five microbes, molecular docking

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi aktivitas antimikroba turunan benzoil dialkilurea untuk memenuhi kebutuhan global akan antibiotika baru. Empat senyawa diuji secara in vitro terhadap Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, dan Candida albicans dengan metode pengenceran agar menggunakan siprofloksasin dan nistatin sebagai standar. Simulasi docking dilakukan untuk target protein esensial termasuk DNA gyrase, FabH, RNA polimerase pada sel bakteri dan DHFR pada C. albicans. Senyawa BDMU, BEU1, BEU2, BEU3

menunjukkan aktivitas antibakteri, sedangkan BEU1 dan BEU2 menunjukkan aktivitas antijamur yang lemah terhadap C. albicans. BDMU, BEU2, dan BEU3 dianggap mampu menghambat pertumbuhan P. aeruginosa. Docking molekuler in silico pada DNA gyrase dari P. aeruginosa (PDB. 6M1S) direkomendasikan sebagai model untuk mekanisme kerja dalam sel bakteri. Monobenzoil dialkilurea yang mengandung gugus fungsi urea dengan substituen kloro posisi-2 dan 4 pada cincin aromatik berpotensi sebagai senyawa utama untuk menghasilkan agen antibiotika baru.

Kata kunci: *Benzoil-N, N'-dialkilurea, aktivitas antimikroba, lima mikroba, docking molekuler*

INTRODUCTION

Antibiotics resistance is a global problem, therefore it is very important to search new chemical structure scaffoldings that are potential to be developed as the new antimicrobial drugs (Li et al, 2011). Urea derivatives have diverse biological activities including antibacterial (alkylurea derivatives (Zheng et al, 2010; Umadevi et al, 2012), anticonvulsant (Librowski et al, 2007), antiparkinson (Azam et al, 2012), and anticancer (El-Sawy et al, 2012; Lokwani et al, 2011; Petitclerc et al, 2004). Various urea derivatives were also possessed inhibitory effect on acyl-coenzyme A-cholesterol acyltransferase (ACAT) (Higley et al, 1994), NADH oxidase (Morre et al, 1994), HIV protease enzyme (Deeb et al, 2012), receptor tyrosine kinases (Sammond et al, 2005; Mitchell et al, 2009), raf kinases (Zhan et al, 2012), and cytokinin oxidase (Kopečný et al, 2010).

Since urea functional group was a central moiety in many drug candidates, this research was focused on develop antimicrobial agents containing the urea functional groups, in this case benzoyl diethylurea derivatives which was not difficult to be synthesized by a single step reaction (Diyah et al, 2017).

Previous studies reported the synthesis and activity of some benzoyl dialkylureas against human breast cancer cells (Diyah et al, 2014; Diyah, 2014). It was found that the compounds with one aromatic ring (mono-benzoyl derivatives) showed lower activity than two rings aromatic compounds. Considering that mono-benzoyl derivatives have some degree of cytotoxicity against tumor cells, it is necessary to study their toxicity towards prokaryotic cells as the basis for design of new compounds that active as antibacterial agents.

This exploration of antimicrobial candidates focuses on its molecular interactions with enzymes that are essential for metabolic process due to the one of antimicrobial mechanism: by inhibiting bacterial growth through the inhibition of enzymes that are essential for metabolic process such as: DNA gyrase, β-ketoacyl-acyl-carrier protein synthase III (FabH) (Li et al, 2011; Champoux, 2001); prokaryotic RNA polymerase (Pommier et al, 2010); and dehydrofolate reductase (DHFR) (Choi et al, 2000). The previous studies showed that some arylureas inhibit FabH, a core enzyme in fatty acid biosynthesis and that is highly conserved among Gram-positive and Gram-negative bacteria (Li et al, 2011; Choi et al, 2000). Prokaryotic RNA polymerase is also an important target for antibacterial agents because it is essential for bacterial growth (Esteves-Souza et al, 2006). On the other hand, the effectiveness of DHFR inhibition has been demonstrated by DHFR inhibitor drugs which are used as anticancer, antiprotozoa, and antibacterial (Choi et al, 2000). The drugs act as DHFR inhibitor targeting pathogenic microbes must also be selective on the microbial cells to avoid toxicity against host cells. There has been some effort to develop DHFR inhibitors of *C. albicans* (Liu et al, 2008).

This research intends to explore the potency of selected derivatives as antimicrobial agents by screened their bioactivity against *S. aureus*, *B. subtilis, E. coli, P. aeruginosa,* and *C. albicans*. Interactions of these compounds with target proteins was performed by molecular docking on some enzymes from microbes similar to the strains used in the *in vitro* antimicrobial assay, e.g DNA gyrase from *E. coli, S. aureus,* and *P. aeruginosa*, FabH from *E. coli* and *S. aureus*, RNA polymerase from *B.subtilis*, and DHFR of *C. albicans*.

MATERIAL AND METHODS

Materials

The test compounds including (1) N,N'-carbonylbis(N-methylbenzamide (DBMU); (2) 3,5-dichloro-N-ethyl-N-(ethylcarbamoyl) benzamide (BEU1); (3) 2,4-dichloro-N-ethyl-N-(ethyl carbamoyl)benzamide (BEU2); and (4)4-

nitro -N-ethyl-N-(ethylcarbamoyl) benzamide (BEU3) DBMU, DBEU1, DBEU2, BEU1, BEU2, and BEU3 (Fig. 1) were synthesis products and available in our laboratory. Before being used for this study, the compounds were recrystallized and identified by TLC and melting points test was carried out using Fischer-Johns apparatus. The results obtained were compared to previous data (Li et al, 2011; Diyah et al, 2017; Diyah et al, 2014).

Nutrient agar media was used for sub culturing and preliminary antibacterial activity, while nutrient broth media was used for antimicrobial assay. Saboraud-dextrose agar and broth media were used for *C. Albicans* (Khadka et al, 2017). All the culture media were prepared and treated according to the manufacturer guidelines.

Antimicrobial assay

Antimicrobial activities were tested against Gram-positive bacteria (*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633), Gram-negative bacteria (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853), and a fungal strain *C. albicans* ATCC 2091. Antimicrobial activities were determined by conventional broth dilution method (EUCAST, 2003), using ciprofloxacin and nystatin as standards of antibacterial and antifungal respectively. The Antimicrobial activities were expressed as minimum inhibitory concentration (MIC), which is defined as the lowest concentration at which no visible microbial growth is observed (Andrews, 2001).

The test microorganisms were sub cultured before used for antimicrobial assay. The culture was inoculated onto slant agar medium and incubated at 37^{o}C, 24 hours and 27^{o}C, 48 hours for bacterial cells and *C. albicans* respectively. 10 mL of sterile saline solution (NaCl 0.9% m/v) was added to the slant agar tube and the colonies were remove from the agar media by vortex. The turbidity of the suspensions was adjusted to obtain a transmittance of 25.0% at 580 nm (Depkes RI, 1995), which is equivalent to approximately 3.0x10^{8} CFU/mL (Rojas et al, 2006).

The stock solutions of the tested compounds and reference drugs were prepared at 10000 µg/ml in methanol-water (1:1) followed by twofold serial dilution at range concentrations of (1000 to 50µg/ml) using the proper broth media. Series of tubes were inoculated with the 10µL microbial inoculum in 1 mL suitable broth media, then each tube was added by 1 mL of the test solution. All the tube and control test (solvent and uninoculated media) were incubated at the same condition in inoculum preparation. Microbial growth was observed visually and all tests were conducted in triplicate.

Molecular docking studies

In silico molecular docking aims to investigate the ligand-biomolecular interactions and the feasible binding geometries of an assumed ligand with a target protein (Dar A.M and Mir S, 2017). To find the best target in microbial cells, three-dimensional (3D) crystal structure of target proteins including (1) DNA gyrase from *E.coli* (PDB:1S14), (2) from *S.aureus* (PDB:2XCT), and (3) from *P. aeruginosa* (PDB:6M1S); (4) RNA polymerase from *B. subtilis* (PDB:4NC7); (5) DHFR from *C. albicans* (PDB:1AOE); (6) FabH from E. coli (PDB:5BNM) and (7) from S. aureus (PDB:6KVS) were used and retrieved from www.rcsb.org. Docking simulation was proceed using the Molegro Virtual Docker (MVD) version 6.0 program and further inspection of molecular interactions was observed by Discovery Studio Visualizer (DSV) 2016.

Two-dimensional (2D) structure of ligands were built using ChemDraw Pro 16.0 to obtain physicochemical properties such as logP, water solubility (logS), and topological polar surface area (tPSA), as was shown in Fig. 1. The structures were converted to 3D to calculate energy of optimal geometry (E_{total}) using MMFF94 method, then were saved in *.mol2 format.

To prepare the proteins prior to docking, the 3D structure of target proteins in pdb format was imported into the workspace of MVD program and then binding site containing co-crystallized ligand was detected as cavity. 3D structure of test ligand was imported into cavity by align method to the co-crystallized ligand which served as reference ligand. Molecular docking was executed using the MVD protocol and default parameters. The MolDock score (MDS) was selected as representation of binding energy. Seven reference ligands were used in separated docking validation according to each protein targets.

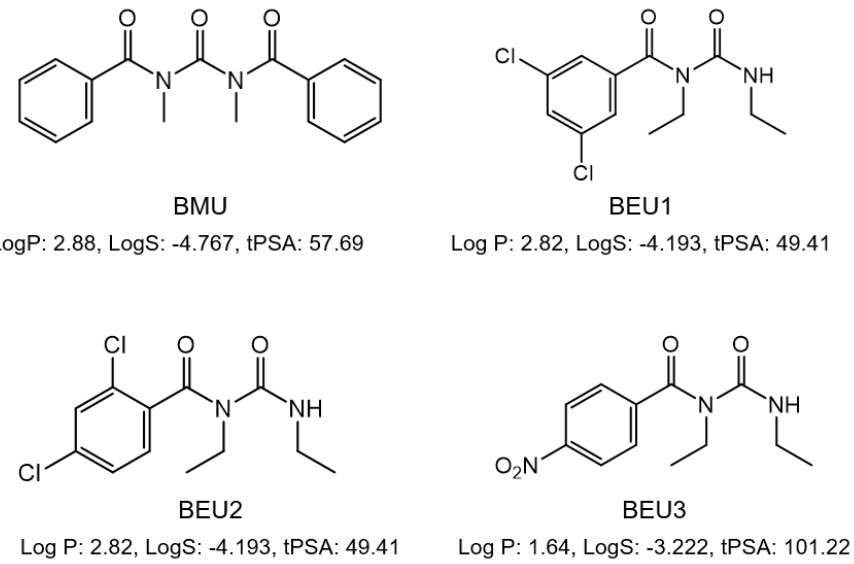


Figure 1. Chemical structure and physicochemical properties of test compounds.

RESULTS AND DISCUSSION

Antimicrobial activity

The MIC against four strain microbes were displayed in Table 1. The BDMU, BEU1, BEU2, BEU3 exhibited antibacterial activity, while BEU1 and BEU2 showed weak antifungal activity against *C. Albicans*. BEU1 and BEU2 were mono-benzoyl derivatives containing two chloro-substituents in a benzene ring.

Table 1. Antimicrobial activity (MIC) of the compounds

Compound	Minimum inhibitory concentration (μg/mL)* against				
	S.aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*
BMU	100	250	100	75	>500
BEU1	500	150	250	125	300
BEU2	100	100	87.5	37.5	250
BEU3	100	150	87.5	50	>500
Ciprofloxacin	0.5	12.5	10	3	-
Nystatin	-	-	-	-	2

* *average from triplicate, (-): not active*

Data in Table 1 showed the first four compounds were more active against *E. coli* and *P. aeruginosa*, different from most of urea derivatives that revealed better activity against the Gram-positive rather than the Gram-negative bacteria (Yavuz S and Yildirim H, 2013). These results indicated the test compounds have potency to be developed as antibacterial agents against Gram-negative bacteria, especially to *P. aeruginosa* which was known as nosocomial bacteria. Ciprofloxacin, a synthetic flouroquinolone derivatives, which served as reference drug showed potent activity against *S. aureus*, *S. pneumonia*, *E. coli*, and *P. aeruginosa*.

Regarding physicochemical aspect, the results indicated that *in vitro* antibacterial activities were in accordance with LogP and LogS (water solubility). Lipophilicity is an important factor for exploring the mechanism of action of antimicrobial agent against pathogenic bacteria (Shi et al, 2018). Compounds BEU2 and BEU3 which showed high antimicrobial activity had lower

lipophilicity. The similar result also found in auranofin analogs with lower logP values (1–2) that exhibited higher activity than some analogs with higher logP (>3) against *S. aureus, E. coli,* and *P. aeruginosa* (Wu et al, 2019). However, DBMU (log P= 2.88) still generated antibacterial activity due to its polar surface available to intermolecular interaction was lower than BEU3. Clog P between 2 and 3 are often considered optimal for oral drugs. It has been considered that compounds with Clog P< 5 have more promising drug-likeness characteristic (Schübler et al, 2017). The four compounds in this experiment possessed log P value in a range of 1–3.

Docking Results

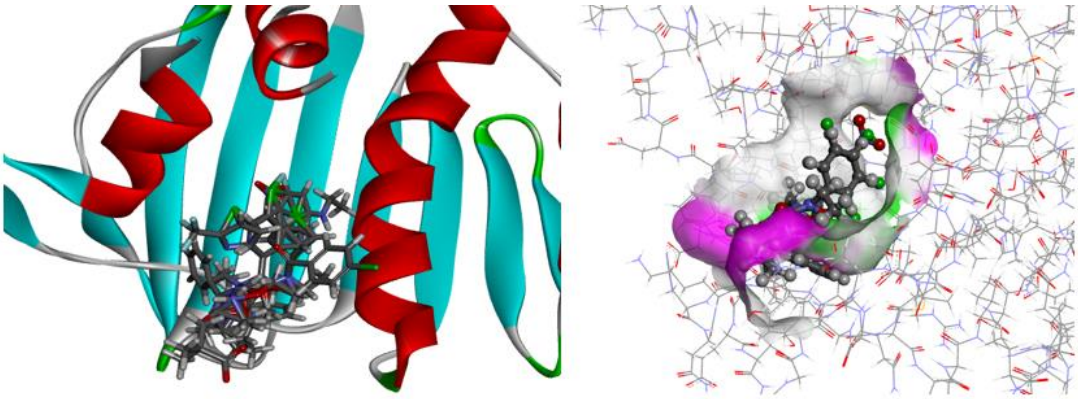
The docking experiments performed against seven target proteins were carried out to investigate the interaction responsible for antibacterial activity and to find out the most dominant type of protein. Validation of docking were done by re-docking each of reference ligands on their binding sites and re-docking was accepted if the RMSD< 2.0Aº.

Based on statistical analysis applied to docking score in Table 2, the MDS was not significantly different (P>0.05), by which indicated that compounds could interacted with all enzyme models. In binding site of *S. aureus* gyrase, both CPF and test ligands didn't form hydrogen bonds and their interaction patterns were different. This evidence inferred the binding mode of test compounds were not same as ciprofloxacin. Docking study exploring the conformation of all docked molecules within the binding site of gyrase (PDB. 6M1S) showed that ligands confined within the binding site and take similar conformation with reference ligand (Fig. 2).

Table 2. Binding energy, number of hydrogen bonds, and amino acids interacting with compounds in each binding sites of target proteins.

Compound	Docking Result	Target Protein (PDB ID)						
		2XCT	1S14	6M1S	4NC7	1AOE	6KVS	5BNM
DBMU	MDS	−82.67	−103.96	−89.53	− 104.62	−108.70	−91.60	−83.13
	H-bond	no	2: Ser1043, Thr1163	2: Arg138	1: Asn62	no	no	no
BEU1	MDS	−84.99	−90.03	−95.98	−100.787	−94.848	−87.783	−56.975
	H-bond	no	1: Ser1043	2: Arg78, Gly78	no	no	no	no
BEU2	MDS	−81.26	−99.93	−97.397	−93.615	−101.538	−78.746	−79.578
	H-bond	no	2: Asp1069, Ser1043, Thr1163	2: Arg138, Glu52	1: Leu70	1: Ile112	no	no
BEU3	MDS	−80.93	−86.40	−91.06	−93.48	−106.22	−85.94	−82.53
	H-bond	no	6: Asp1069 Arg1072, Ser1043, Thr1163	1: Arg78	3: Asn62	no	1: Cys425	no
Reference Ligand*	MDS	−71.21	−145.29	−190.62	−77.66	−110.68	−90.49	−103.18
	H-bond	no	4: Arg1132 Asn1042 Asn1077 Asp1069	3: Arg78 Ser49 Thr167	1: Leu68	3: Glu32 Ile9 Ile 112	3 : Cys425 Gly613	3: Asn247 Cys112 His244

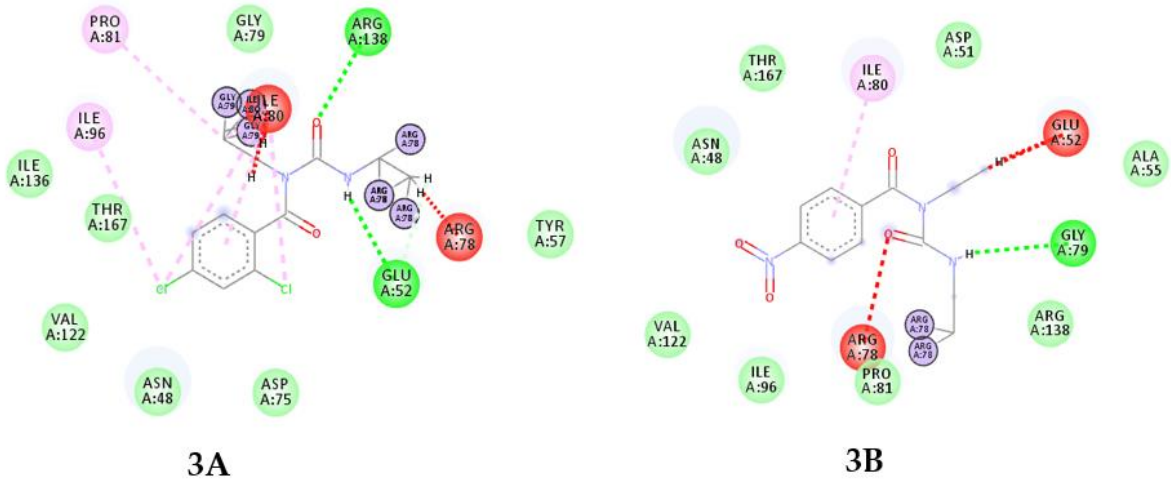
*Notes: reference ligands were CPF, NOV, EZ9, , I3C, GW3, OAX , and 4VK respectively to the right side



2A 2B

Figure 2. Docked conformation of test compounds in binding site (cavity-1) of *P. aeruginosa* DNA gyrase (PDB. 6M1S). All ligands in binding site of protein in ribbon format (2A), Three ligands (BEUs) within H-bond environtment of amino acids, molecules as ball and stick format colored by element (2B).

The binding site analysis showed that mono-benzoyl BEU and dibenzoyl dimethylurea (BMU) form two hydrogen bonds with arginine (Arg78 and Arg138), the same amino acid which interacted with reference ligand EZ9 and their docked conformations also similar with EZ9 (Fig. 2). It can be inferred that the compounds have the same binding mode as reference ligand. The most active antibacterial compound BEU2 was used to suggest the binding properties and inspect their binding pattern against DNA gyrase (Fig. 3).



3A 3B

Figure 3. Docking interactions with amino acids in P. aeruginosa DNA gyrase (PDB.6M1S). BEU2 shows two H-bonds and more covalent interactions (3A) than BEU3 (3B). Green dashed-line denoted H-bond, purple circle is covalent bond, pink dashed-line pointed π-alkyl interaction, red dashed-line depicted unfavorable proton transfer from amino acid.

Results displayed BEU2 binds with amino acid residues, couple of hydrogen bonds were observed at Arg138 and Glu52 with different bonding distances. The oxygen atom of carbonyl linked to benzene ring held hydrogen bond with Arg138 with bond length 2.38 Å, whereas hydrogen atom at secondary amino group was involved in hydrogen bond at Glu52 through bond length 2.27 Å. Three amino acids Ile80, Ile96 and Pro81 bind BEU2 through hydrophobic interactions, which are alkyl-alkyl interaction between atom carbon of ethyl in BEU2 and amino acid Pro81, phi-alkyl interaction between carbon of aromatic ring and amino acid Ile80, interaction of lone pair electron of both chloro-substituent at 2 and 4-position with Ile80, and chlorine at 4-position with Ile 96. These interactions along with hydrogen bonding indicated good stability in BEU2-6M1S complex.

To select the mechanism of interaction which may underlaid the antibacterial activity, the following criteria were used: (1) RMSD of test compound must be less than RMSD of reference ligand, (2) the compound formed hydrogen bond with amino acid in the binding site, (3) the same interacting amino acid as the reference ligand that meant compound has similar binding mode, (4) binding energy rank was parallel with MIC of antibacterial activity. Per these requirements, the best suitable pattern was found in the interaction with *P. aeruginosa* gyrase (PDB. 6M1S). The docking results on this gyrase (PDB. 6M1S) showed that BEU1–3 was justified to possessed good binding energy (–97.4 to –91.0 kcal/mol). The structural feature of these mono-benzoyl derivatives was comparable therefore their binding energy were also similar. The predicted energy values were not higher than 2.5 kcal/mol that indicates the fitness of ligands with the active site of the target enzyme. This finding revealed a concordance with in vitro antibacterial assay that indicated compounds BEU1–3 was more active against Gram-negative bacteria than Gram-positive.

CONCLUSIONS

All test compounds exhibited higher potential antibacterial activity against *P. aeruginosa* than *S. aureus, B. subtilis, and E. coli*. The BEU1 and BEU2 were found as weak antifungals against *C. albicans*. The proposed mechanism of action in bacterial cell by molecular docking was inhibition of *P. aeruginosa* DNA gyrase. The mono-benzoyl derivatives of diethyl urea containing chloro-substituent at the position- of 2 and 4 on aromatic rings might be promoted to generate the new antibacterial agent.

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