Eco. Env. & Cons. 27 (4) : 2021; pp. (1491-1496)
Copyright@ EM International
ISSN 0971–765X

Exploration of Target Assay as Guidance for Potencial Cytotoxic Compound Isolation from *Streptomyces* Sp. Gmy01 Bacteria Using in Silico and *In vitro* Study

Rifki Febriansah[1], Triana Hertiani[2]*, Jaka Widada[3], Mustofa[4] and Puspita Lisdiyani[5]

[1]School of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Indonesia
[2]Faculty of Pharmacy, Universitas Gadjah Mada Yogyakarta, Indonesia
[3]Faculty of Agriculture, Universitas Gadjah Mada Yogyakarta, Indonesia
[4]Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada Yogyakarta, Indonesia
[5]Indonesian Institute of Sciences, Jakarta, Indonesia

(Received 5 February, 2021; Accepted 15 March, 2021)

ABSTRACT

GMY01 bacteria was known to have good activity as cytotoxic agents in some cancer cells. Methanol extract from *Streptomyces sp.* GMY01 has potential cytotoxic activity against breast cancer cells lines MCF-7 and T47D with IC_{50} values of 0.8 and 127 µg/ml, respectively. Methanol extract of *Streptomyces sp.* GMY01 also proved to be non-toxic in normal NIH-3T3 cells line with IC_{50} values of 3707 µg/ml (Farida *et al*., 2007). As part of the effort to isolate the active compound, the appropriate screening methods for appropriate guidance bioassay are needed, so an *in silico* approach is needed using molecular docking methods. The aim of this study was to analyze the best docking score of some possibilities active compounds which maybe containing in the GMY01 bacteria by Autodock Vina method and the cytotoxic activity of GMY01 methanol extract in MCF-7 breast cancer cells line. *In silico* study was carried out using molecular docking method by Autodock Vina software. Some amina peptide of the active compounds contained in *Streptomyces sp.* which are mitomycin C, mutalomycin and scabichelin compounds were docked in several target proteins (cyclin D1, cyclin E, COX-2, BCl-2 and VEGF) with the doxorubicin as comparative compound. *In vitro* study was done by cytotoxic MTT Assay in MCF-7 cells line and LC-MS analysis to know the active compounds profile containing in GMY01 methanol extract. The results of the *in silico* study obtained the target proteins with PDB ID codes, namely Cyclin D1 (5VZU), cyclin E (1W98), COX-2 (5IKQ), BCl-2 (4IEH) and VEGF (5XV7). All active and native ligand compounds have RMSD values that meet the requirements of less than 2.0 A. Molecular docking results showed that the mutalomycin compound had the lowest docking score compared to other active compounds in some of the target proteins except for cyclin D1 protein (-8.9 in cyclin E; -8.0 in p53; -7.5 in BCl-2; -8.6 in COX-2 and -8.8 in VEGF proteins). Based on *in vitro* study showed that GMY01 extract has IC_{50} value was 4 ug/ml in MCF-7 cells line. From LC-MS result showed that the extract containing the different compounds with the predicted compounds because the differentiation of the molecular weight (MW) values profile. From the study it can be concluded that the mutalomycin has the best potential in inhibiting some target proteins and GMY01 may has different active compounds compared with the predicted compounds.

Key words : *Sptreptomyces sp. GMY01, Cytotoxic, In silico, In vitro, Amine peptide.*

Introduction

Cancer is one of the deadliest diseases in the world. Cancer death rate remains high, reaching about 8.2 million people worldwide in 2012 and 347,792 people in 2013 in Indonesia. Breast cancer is one of the cancers with the highest prevalence in Indonesia in 2013, amounted to 0.5%. Yogyakarta has the highest prevalence of cancer in general and breast cancer in particular among all provinces in Indonesia, which is about 4.1% for all cancers and 2.4% for breast cancer (Balitbangkes Kemenkes, 2013). The persistently high rate of cancer incidence has encouraged researchers to discover anticancer compounds, especially for breast cancer.

Wide range of bioactive compounds metabolites were isolated and identified from soil actinomycetes. Recently, the rate of new metabolites discovery from terrestrial *actinomycetes* decreased, whereas the rate of re-isolation of known compounds increased (Dharmaraj, 2010). Thus, it is crucial that new group of *actinomycetes* from unexplored habitats to be pursued for new sources of bioactive compounds, one of which is marine actinomycetes (Magarvey *et al*., 2004). Mitomycin C is one of the drugs used in the chemotherapy of various types of cancers (Bradner, 2001). At first, the compound was isolated from the extract of secondary metabolites of gram-positive bacterium *Streptomyces lavendulae* growing on land (Mao *et al*., 1999).

Previous study had isolated several marine *actinomycetes* from Krakal Beach, Gunung Kidul, Yogyakarta, Indonesia and proved the cytotoxic effects of the marine *actinomycetes* extracts (Farida *et al.*, 2007). That research results also showed that the cytotoxic compounds from marine *actinomycetes* suggested maybe belong to the polyketide or nonribosomal peptides based on the presence of NRPS or PKS gene. Farida *et al.* (2007) then isolated marine *actinomycetes* named *Streptomyces sp*. GMY01. The sequence analysis of this isolate's NRPS apparently showed a similarity index by 79% relative to *Streptomyces lavendulae* (Widowati, 2010). This high similarity index value leads to an idea that *Streptomyces sp*. GMY01 can also produce anticancer compounds. Furthermore, the ethyl acetate extract from secondary metabolites of *Streptomyces sp*. GMY01 shows cytotoxic effects on MCF-7 cells line. The cytotoxicity effect of this extract is indicated by the IC_{50} value of 118 ng/ml in T47D cells line and 60 ng/ml in MCF-7 (Widowati, 2010). The NRPS and PKS gene analysis of GMY01 bacteria showed that the bacteria had 10 unique NRPS genes and different with other cluster gene, with the nearest similarity percentage (90%) with scabichelin gene cluster (Herdini *et al*., 2015). Analysis of 16S rRNA sequences of *Streptomyces sp*. GMY01 showed a similarity of 97% with *S. mutabilis* that contain mutalomycin as active compound (Hartanto, 2012).

Computational bioinformatics has the potential not only accelerated the drug discovery progress thus reducing the costs, but also of changing the way drugs are designed. One such method is the docking of the drug molecule with the receptor target. Autodock Vina is the example of docking method that could be use easily and free to download in their website. This research aimed to analyze the ligand-protein interaction between predicted active compounds containing in GMY01 bacteria (Mitomycin C, Mutalomycin and Scabichelin) in several target proteins (Cyclin D1, Cyclin E, COX-2, BCL-2 and VEGF) compared with the result of cytotoxic activity using MTT assay and compound analysis by LC-MS method.

Materials and Methods

Materials

In the present study, used different biological databases like PubChem and PDB (Protein Data Bank) and software's like Autodock Vina and Chem Draw. The PDB (Protein Data Bank) contains structural evidence of the macromolecules determined by X-ray crystallographic, NMR methods and others. MTT reagents, SDS, methanol for cytotoxic assay. H_2O and formic acid for LC-MS analysis.

Methods

Preparation of Protein Structure: Protein objective were downloaded from Protein Data Bank (PDB) database. Cyclin D1 (5VZU), cyclin E (1W98), COX-2 (5IKQ), BCl-2 (4IEH) and VEGF (5XV7) were PDB ID of each target proteins. All water molecules were detached and on final stage hydrogen atoms were added to receptor molecule.

Preparation of Ligand Structure: Three predicted active compounds (Mytomycin C, Mutalomycin and Scabichelin) were selected as ligand considering their closest characteristic, and doxorubicin as standard anticancer drug. The 3D structures of these

active compounds were found by using "Chem Draw". The docking analysis of predicted active compound and oncogene proteins were conceded by Autodock Vina software. Docking permits virtually screening a database compounds and predicting the solidest binders based on their scoring functions. It explores ways in which two molecules, such as drugs and an oncogene protein receptor fit together and dock each other well. The molecules binding to a receptor, hinder its function and thus act as a drug. Anticancer drugs and oncogene protein receptor were identified by docking and their relative strengths were evaluated using molecular dynamics and their binding affinities using free energy simulations. Predicted active compounds were docked with the oncogene protein receptor using parameters by default in Autodock Vina software.

Protein Ligand Interaction using Autodock Vina: Autodock is the electronic structure program that is based on the quantum mechanics, it foretells the potential energies, molecular structures; geometry optimization of structure, vibration frequencies of coordinates of atoms, bond length, bond angle and reactions pathway (Jimmy *et al*., 2005). Oncogene proteins receptor were docked against the obtained ligand using Autodock Vina to find the reasonable binding geometries and discover the protein ligand connections. Docking of the protein ligand complex was mainly targeted only on to the predicted active site. The selected residues of the receptor were defined to be a part of the binding site.

Interaction Studies: The goal of ligand-protein docking is to predict the principal binding model(s) of a ligand with a protein of known three dimensional structures. To study the binding modes of bioactive compounds in the binding site of target proteins, intermolecular flexible orders were assigned to residues of proteins, hydrogen docking simulations were performed and energy values were considered from the docked conformations of the target protein-inhibitor complexes. Docking studies yielded crucial information regarding the orientation of the inhibitors in the binding pocket of the objective protein (Umamabeswari *et al*., 2011). Docking results of the ligands and its derivatives via Autodock software reveals that the binding energy of these drugs can be compared with the receptor.

Cytotoxic Activity by MTT Assay

Prepared the solution of GMY01 extract with concentration $1x10^5$ µg/ml and several series concentrations were made. Cells with a density of $1x10^4$ cells/well are distributed into 96 wells plate and incubated for 24 hours to adapt and stick to the bottom of the well. Then, medium was taken, washed with PBS and then added 100 µl culture medium containing only 0.2% DMSO (control) or single test sample (extract and doxorubicin) incubated for 24 hours. At the end of the incubation, culture medium containing the sample was removed, washed with 100 µl PBS. Then added 100 µl culture medium containing 5 mg/ml of MTT reagen into each well, incubating again for 4 hours at 37 °C. The living cells react with MTT to form a purple formazan crystals. After 4 hours, the medium containing MTT was removed, washed with PBS then added 200 µl SDS stopper solution in 0.1% HCl to dissolve the formazan crystals. Rocked over a shaker for 10 minutes then read with an ELISA reader at a 595 nm wavelength. The absorption value is converted into live cell percentage then calculated IC_{50} value with linear regression equation.

Compound analysis using LC-MS Method

The chemical constituents of the GMY01 methanol extracts were determined using LC-MS method. The HPLC was interfaced with a Q-TOF mass spectrometer fitted with an ESI source. Full-scan mode from m/z 50 to 1350 was performed with a source temperature of 65 °C. HPLC column ACQUITY UPLC 1.7 ìm C8 (100 × 2.1 mm) was used for the analysis. The solvent was methanol with 0.1% formic acid. Solvents were delivered at a total flow rate of 0.4 ml/min. The solvent was run by gradient elution. The MS spectra were acquired in the positive ion mode.

Results

Docking Score Result

Molecular docking was performed by autodock vina method. This is free software for docking and could be operated in windows operating system. The predicted ligand that used for docking were mitomycin C, mutalomycin and scabichelin. Based on the previous study showed that 3 ligands maybe containing in the bacteria because of the similarity of the gene and gene cluster. Doxorubicin was used as standard drug for comparing the docking score result. The result showed that mutalomycin has the strongest

interaction in the most of the protein targets (COX-2, BCl-2, cyclin E and VEGF) except with Cyclin D1 which is the mitomycin as the strongest interaction with the ligand.

Cytotoxic Activity of GMY01 Methanol Extract

The cytotoxic activity was performed by MTT assay. The MCF-7 breast cancer cells line was treated by GMY01 methanol extract with the concentration range from 6.25 – 200 ug/ml. Cells were incubated in CO_2 incubator for 24 h. The treatment result of the IC_{50} value was 4 ug/ml.

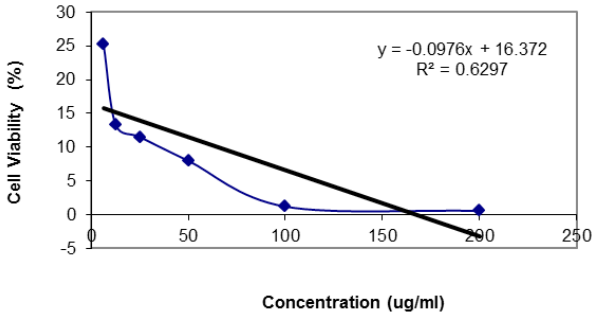

Fig. 1. Graphic of cytotoxic activity of GMY01 methanol extract in MCF-7 cells line

Table 2. The result of time retention and MW value of the GMY01 methanol extract

Peak Number	Retention Time (t_R)	MW Compound
1	0.46	166
2	2.30	211
3	3.52	397.03
4	8.87	200.33
5	15.81	359.55
6	17.89	225.51
7	19.19	500.21
8	21.16	200.22

LC-MS Compound Analysis Result

The GMY01 methanolic extract was injected to the UPLC column and run with the mobile phase by gradient elution. The result showed that the extract contains 8 dominant compounds with the MW range from 166 – 200,22 g/mol with the time retention (t_R) from 0.46 – 21.16 minutes. The MW value of the result showed that the extract had different active compounds compared with the predicted compounds because of the different of the MW values.

Table 1. RMSD value and docking score of the ligands to several target proteins

Protein	PDB ID	Ligan	RMSD Value	Docking Score
COX-2	5IKQ	Doxorubicin	1.817	-7.7
		Mitomycin	1.630	-7.0
		Mutalomycin	1.794	-8.6
		Scabichelin	1.918	-6.2
		Native Ligand	1.578	-7.4
Cyclin D1	5VZU	Doxorubicin	1.590	-7.3
		Mitomycin	1.898	-6.7
		Mutalomycin	1.463	-4.1
		Scabichelin	1.645	-5.2
		Native Ligand	-	-
BCL-2	4IEH	Doxorubicin	1.566	-6.5
		Mitomycin	1.985	-5.7
		Mutalomycin	1.111	-7.5
		Scabichelin	1.389	-5.5
		Native Ligand	1.862	-7.2
Cyclin E	1W98	Doxorubicin	1.112	-8.9
		Mitomycin	1.514	-6.2
		Mutalomycin	1.334	-8.9
		Scabichelin	1.674	-5.5
		Native Ligand	-	-
VEGF	5XV7	Doxorubicin	1.538	-4.5
		Mitomycin	1.594	-7.2
		Mutalomycin	1.546	-8.8
		Scabichelin	1.774	-6.2
		Native Ligand	1.950	-10.5

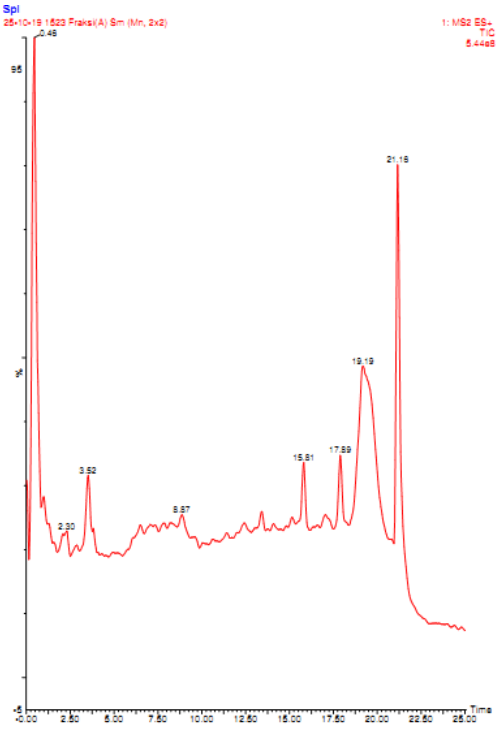

Fig. 2. The LC-MS spectra of the GMY01 methanol extract

Discussion

Streptomyces Sp. GMY01 bacteria is believed to be a new type of bacteria that is different from the species of bacteria that have been previously identified. Sequence analysis of NRPS gene *Streptomyces sp.* GMY01 showed that the isolate had a high similarity to *S. lavendulae* at 79% which was known to contain mitomycin C compound which is a amine peptide group compound, and an analysis of 16S rRNA sequence of *Streptomyces sp.* GMY01 shows a similarity of 97% with *S. mutabilis* that contain mutalomycin as active compound (Hartanto, 2012). The NRPS and PKS gene analysis of GMY01 bacteria showed that the bacteria had 10 unique NRPS genes and different with other cluster gene, with the nearest similarity percentage (90%) with scabichelin gene cluster (Herdini *et al.*, 2015). Molecular docking result showed that mutalomycin has the strongest affinity in almost target proteins (COX-2, BCl-2, cyclin E and VEGF) compared with the other compounds. Mutalomycin is a new metal-complexing polyether antibiotic produced by a strain of *Streptomyces mutabilis*. Mutalomycin contains six heterocyclic rings and is structurally related to nigericin. The metabolite is active against gram-positive bacteria and *Eimeria tenella* (chicken coccidiosis) (Fehr *et al.*, 1977).

Cytotoxicity test of the methanol extract of GMY01 bacteria was done in MCF-7 cells line. The result showed that the IC_{50} value of the extract was 4 ug/ml. Based on previous study, extract with the IC_{50} value < 10 ug/ml were categorized to have strong activity (Jabit *et al.*, 2009). This strong activity maybe caused by several active compounds that contain in the extract with different mechanism in prohibited the cell cycle progression.

The analysis of the active compound that containing in the extract was done by LC-MS method. The result showed that the extract contains 8 dominant compounds with time retention (t_R) from 0.46 – 21.16 minutes and the MW range from 166 – 200,22 g/mol. The result showed different compounds compared with the predicted compounds from the MW value result. Previous study showed that scabichelin, mitomycin and mutalomycin have MW value of 519; 334,33 and 775 g/mol, respectively (pubchem data, 2020). It is means that GMY01 methanol extract may be contain new active compound that has strong activity as cytotoxic in MCF-7 breast cancer cells line.

Conclusion

Based on autodock Vina result, mutalomycin has the strongest interaction with the almost of protein targets (COX-2, BCl-2, cyclin E and VEGF) than others compound candidates. From the MTT assay, the GMY01 methanolic extract showed high cytotoxic effect with the IC_{50} value was 4 ug/ml in MCF-7 breast cancer cells line. Based on LC-MS result showed that the extract contain different compounds with the predicted compounds because of the different of MW value and LC-MS spectra profile.

Acknowledgement

We would like to thank to ministry of Research, technology and higher education of Indonesia and LPDP RI for funding this research and the doctoral study of the first author.

References

Balitbangkes. 2013. Basic Health Research. Indonesian Health Ministry: Jakarta.

Bradner, W.T. 2001. Mitomycin C: a clinical update. *Cancer Treatment Rev*. 27 : 35-50.

Farida, Y., Widada, J. and Meiyanto, E. 2007. Combination methods for screening marine actinomycetes producing potential compounds as anticancer. *Indonesian Journal of Biotechnology.* 12(2) : 988-997.

Fehr, T., King, H.D. and Kuhn, M 1977. Mutalomycin, a New Polyether Antibiotic Taxonomy, Fermentation, Isolation and Characterization. *J Antibiot (Tokyo)*. 30 (11): 903-7. doi: 10.7164/antibiotics.30.903.

Hartanto, S. 2012. Keragaman Gen NRPS pada Isolat-isolat Aktinomisetes Laut yang Berpotensi Menghasilkan Senyawa Antikanker, *Tesis*, Universitas Gadjah Mada.

Herdini, C., Hartanto, S., Mubarika, S., Hariwiyanto, B., Wijayanti, N. and Hosoyama, A. 2015. Diversity of non-ribosomal peptide synthase genes in the anticancer producing Actinomycetes isolated from marine sediment in Indonesia. *Indonesian Journal of Biotechnology*, 20(1): 34-41.

Jabit Md. Lip, Fatma Sri Wahyuni, Rozida Khalid, Daud Ahmad Israf, Khozirah Shaari, Nordin H. Lajis and Johnson Stanslas, 2009. Cytotoxic and nitric oxide inhibitory activities of methanol extracts of *Garcinia* species. *Pharmaceutical Biology*. 47 : 11. 1019-1026, DOI: 10.3109/13880200902973787.

Jimmy, S., Joel, T., McCarthy, S., Enkemann, S., Gong Li, C., Yan, P., Huang, T. and Timothy, J. 2005. Yeatman. Oncogene regulation of tumor suppressor genes in tumorigenesis. *Carcinogenesis*. 26(2): 487-494.

Magarvey, N. A., Keller, J., Bernan, V., Dworkin, M. and Sherman, D. 2004. Isolation and characterization of novel marine-derived actinomycetes taxa rich in bioactive metabolites. *Appl Environ Microbiol.* 70 : 7520-7529.

Mao, Y., Varoglu, M. and Sherman, D.H. 1999. Genetic localization and molecular characterization of two key genes (mit AB) required for biosynthesis of the antitumor antibiotic mitomycin C. *J Bacteriol*. 181 : 2199-208.

Sheeba, J.M.D.J., Mohan, C.M., Marine, H., Gauri, D., Neeraj, K. and Mukund, L.A. 2016. Estrogen-regulated extracellular matrix remodelling genes in MCF-7 breast cancer cells. *Gene Reports*. 3 : 14–21.

Umamaheswari, M., Madeswaran, A., Asokkumar, K., Sivashanmugam, T., Subhadradevi, V. and Jagannath, P. 2011. Discovery of potential xanthine oxidase inhibitors using in silico docking studies. *Der Pharma Chemica*. 3 : 240-247.

Widowati, E.W. 2010. *Toxic compounds in various actinomycetes GMY01 extracts against breast cancer cells T47D and MCF7,* Thesis, Gadjah Mada University.