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Cytotoxicity and molecular mechanism of marine-derived *Streptomyces* sp. GMY01 on human lung cancer cell line A549

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ABSTRACT

Marine-derived *Streptomyces* sp. GMY01 is a biotechnologically potential bacterium which has anticancer activity. This research aimed to investigate the anticancer activity of GMY01 extract on human lung cancer cell line A549 and its mechanism using Western blot analysis and *in silico* molecular docking. Ethyl acetate extract was obtained from the supernatant of an 11-day fermented product and it was fractionated using flash chromatography. An *in vitro* assay of the selected fractions (F5, F6, F7, F8, and F14) on the A549 cell line showed moderate activity (IC_{50} value of 18.41–231.6 µg/ml), whereas the IC_{50} value of the crude extract (CE) was 34.26 µg/ml. Western blot analysis revealed the mode of action of GMY01 fractions F6 and F7 to be that of an autophagy induction mechanism, similar to that of the CE. Targeted liquid chromatography high-resolution mass spectrometry analysis of GMY01 extract detected eight compounds: herboxidiene ($C_{25}H_{42}O_6$), vazabitide A ($C_{12}H_{21}N_3O_4$), albaflavenone ($C_{15}H_{22}O$), grincamycin ($C_{49}H_{62}O_{18}$), isorenieratene ($C_{40}H_{48}$), geosmin ($C_{12}H_{22}O$), hopene ($C_{30}H_{50}$), and saframycin A ($C_{60}H_{68}N_{10}O_{14}$). A molecular docking study on target proteins of antiapoptotic proteins (BCL-2 and BCL-XL) and autophagy proteins mammalian target of rapamycin (mTOR-C1 and mTORC2) showed that these compounds had a high affinity. Among all the compounds, grincamycin ($C_{49}H_{62}O_{18}$) had the highest affinity on antiapoptotic proteins (−11.8 and −10.4) and on autophagy proteins (−12.1 and −11.5) within all binding domains. These results indicate that *Streptomyces* sp. GMY01 has a promising anticancer agent, grincamycin, for human lung cancer.

INTRODUCTION

Lung cancer is a type of cancer with the highest incidence after prostate cancer in men and after breast cancer in women (Dela Cruz *et al*., 2011). However, lung cancer still has the highest mortality among cancer patients worldwide (Bade and Dela Cruz, 2020). The risk factors for lung cancer are industry emissions, air pollution, cigarette smoking, chronic infectious lung diseases, and living lifestyle (Barta *et al*., 2019). In Indonesia, lung cancer ranks first in men and third in women (The Global Cancer Observatory, 2019) with a mortality rate of 12.6% from 207,210 deaths caused by cancer in 2018 (WHO, 2020).

For more than 40 years, small organic molecules (<3,000 Daltons) derived from microbes and plants have been used as chemotherapy agents for cancer (Kinghorn *et al*., 2009). The *Streptomyces* bacteria are the main source of medicinal compounds derived from microbes for nearly 80% of drugs, especially antibiotics (Weber *et al*., 2015) and anticancer agents (Dhaneesha *et al*., 2017; Nguyen *et al*., 2020). In our previous research, we discovered a marine bacterium, *Streptomyces* sp. GMY01, which has a very high potential active principle to be

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developed as a therapeutic agent (Herdini *et al*., 2015). This bacterial extract was also found to contain anticancer activities against T47D and MCF7 breast cancer cell lines (Farida *et al*., 2007; Werdyani *et al*., 2017).

In this research, we studied the cytotoxicity of *Streptomyces* sp. GMY01 extract on human lung cancer cell line A549 and its potential mechanism of action on anticancer drugs during apoptosis and autophagy. In the apoptosis mechanism, poly(ADP-ribose) polymerases (PARPs) are proteins (enzymes) that play a role in the catalytic process of transferring (adenosine diphosphate) ADP- ribose to the target protein. PARP is known to have many important roles in cellular mechanisms, including modulation of chromatin structure, transcription, replication, recombination, and (deoxyribonucleic acid) DNA repair. Meanwhile, in autophagy, the (light chain 3) LC3-II protein indicates the presence of a compound that acts as a trigger for autophagy mechanism. Autophagy plays a critical role in cellular development and differentiation, the response to stress conditions, and tumor suppression (Yang and Klionsky, 2009). To determine the anticancer activities of *Streptomyces* compounds, we also carried out molecular docking studies to active compounds detected in GMY01 extract. Molecular docking was carried out for the two important protein targets as antiapoptotic, B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra large (BCL-XL) (Sathishkumar *et al*., 2012) and for the two autophagy proteins, namely, mammalian target of rapamycin (mTOR), which consist of two complexes: mTORC1 and mTORC2 (Yun and Lee, 2018). Thus, molecular docking of antiapoptotic and autophagy proteins is one of the methods for finding new anticancer drug candidates.

MATERIALS AND METHODS

Biological materials

Marine actinobacteria *Streptomyces* sp. GMY01 were isolated from a marine sediment sample collected from Krakal Beach (8°8′44′′′S 110°35′59′′′E), Gunungkidul, Yogyakarta, Indonesia (Farida *et al*., 2007). GMY01 was deposited in the Indonesian Culture Collection (WDCM 769), Indonesian Institute of Sciences as InaCC A147, and NITE Biological Research Center (NBRC, WDCM 825), Japan, with registration number NBRC 110111. The whole-genome shotgun project of *Streptomyces* sp. GMY01 has been deposited at DNA Data Bank of Japan/European Nucleotide Archive/GenBank with GenBank accession number: JABBNA000000000. Human lung cancer cell line A549 was obtained from the Department of Cancer Cell Biology, Faculty of Pharmaceutical Sciences, University of Toyama, Japan.

Fermentation, extraction, and fractionation

GMY01 bacterium was maintained in International *Streptomyces* Project-2 (ISP-2) agar medium (Difco, Sparks, MD). GMY01 was cultured at 28°C and agitated at 180 rpm for 3 days in a 250 ml Erlenmeyer flask containing 100 ml of tryptic soy broth (Difco, Sparks, MD) as the seed medium. For fermentation, the cell culture was transferred into four 1,000 ml flasks containing 500 ml of starch nitrate broth (SNB) medium, incubated for 11 days at 28°C, and agitated at 180 rpm in a shaking incubator (Ghanem *et al*., 2000). The SNB medium contained 0.5 g of NaCl, 1 g of KNO_3, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 20 g of soluble starch in 1,000 ml of distilled water. Secondary metabolites were obtained by separating the cell biomass from the liquid using refrigerated centrifuge at 4,137 × g at 4°C for 15 minutes (Farida *et al*., 2007). The supernatant was extracted twice with an equal volume of ethyl acetate (Merck, Germany) and evaporated using an evaporator machine (Buchi, Switzerland) to obtain the crude extract (CE). The crude ethyl acetate extract was dissolved in methanol (Merck, Germany) and fractionated using an equal volume of *n*-hexane (Merck, Germany) to separate the polar and nonpolar fractions. The polar fraction was refractionated using flash chromatography (Reveleris™, Buchi, Switzerland) using a 30 µm C-18 column (FlashPure, Buchi, Switzerland) with water-acetonitrile (Merck, Germany) as the mobile phase to obtain fractions. The ethyl acetate–methanol fraction was dissolved in methanol impregnated into celite (Merck, Germany), with a fraction:celite ratio of 1:3 (*w/w*), and dried. Flash chromatography was carried out on the ethyl acetate–methanol fraction based on the procedure manual for dry loading samples. All the targeted fractions were weighed and evaluated for anticancer assays.

Cytotoxicity assay

The water-soluble tetrazolium salt (WST) assay was employed to evaluate anticancer activity *in vitro* (Tominaga *et al*., 1999) with minor modifications. Selected lung cancer cell line A549 was grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium. Cells were seeded into a sterile flat bottom 96-well microplate at a density of 1.3×10^4 cells/well and allowed to adhere overnight at a total volume of 100 µl in a humidified incubator (5% CO_2, 37°C). One hundred microliters of the sample solution (in 0.1% dimethyl sulfoxide, DMSO) was added to the cells ranging from 0, 12.5, 25, and 50 to 100 µg/ml and then incubated for 24 hours before carrying out the WST-1 assay. Negative control was made using 0.1% DMSO (1 µl DMSO in 1,000 ml RMPI), while positive control was made using a cisplatin standard of 1.8 µl in 1.8 ml (1 mM). All treatments were carried out in triplicate. After aspiration of the liquid medium from the plate, 99 µl of WST-1 solution (90 RPMI 1,640: 1 WST-1) was then added to each well and incubated at 37°C in a CO_2 incubator for 4 × 10 minutes. The reaction product was observed spectrophotometrically at 420 and 660 nm using a microplate reader. The collected data were calculated to obtain the ratio of the control (RPMI media) and the percentage of cell growth inhibition according to the following formula:

$$Inhibition\ (\%) = \frac{A - B}{A}\ x\ 100$$

where A is the mean absorbance of the control (RPMI medium without drug) and B is the mean absorbance of the treated cells.

IC_{50} values were obtained using nonlinear regression analysis between the $\log_{10}$ concentrations of the extract versus the percent of cell inhibition using GraphPad Prism 8.4.3 software. All the experiments were repeated twice independently.

Protein extraction and Western blot analysis

The protein extraction procedures and Western blot analysis were carried out to evaluate both apoptosis and autophagy mechanisms (Kamal *et al*., 2015; Williams, 2013). A549 cells were treated with 1 µl of the 100 µg/ml fraction for 6 hours. The

cells were separated from the medium and washed with phosphate buffered saline (PBS). The cell lysates were obtained by lysing the cells in 100 µl ice-cold (whole cell extract) WCE option (reductant/inhibitor) consisting of WCE buffer 1.7 ml and WCE + option: 1 M dithiothreitol (DTT) (×1,000) 1.7 µl, sodium orthovanadate (1 M × 1,000) 1.7 µl, B-glycerophosphate (1M × 50) 34 µl, phenylmethylsulfonyl fluoride (0.1M × 100) 17 µl, leupeptin (10 mg/ml × 1,000) 1.7 µl, and aprotinin (10 mg/ml × 1,000) 1.7 µl. After centrifugation at 14,000 rpm, 4°C for 10 minutes, the protein in the supernatant was quantified by the XL Bradford method by using a microplate reader at 595 nm. The concentration of the protein sample was set to the same concentration by the addition of WCE buffer. The sample solution (150 µl) was added, homogenized, and heat shocked at 95°C for 5 minutes. The protein (10 µg per lane) was applied to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, at 500 volts and 120 mA for 75 minutes. After electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane at 400 V and 100 mA for 100 minutes. The membrane was blocked overnight in PBS with 0.1% Tween 20 (phosphate buffer saline – Tween 20) (PBST) and washed with PBST three times. The primary antibody was added to the membrane and incubated on a hot plate for 3 hours for PARP (Cell Signaling Technology) and 2 hours for α-tubulin (Santa Cruz Biotechnology). The membrane was washed again in PBST for 15 minutes three times. The membrane was incubated for 1 additional hour with the corresponding horseradish peroxidase-labeled secondary antibody. The membrane was washed with PBST 0.1%. The protein blots were visualized with enhanced chemiluminescence (ECL) solution 1 and 2 (1:1 ratio) and incubated for 10–20 minutes. The membrane was exposed to a film negative for 10–20 minutes and washed with solution 1 and NaCl 0.3%. Finally, the membrane was fixed with fixative solution and then washed with aqueous solution. Images were captured by using a scanner.

Liquid chromatography high-resolution mass spectrometry (LC-HRMS)

Metabolomic analysis of the CE was carried out using a ultra-high-performance liquid chromatography coupled to targeted high-performance mass spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC paired with Thermo Scientific Q Exactive) (Thermo Fisher Scientific, Bremen, Germany). Targeted LC-HRMS was based on a predicted compound formula which was obtained from the genome mining analysis of *Streptomyces* sp. GMY01 whole-genome sequence using antiSMASH version 5 (Blin *et al*., 2019). HRMS was carried out with mobile phases A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). The column used was a Hypersil Gold aQ analytical column (50 × 1 mm × 1.9 µm) (Thermo Fisher Scientific, Waltham, MA) with a flow rate of 40 µl/minutes, an injection volume of 5 µl, and a gradient with an analysis time of 30 minutes. The gradient was programmed as follows: 2 minutes, 5% B; 15 minutes, 60% B; 22 minutes, 95% B; 25 minutes, 95% B; 25.1 minutes, 5% B; and 30 minutes, 5% B. Experiments were carried out in parallel reaction monitoring at 35,000 the full width at half maximum resolution, heated electrospray ionization, positive ionization, and data processing with Thermo Scientific XCalibur.

In silico molecular docking studies

Molecular docking was used to predict the binding affinity of several compounds with proteins that play a role in the apoptosis mechanism. The active compounds of *Streptomyces* sp. GMY01 were detected using targeted LC-HRMS based on a genome mining study. This study used apoptosis proteins in cancer cells BCL-2 (2w3l) and BCL-XL (2yxj) and autophagy proteins in cancer cells mTORC1 (6BT0) and mTORC2 (6zwm) which were obtained from RCSB database. The 2D structure of the compounds was retrieved from the ChemSpider webserver and converted to an optimized 3D structure using Marvinsketch tools (https://www.chemaxon.com). Both proteins and compounds were prepared using AutoDock tools. The proteins were purified from water, ions, and other small molecules. Hydrogen atoms were added to the polar groups of the proteins to minimize errors caused by ionization and tautomeric states of amino acid residues. Molecular docking was carried out on AutoDock Vina (Trott Oleg and Olson, 2010), while visualization of binding interactions was carried out using the DisCoVery studio visualizer (DS visualizer) tool (Studio, 2015). The computational simulation was carried out on a Windows 10 operating system, with an AMD A8 7410 (Quad-core; 2,2 GHz) processor and 4 GB of RAM. The molecular docking study was observed from the affinity (kcal/mol) value with a root mean square deviation score less than 2 A, which expressed a visualization of the binding interaction between the compounds and the active site of the proteins.

RESULTS AND DISCUSSION

Anticancer activity of *Streptomyces* sp. GMY01

The fermentation process of *Streptomyces* sp. GMY01 in 6 l of SNB medium for 11 days obtained a 4.78 g of crude ethyl acetate extract. The results of the refractionation using *n*-hexane, methanol, and ethyl acetate solvents selected 177.8 mg of methanol-ethyl acetate as an active extract**.** The refractionation using flash chromatography obtained 17 fractions, five of which were active as anticancer agents. The cytotoxicity assay of *Streptomyces* sp. GMY01 extract on the lung cancer cell line A549 showed that F5, F6, F7, F8, and F14 fractions and CE had cytotoxic activities at concentrations of 12.5–100 µg/ml (Table 1). At concentrations of 50 and 100 µg/ml, F5 showed the highest inhibitory activity, which was lower than that of 1 mM cisplatin as a positive control. By regression analysis, the CE and two fractions (F5 and F6) showed IC_{50} value lower than 100 µg/ml. The lowest IC_{50} value (18.41 µg/ml) was revealed by F5. From these results, the obtained fractions from *Streptomyces* sp. GMY01 extract had potency as anticancer agents, especially on human lung cancer cells.

In a previous bioassay study, GMY01 exhibited anticancer activity against human breast cancer T47D cells (IC_{50} value of 19 µg/ml) and MCF7 cells (IC_{50} value of 7 µg/ml) (Farida *et al*., 2007). In a previous genome mining study, a whole-genome sequence of *Streptomyces* sp. GMY01 had 10 non-ribosomal polyketide synthase (NRPS) gene clusters (Herdini *et al*., 2015). This indicates that *Streptomyces* sp. GMY01 is a marine organism with biotechnological potential as a drug source.

Molecular mechanism of anticancer

Western blot analysis was carried out to evaluate the fractional effect on protein expression in a human lung cancer cell

Table 1. Inhibitory activity and IC_{50} value of *Streptomyces* sp. GMY01 fractions against lung cancer cell A549.

Concentration (µg/ml)	% Inhibition of fraction/extract					
	F5	F6	F7	F8	F14	CE
12.5	40.09 ± 19.39	15.23 ± 0.82	14.64 ± 2.85	9.25 ± 1.94	12.26 ± 1.38	42.78 ± 1.52
25	59.29 ± 15.92	20.20 ± 2.12	20.23 ± 1.53	11.50 ± 0.88	17.94 ± 3.17	49.68 ± 1.55
50	70.32 ± 9.80	30.92 ± 1.95	28.52 ± 3.93	22.61 ± 1.17	25.89 ± 1.34	50.89 ± 2.26
100	93.78 ± 0.36	59.13 ± 0.49	42.39 ± 1.33	32.94 ± 18.71	44.32 ± 1.47	57.47 ± 1.15
IC_{50} (µg/ml)	18.41	81.81	160.4	231.6	138.2	34.26

Cisplatin 1 mM was used as a positive control and has 59.61% ± 2.15% of inhibition.

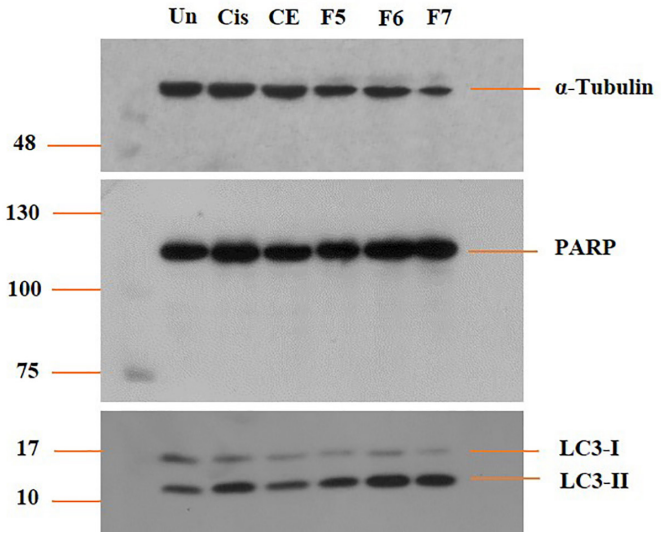

Figure 1. Effect of *Streptomyces* sp. GMY01 fraction extract on LC3-I and LC3-II and PARP protein expression. A549 cells were treated with the fraction extract at 1 µl (100 µg/ml) concentration in ml media for 6 hours. LC3-I, LC3-II, and PARP protein levels were examined by Western blot analysis. α-Tubulin was used as an equal loading control.

line (A549). An α-tubulin was used as the control protein in this study. Three selected fractions (F5, F6, and F7) were used for the protein expression assay using Western blot analysis. The results showed that none of the samples induced cleavage of the PARP protein (Fig. 1). On the other hand, all the samples exhibited slight inhibition against LC3-I and an increase in the LC3-II protein level, similar to cisplatin as a positive control. Fractions 6 and 7 showed the highest level of LC3-II protein expression, followed by fraction 5. However, we investigated the apoptosis and autophagy mechanisms in A549 cancer cells.

PARP inhibitors have been widely investigated in clinical trials to determine the benefits of this approach in cancer therapy. PARP is often associated with programmed cell death mechanisms characterized by decreased nicotinamide adenine dinucleotide (NAD) ± adenosine triphosphate (ATP), loss of mitochondrial membrane potential, and the presence of apoptosis-inducing factors (Morales *et al*., 2014). Figure 1 shows that all the cells treated with the fractions, CE, or cisplatin as the positive controls did not display significant differences in the PARP expression level compared with the untreated cells. It can be said that the mechanism of action of the compounds produced by *Streptomyces* sp. GMY01 is not related to the apoptosis mechanism. In this study, the apoptosis mechanism cannot be seen clearly because there were no smaller PARP proteins as a product of PARP cleavage. Another study mentioned that 4b cinnamido-linked podophyllotoxins were confirmed as anticancer agents in lung A549, with the apoptosis mechanism indicated by cleavage of 116 kDa PARP to cleaved PARP 89 kDa (Kamal *et al*., 2015).

An anticancer mechanism apart from apoptosis is autophagy, which is a normal process in cells to maintain homeostasis in times of limited nutritional conditions. In autophagy, cells break down unnecessary cell components into metabolites which are useful for the availability of cell nutrients. Microtubule-associated protein 1 light chain 3 (MAP1 LC3) is a very common indicator as a process of autophagy in diseased cells. One of the important markers in the autophagy process is the upregulation and localization of LC3 protein. The carboxyl terminal region of the LC3 is cleaved, showing glycine residue forming LC3I. LC3II is a modified form of LC3I through the addition of phosphatidylethanolamine. The LC3II becomes bound and accumulated in the membrane of the autophagosome (Williams, 2013). In this study, cell treatment was carried out for 6 hours of incubation. The autophagy mechanism was clearly seen in the cells treated with F6 and F7 and slightly seen in F5. Thus, the apoptosis mechanism might not yet be clearly seen because the 6-hour incubation was not sufficient to trigger apoptosis. Autophagy avoids apoptotic mechanism; however, under long-term stress conditions, it can lead to apoptosis (Williams, 2013).

In silico molecular docking

To predict the compounds produced by GMY01 bacteria, we carried out targeted LC-HRMS analysis based on a genome mining study of *Streptomyces* sp. GMY01 (Supplementary Table S1). Targeted HRMS detected eight compounds in CE (Supplementary Table S2). These compounds are known as antitumor and herbicidal (herboxidiene) (Miller-Wideman *et al*., 1992; Pokhrel *et al*., 2015), antibiotic (albaflavenone and grincamycin) (Lai *et al*., 2018; Zhao *et al*., 2008), antitumor antibiotic (saframycin A) (Li *et al*., 2008), aromatic carotenoid (isorenieratene) (Krügel *et al*., 1999), peptide metabolite (vazabitide A) (Yashiro *et al*., 2019), and volatile compounds (geosmin and hopene) (Jiang *et al*., 2006; Seipke and Loria, 2009).

The molecular docking affinity of these detected compounds on the target protein of antiapoptotic proteins BCL-2 and BCL-XL and autophagy proteins mTORC1 and mTORC2 is shown in Tables 2 and 3. Three compounds, namely, grincamycin, isorenieratene, and hopene, showed a higher affinity than other

Table 2. Docking scores of detected compounds in *Streptomyces* sp. GMY01 extract toward potential binding domain of apoptosis protein.

Detected Compounds	Molecule formula	Molecular mass (Da)	BCL2 (2w3l)			BCL XL (2yxj)		
			Affinity	RMSD		Affinity	RMSD	
				Lower bond	Upper bond		Lower bond	Upper bond
Hopene	$C_{30}H_{50}$	410.73	−8.2	1.949	8.292	−9.2	1.445	4.017
Albaflavenone	$C_{15}H_{22}O$	218.34	−6.6	1.788	2.969	−6.8	1.796	3.242
Geosmin	$C_{12}H_{22}O$	182.303	−6	1.339	3.794	−6.9	1.512	2.986
Isorenieratene	$C_{40}H_{48}$	528.809	−8	1.698	2.169	−9.1	1.387	2.727
Grincamycin	$C_{49}H_{62}O_{18}$	938.393	−11.8	1.616	2.592	−10.4	1.55	2.273
Vazabitide A	$C_{12}H_{21}N_3O_4$	271.313	−5.6	1.514	2.038	−5.5	1.483	1.875
Herboxidiene	$C_{25}H_{42}O_6$	438.605	−6.7	1.551	2.372	−7.4	1.513	2.125
Saframycin A	$C_{60}H_{68}N_{10}O_{14}$	1,153.26	−9.3	1.655	2.019	−8	1.812	3.976

Affinity [Energy Gibbs (kcal/mol)]; RMSD: Root Mean Square Deviation

Table 3. Docking scores of detected compounds in *Streptomyces* sp. GMY01 extract toward potential binding domain of autophagy protein.

Detected Compounds	Molecule formula	Molecular mass (Da)	mTORC1 (6BT0)			mTORC2 (6zwm)		
			Affinity	RMSD		Affinity	RMSD	
				Lower bond	Upper bond		Lower bond	Upper bond
Hopene	$C_{30}H_{50}$	410.73	−9	1.533	8.196	−8.7	1.203	1.719
Albaflavenone	$C_{15}H_{22}O$	218.34	−7.1	1.305	2.003	−7	1.951	3.992
Geosmin	$C_{12}H_{22}O$	182.303	−6.3	1.41	2.892	5.8	1.242	3.634
Isorenieratene	$C_{40}H_{48}$	528.809	−7.8	1.392	2.979	−8.6	1.236	2.036
Grincamycin	$C_{49}H_{62}O_{18}$	938.393	−12.1	1.376	2.196	−11.5	1.264	1.746
Vazabitide A	$C_{12}H_{21}N_3O_4$	271.313	−5.8	1.835	5.278	−5.5	1.54	5.105
Herboxidiene	$C_{25}H_{42}O_6$	438.605	−6.5	1.761	2.236	−6.9	1.984	3.277
Saframycin A	$C_{60}H_{68}N_{10}O_{14}$	1153.26	−8.6	1.851	2.461	−7.9	1.403	2.149

Affinity [Energy Gibbs (kcal/mol)]; RMSD: Root Mean Square Deviation

compounds. Among all these compounds, grincamycin ($C_{49}H_{62}O_{18}$) had the highest affinity with BCL-2 and BCL-XL (−11.8 and −10.4) and with mTORC1 and mTORC2 (−12.1 and −11.5). A three-dimensional illustration of the binding between grincamycin and the target proteins is shown in Figure 2 and Figure 3.

BCL-2 and BCL-XL are known as antiapoptotic proteins that bind with proapoptotic proteins to induce apoptosis mechanism. This protein family plays a role as key regulators of programmed cell death or apoptosis (Goreshnik *et al*., 2011). Several cancers are affected by overexpression of these antiapoptotic proteins (Sathishkumar *et al*., 2012). High expression of antiapoptotic agents such as BCL-2 and BCL-XL which are commonly found in human cancer causes neoplastic cell expansion and interferes with the therapeutic action of chemotherapy drugs (Huang, 2000). In another study, molecular docking analysis between ginsenosides from *Panax ginseng* against BCL-2 and BCL-XL resulted in binding energy of −5.62 to −9.77 kcal/mol (Sathishkumar *et al*., 2012). The result of that study was lower than the binding affinity affinity produced by grincamycin in this study (−10.4 to −11.8 kcal/mol).

mTOR is a protein associated with cell proliferation, stress, and cancer progression. This protein controlled the autophagy mechanism. mTOR protein consists of mTORC1 and mTORC2 as a complex. Activated mTORC1 leads to the inhibition of autophagy (Yun and Lee, 2018). Inhibition of mTORC1 protein is associated with enhanced autophagy. Another study showed that silencing mTORC2-induced apoptosis (Chawsheen and Dash, 2021). The mTORC1 and mTORC2 inhibitor compounds are used as models in the design and development of lung cancer treatments (Chaube *et al*., 2021). Two compounds that have been shown to be mTORC inhibitors include tetrahydroquinoline derivatives (Chaube *et al*., 2021) in lung cancer and MG-2477 in A549 cell (Viola *et al*., 2012).

In this study, grincamycin compounds had a higher affinity for autophagy proteins than for apoptosis-related proteins. This is in line with the results of the analysis of protein expression using Western blot where the LC3-I and LC3-II apoptosis-related proteins were higher in expression than the PARP-related apoptosis-related proteins. This was explained in a previous study which states that when mTORC1 is inhibited, the protein complex associated with autophagy activation recruits LC3 protein which will be converted into active cytosolic isoform LC3-I and transformed into LC3-II. LC3 II protein is located in the autophagosome membrane and allows the binding of degraded substrates. Autophagosomes will fuse with lysosomes and form autolysosomes which play a role in the autophagy process (Yun and Lee, 2018).

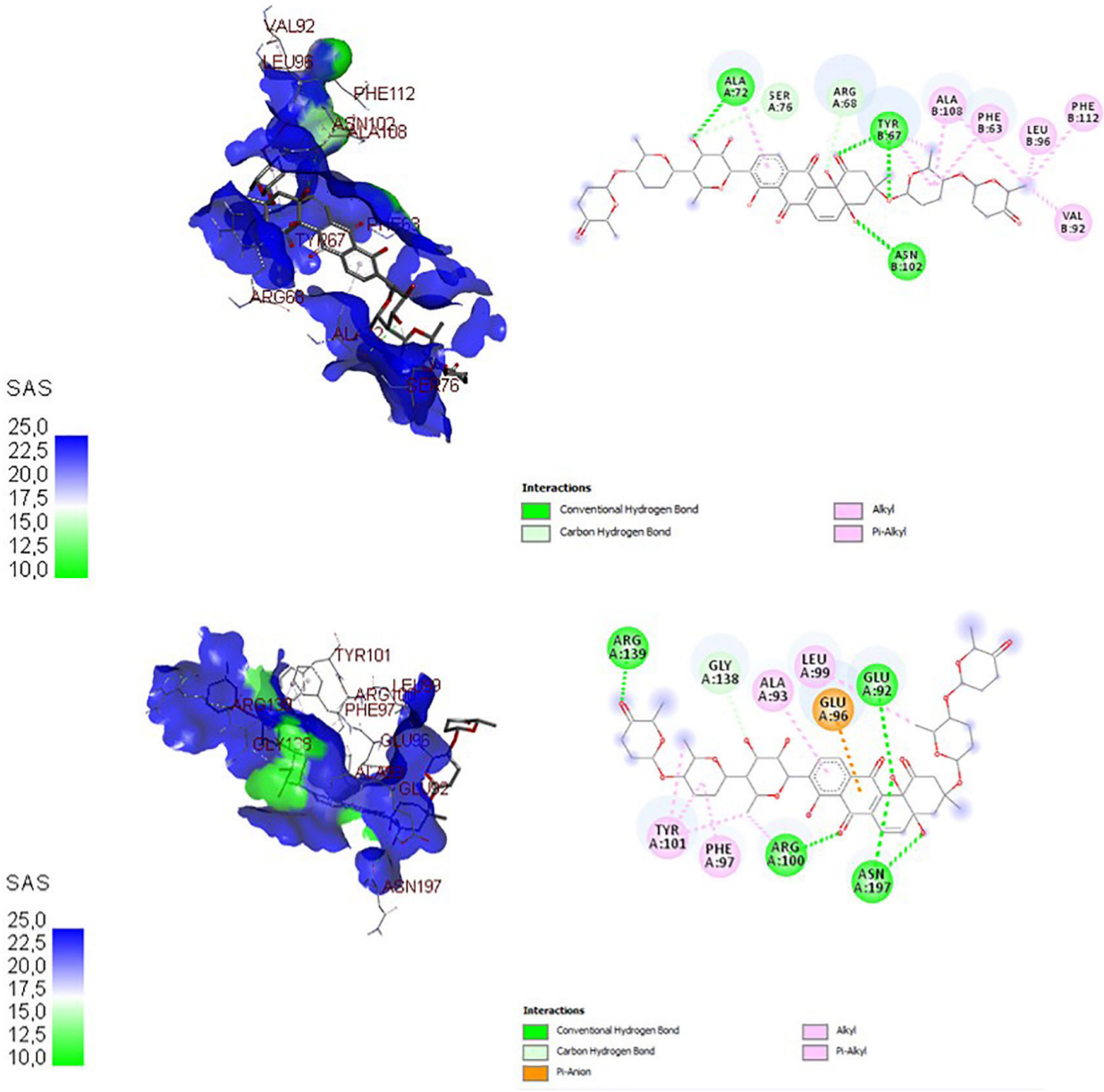

Figure 2. Three-dimensional and two-dimensional docking visualization of grincamycin on BCL-2 (top) and BCL-XL (bottom) proteins.

The docking visualization of grincamycin on antiapoptosis proteins BCL-2 and BCL-XL is shown in Figure 2 and on autophagy proteins mTORC1 and mTORC2 is shown in Figure 3. In three- and two-dimensional docking visualization, grincamycin binds with alanine (72), serine (76), arginine (68), tyrosine (67), asparagine (102), alanine (108), phenylalanine (63), leucine (96), phenylalanine (112), valine (92) amino acids of BCL-2 protein and binds with arginine (139), glycine (138), alanine (93), glutamic acid (96), leucine (99), glutamic acid (92), tyrosine (101), phenylalanine (97), arginine (100), and asparagine (197) amino acids of BCL-XL. This compound also binds isoleucine (90), lysine (9,151), leucine (170), histidine (9,100), leucine (9,104), lysine (169), methionine (125), glutamic acid (126), threonine (88), serine (89), lysine (91) of mTORC1, arginine (1,665), isoleucine (1,629), histidine (1,454), tryptophan (1,456), proline (1,426), arginine (2,117), arginine (2,322), glycine (1,425), lysine (2,218), tyrosine (1,393), phenylalanine (2,314), and leucine (1,423) of mTORC2. This finding showed that grincamycin had high affinity antiapoptotic proteins and autophagy proteins. The presence of grincamycin binds with BCL-2, BCL-XL, mTORC1, and mTORC2 areas expected to inhibit these proteins, leading to apoptosis and autophagy induction.

In previous studies, the grincamycin compounds from an actinomycete from the sea, namely *Streptomyces lusitanus* SCSIO LR32, were found. Grincamycin compounds show antitumor activity against several human cancer cells, namely MDA-MB-435, MDA-MB-231, NCI-H460, HCT-116, HepG2, and MCF10A normal human breast epithelial cells with IC_{50} values ranging from 0.4 to 6.9 µM (Lai *et al*., 2018). In another study, grincamycins B–F also were isolated from *S. lusitanus* SCSIO

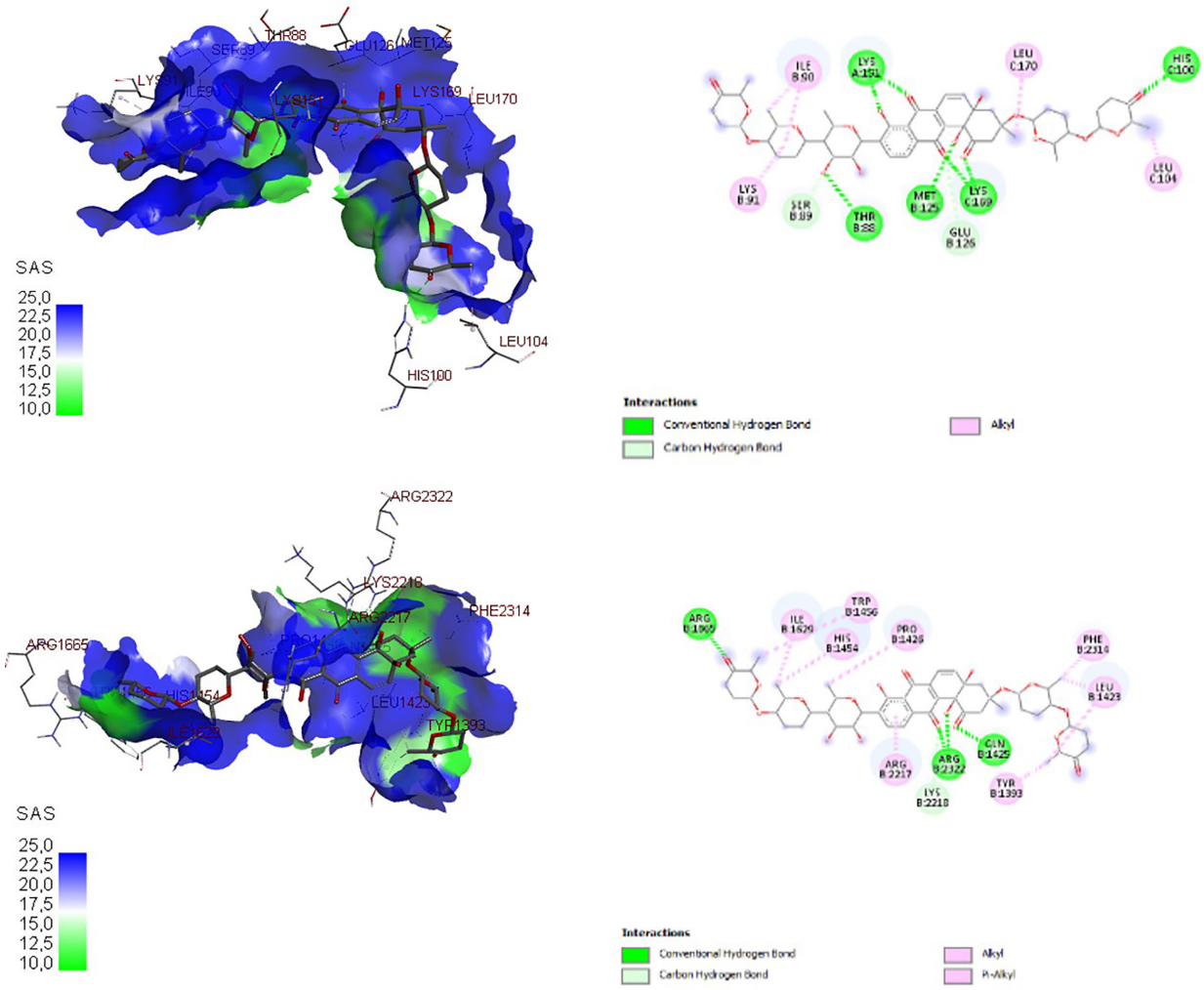

Figure 3. Three-dimensional and two-dimensional docking visualization of gricamycin on mTORC1 (top) and mTORC2 (bottom) proteins.

LR32 and havehad cytotoxicity on human cancer cell lines: liver carcinoma HepG2, pancreatic carcinoma SW-1990, cervixal carcinoma HeLa, and breast carcinoma NCI-H460 and MCF-7, and also the mouse melanoma cell line B16, with IC_{50} being in the range of 1.1–31 µM (Huang *et al*., 2012). Previous findings also showed that *Streptomyces dengpaensis* XZHG99 produces a high level of compounds: grincamycins L, M, and N (Patent CN108949610A) available online at https://patents.google.com/patent/CN108949610A/en?q=grincamycin&oq=grincamycin (accessed on 16 January 2021). In another medical field, it is known that the application of grincamycin B compounds functions as an antiglioma drug. This compound has a selective activity on glioblastoma cells. In the cell cycle, grincamycin is able to induce apoptosis in tumor cells and tumor stem cells, block cells at stages G0–G1, and inhibit glioma stem cell regeneration. Thus, grincamycin has been claimed to be a potential anticancer drug with a new mechanism of action for targeted cancer stem cell oncotherapy (Patent CN103550231B) available online at https://patents.google.com/patent/CN103550231B/en?q=grincamycin&oq=grincamycin (accessed on 16 January 2021). However, in these studies, grincamycin has never been tested as an anticancer compound in lung cancer cell lines, especially A549. Thus, this finding becomes important for the possible use of grincamycin as a candidate for lung cancer anticancer drugs in humans.

CONCLUSION

Marine-derived *Streptomyces* sp. GMY01 exhibited potential anticancer activity against lung cancer cell line A549-induced autophagy mechanism. *In silico* molecular docking also showed that detected compounds on GMY01 extract have an affinity with autophagy proteins mTORC1 and mTORC2 higher than with antiapoptotic protein BCL-2 and BCL-XL of cancer cells. This finding revealed that detected compound grincamycin from *Streptomyces* sp. GMY01 has the highest affinity on autophagy and antiapoptotic protein and has a promising anticancer agent against human lung cancer.

SUPPLEMENTARY MATERIALS

The following are available online: Table S1: Detected compounds on targeted LC-HRMS of the ethyl acetate extract produced by *Streptomyces* GMY01. Table S2: Genome mining analysis of the *Streptomyces* sp. GMY01 genome using antiSMASH 5.0.

AUTHORS' CONTRIBUTIONS

Concept and design: ED, KN, M, and JW; data acquisition: ED, KN, SH, and RF; data analysis/interpretation: ED, KN, SH, RTD, and RF; drafting manuscript: ED and KN; critical revision of the manuscript: KN, SH, M, AD and JW; statistical analysis: ED; funding: JW and KN; admin, technical, and material support: ED, KN, RTD, and JW; supervision: KN, M, AD, and JW; final approval: KN, M, AD, and JW.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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Supplementary Table S1. Genome mining analysis of *Streptomyces* sp. GMY01 genome using antiSMASH 5.0.

Region	Biosynthesis gene cluster(BGC) type	Most similar known cluster	Similarity(%)	Length (bp)	Origin organism
4.1	NRPS-like	Rhizomide A/B/C	100	22,977	*Paraburkholderia rhizoxinica*
13.1	NRPS, Lanthipeptide	Lysocin	9	71,777	*Lysobacter* sp. RH2180-5
21.1	Type 3 PKS	Herboxidiene	6	159,472	*Streptomyces chromofuscus*
21.2	NRPS	Stenothricin	13	54,895	*Streptomyces roseosporus*
24.1	Terpene	Geosmin	100	2,181	*Streptomyces coelicolor*
24.2	Bacteriocin, NRPS	Streptobactin	47	26,478	*Streptomyces* sp. ATCC 700974
24.3	Type 1 PKS (T1PKS), NRPS-like, Butyrolactone, NRPS	Microansamycin	70	69,750	*Micromonospora* sp. HK160111
24.4	Siderophore	No matches found	–	–	–
25.1	Ectoin	Ectoine	100	3,366	*Streptomyces anulatus*
26.1	NRPS	Deimino–antipain	66	15,484	*Streptomyces albulus*
27.1	Lanthipeptide	Venezuelin	50	5,339	*Streptomyces venezuelae*
27.2	Bacteriocin	No matches found	–	–	–
27.3	NRPS, other	S56-p1	17	67,922	*Streptomyces* sp. SoC090715LN-17
28.1	NRPS	Mirubactin	50	27,717	*Actinosynnema mirum*
29.1	T1PKS, NRPS	Scabichelin	90	30,020	*Streptomyces scabies*
30.1	Lanthipeptide	No matches found	–	–	–
36.1	Terpene	Albaflavenone	100	2,468	*S. coelicolor* A3(2)
38.1	Siderophore	Grincamycin	5	41,909	*S. lusitanus*
42.1	Butyrolactone	Scleric acid	23	18,478	*Streptomyces sclerotialus*
45.1	Terpene	Isorenieratene	15	9,777	*Streptomyces argillaceus*
48.1	NRPS	Saframycin A	12	62,804	*Streptomyces lavendulae*
49.1	Terpene	Hopene	53	13,757	*S. coelicolor* A3(2)
53.1	T1PKS, hgIE-KS	Vazabitide A	17	40,455	*Streptomyces* sp. SANK 60404
53.2	Bacteriocin, Lanthipeptide	Informatipeptin	8	14,866	*S. viridochromogenes* DSM 40736
55.1	Butyrolactone	No matches found	–	–	–
60.1	T1PKS	Abyssomicins M-X	56	70,308	*Streptomyces* sp. LC-6-2
63.1	Type 2 PKS	Spore pigment	83	11,118	*Streptomyces avermitilis*

Supplementary Table S2. Metabolite profile from targeted LC-HRMS of the ethyl acetate extract produced by *Streptomyces* GMY01.

Formula	Compound	Molecular mass (Da)	Area	Rt[a]	Similarity with known BGCs (%)[b]
$C_{30}H_{50}$	Hopene	410.73	81,440,490	0.90	53
$C_{15}H_{22}O$	Albaflavenone	218.34	163,449,138	7.19	100
$C_{12}H_{22}O$	Geosmin	182.303	1,350,339,203	7.34	100
$C_{40}H_{48}$	Isorenieratene	528.809	409,344,114	20.44	75
$C_{49}H_{62}O_{18}$	Grincamycin	938.393	82,450,007	20.97	5
$C_{12}H_{21}N_3O_4$	Vazabitide A	271.313	108,669,018	23.64	17
$C_{25}H_{42}O_6$	Herboxidiene	438.605	1,946,046,629	24.83	6
$C_{60}H_{68}N_{10}O_{14}$	Saframycin A	1,153.26	68,211,570	25.77	12

[a]Retention time (minutes).

[b]Based on genome mining analysis using antiSMASH 5.0.