Bioactive Compounds from Sponge Associated Bacteria: Anticancer Activity and NRPS-PKS Gene Expression in Different Carbon Sources

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Abstract: Sponge associated bacteria with diverse NRPS-PKS genes could be the alternative source of bioactive compounds. This study aimed to analyze the activity as well as the regulatory mechanism regarding the anticancer properties derived from these bacteria against cancer cell lines. Extraction of bacterial metabolites using ethyl acetate solvent resulted yield ranging from 0.003 to 0.016% (w/v). Six extracts of bacterial metabolites showed markedly cytotoxic activity against 8 human hematopoietic cancer cell lines, with IC50 value 27.24 to 533.73 µg mL−1 in a dose dependent response, as assessed by MTT test. Supporting those results, reduction of cell density, morphological changes including cell shrinkage and formation of the apoptotic body of cancer cells lines, were found. As it might be due to the inhibitory effect of bacterial extract treatment. Apoptosis assay determined by annexin V-FITC apoptosis analysis showed that apoptotic cells were induced by bacterial extract after 6 h of treatment. Thus suggesting that the extract could be one of the external signals that activate the signal transduction pathway of apoptosis, especially in MOLT4 cells, acute lymphoblastic leukemic cell lines. Cell cycle analysis using propidium iodide revealed that the extract HAA 01 caused cell cycle arrest, since most of the MOLT4 cells were accumulated in Sub-G1 phase after 24 h of extract treatment. Interestingly, glycerol, lactose and amylose were the more preferable carbon source to produce anticancer compounds, than glucose. Our study indicates that anticancer activity of HAA 01 (identified as Bacillus subtilis based on API identification) extract is highly correlated with NRPS-PKS gene expression which is likely regulated by the type of carbon source.

Keywords: Anticancer Activity, Sponge Associated Bacteria, Nonribosomal Peptide Synthetase (NRPS), Polyketide Synthase (PKS), Carbon Source

Introduction

Cancer is one of the serious diseases causing mortality worldwide. The disease caused by genetic mutations that cause uncontrolled cell growth and can spread throughout the body (Bhatt et al., 2010). World Health Organization (WHO, 2017) reported that cancer is responsible for 8.8 million deaths in 2015. The number of cancer prevalence is expected to increase in the next decade. Therefore, the discovery of a new anticancer drug would be important in cancer treatment. Principles of cancer treatment are to reduce the number of cancer cells by inducing apoptosis and inhibiting cell proliferation (Kuno et al., 2012). Chemotherapy plays an important role to treat cancer, but it still not effective due to many side effects, such as experienced are hyperuricemia, hair loss, nausea and vomiting, depression of bone marrow, anemia, bleeding, alopecia and mucositis (Hassan, 2012). The development of a new anticancer that lack of side effect is essential to effectively reduce the cancer prevalence. Screening of natural products is expected to...
obtain a bioactive compound with high anticancer activity with minimum side effects.

Sponge associated bacteria could be the alternative source of bioactive compounds. In the previous study, exploration of secondary metabolites extracted from bacteria associated with sponges *Callyspongia* sp, *Petrosia* sp and *Stylotella* sp. (from Bira Island, Kepulauan Seribu, Jakarta), *Halilcena* sp. and *Jaspis* sp. (from Raja Ampat Island, Papua) has been done. They have some of medicinal properties, including antimicrobial activity (Tokasaya, 2012; Yoghiiapiressa et al., 2016), protease inhibitor (Wahyudi et al., 2010), antioxidant and anticancer on HeLa cell lines (Utami et al., 2014; Safari et al., 2016) and leukemic cell lines (Karwati et al., 2015). Based on those potential characters, thus in this study, we attempted to understand the activity as well as the regulatory mechanism of the anticancer properties derived from these sponge associated bacteria towards human hematopoietic cancer cell lines. It is worth noting that the genes encoding bioactive compounds, Nonribosomal Peptide Synthetase (NRPS) and Polyketide Synthase (PKS) gene have been detected in some of the bacterial isolates (Safari et al., 2016). Therefore, this study is essential to confirm the involvement of these genes in the synthesis of bacterial anticancer compounds. In addition, synthesis of bioactive compounds can be affected by carbon source in the growth medium (Ruiz et al., 2010). Carbon sources are often involved in transcriptional activation or repression of genes (Uguru et al., 2005), therefore modification of medium carbon source needs to be done to provide the suitable condition to produce anticancer compounds. Thus, the study concerning the effect of carbon source on the expression of the anticancer compound synthetic pathway was conducted.

### Materials and Methods

#### Culture of Sponge Associated Bacteria

Each bacterial isolate was cultured in 1 L Sea Water Complete (SWC) medium (bacto peptone 5 g, yeast extract 1 g, glycerol 3 mL, artificial sea water 750 mL and distilled water 250 mL) and incubated in a shaker incubator at 100 rpm for 3 days at 28°C.

#### Extraction of Bacterial Metabolites

Bacterial extracts were prepared by using ethyl acetate. Extractions were conducted based on the previous methods (Utami et al., 2014; Safari et al., 2016; Karwati et al., 2015) but with several modifications. Following addition of 1 L ethyl acetate to the bacterial culture, the culture and solvent solutions were then shaked continuously for 20 min in a separating funnel. The solvent layer was evaporated at 40°C. Then, the bacterial extracts were stored below 5°C until used. The bacterial extract was solubilized in Dimethyl Sulfoxide (DMSO) before used.

#### Anticancer Activity Assay

Cancer cell lines used in this study were K562 (chronic myelogenous leukemia), Nalm6 (lymphoma), MOLT4 (acute lymphoblastic leukemia), Raji (Burkitt's lymphoma), Daudi (Burkitt's lymphoma), CEM6 (human T leukemic cell lines), HL60 (acute promyelocytic leukemia), U937 (histiocytic lymphoma) from RIKEN Cell Bank, Japan. A cytotoxic property of six potential bacterial extracts was analyzed by Microculture Tetrazolium Test (MTT) against cancer cell lines (Thakur et al., 2005). 50 µL of cell suspension (5×10³ cells/well) was plated into 96-well plates containing 50 µL Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% Fetal Bovine Serum (FBS), 2% L-glutamine and 1% streptomycin penicillin in various extract concentrations (0, 25, 50, 100, 200, 400, 800 µg mL⁻¹) and incubated at 35°C in 5% CO₂ condition for 48 h. The medium without extracts was used as negative control and doxorubicin was used as a positive control. After 48 h, 10 µL of Cell Counting Kit (CCK-8) were added to each well and incubated at 35°C for 4 h, then measured the Optical Density (OD) at 450 nm using microplate reader MTP-450 (Corona Electric, Japan). The Inhibition of cell growth was determined using the following formula:

\[
\text{% Inhibition} = \left(\frac{\text{OD of control cells} - \text{OD of treated cells}}{\text{OD of control cells}}\right) \times 100\%
\]

This assay was performed in triplicate. The IC₅₀ value was determined by the linear regression.

#### Apoptosis Assay

The effect of the extract on cell apoptosis was analyzed by using Annexin V-FITC Apoptosis Detection Kit (ApoScreen®, Southern Biotech) following the previous method with some modifications (Karwati et al., 2015). Approximately 1×10⁶ MOLT4 cells were incubated with 100 µg mL⁻¹ of bacterial extracts and 5 µg mL⁻¹ of doxorubicin for 6 h. Apoptosis assay was carried out according to the manufacturer’s protocol.

#### Cell Cycle Assay

Cell cycle analysis was conducted as described previously with some modifications (Karwati et al., 2015). MOLT4 cells were seeded at a cell density of 1×10⁶ cells/well in 24-well plates and incubated with 100 µg mL⁻¹ of bacterial extracts and 5 µg mL⁻¹ doxorubicin for 24 h at 37°C in 5% CO₂ condition. After treatment, treated cells were centrifuged and the pellets were then washed three times with Phosphate Buffer Saline (PBS) prior to addition of 0.9 mL staining solutions. Staining solution for 10-11 samples were prepared by mixing 4.5 mL propidium iodide stock
solution (0.1 mg mL\(^{-1}\) DDW), 1 mL Na-citrate stock solution (0.012 g mL\(^{-1}\) DDW), then 0.45 mL\(^{-1}\) RNase (10 mg mL\(^{-1}\)), 0.1 mL 10% triton X-100, 3.95 mL DDW). Cells suspension were then mixed by using vortex and incubated for 10 min on ice prior to addition of 0.1 mL 1.5 M NaCl and filtered through a nylon mesh. Samples were analyzed by flow cytometry (AccuriTM C6, USA). This assay was carried out in triplicate. The output data analyzed by FlowJo v10 software.

**Carbon Source Modification and NRPS-PKS Gene Expression Analysis**

The most potential bacterial isolate was cultured in SWC medium containing 0.3% with different carbon sources as glycerol, glucose, lactose and amyloan. The bacterial RNA was extracted by using Nucleospin® RNA Plus Kit following to the manufacturer’s instructions. PKS-NRPS gene expression analysis was carried out by one step quantitative reverse transcription PCR (qRT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Primer GAPDHF: GCT GAT GTC TCA, GAPDHR: CCC TTC ATC TTG CCG GGT TT) were designed using PRIMER 3. PCR mix was performed according to Takara Onestep SYBR RTase mix, 0.4 µL PCR forward primer (10 µM), 0.4 µL PCR reverse primer (10 µM), 0.2 µL ROX reference dye, 1 µl total RNA (100 ng) and adjusted with RNase Free dH\(_2\)O to 10 µL. Cycling parameters for reverse transcription are 42°C for 5 min and 95°C for 10 s, followed by 40 cycles of PCR reaction with DNA denaturation at 95°C for 5 s, primer annealing 55°C for 30 s. The evaluation of expression levels of mRNA was determined by calculating their 2\(^{-\Delta\Delta Ct}\) values.

**Anticancer Activity of Sponge Associated Bacteria**

Cytotoxic properties of 6 bacterial crude extracts were determined by MTT test. The capability of 6 bacterial crude extracts to inhibit the proliferation of cancer cells was found diverse, as shown by the various IC\(_{50}\) value ranging from 27.24±11.57 µg mL\(^{-1}\) to 533.73±29.1 µg mL\(^{-1}\). From the MTT assay, we found four potential extracts with highest IC\(_{50}\) value were detected, including HAA 01, HAL 74, HAL 13, STIL 37. Among them, the extract of HAA 01 showed the best anticancer activity on some of the cancer cell lines, with the highest IC\(_{50}\) value (27.24±11.57 µg mL\(^{-1}\)) against CEM6, human T leukemic cell lines. Anticancer activity of the bacterial metabolites extract could also be observed from the alteration of cell density and morphology of cancer cells. The morphological alteration was observed as cells underwent a change from polygonal to unanimous form, that viable cells to nearly 70.5±0.8%. Whereas, about 27.8±1.3% and 1.7±0.6% cells were found in the early apoptotic and late apoptotic phase, respectively. The cell population was significantly different with untreated cells that have more viable cells (98.7%) and DMSO treatment (98.6%).
Fig. 1. Anticancer activity of 6 bacterial extracts on some of the human hematopoietic cancer cell lines. Anticancer activity was measured by using MTT assay in three independent replications. Doxorubicin was used as positive control. CEM6 (human T leukaemic cell lines), K562 (chronic myelogenous leukemia), MOLT4 (acute lymphoblastic leukemia), U937 (histiocytic lymphoma), Nalm6 (lymphoma), HL60 (acute promyelocytic leukemia), Raji (Burkitt’s lymphoma), Daudi (Burkitt’s lymphoma) Fig. 2. Density and morphology of CEM6 cells following 48 hours of extract treatment. Cell morphology was observed in phase contrast microscope 400.0x. Cells were exposed to 25, 50, 100, 200, 400, 800 µg mL$^{-1}$ of HAA 01 extracts. Doxorubicin (6.25 µg mL$^{-1}$) and without extract treatment were used as the positive and negative control, respectively. Arrow represents apoptotic cells

Table 1. Effect of bacterial extracts on the apoptosis of MOLT4 cells

| Samples     | Viable cells (%)$^a$ | Early apoptotic cells (%)$^a$ | Late apoptotic cells (%)$^a$ | Dead cells (%)$^a$ |
|-------------|----------------------|-------------------------------|-----------------------------|-------------------|
| Untreated   | 98.7±0.2             | 1.1±0.1                       | 0.2±0                       | 0±0               |
| DMSO        | 98.6±0.6             | 1.2±0.7                       | 0.2±0.2                     | 0±0               |
| Doxorubicin | 73.9±2.6             | 25.1±3.6                      | 1.1±1.2                     | 0±0               |
| HAA 01      | 70.5±0.8             | 27.8±1.3                      | 1.7±0.6                     | 0±0               |
| HAL 13      | 87.9±1.8             | 11.8±1.8                      | 0.3±0.1                     | 0±0               |
| HAL 74      | 78.7±2.9             | 20.8±3                        | 0.4±0.1                     | 0.1±0             |
| STIL 37     | 95.2±1.5             | 4.3±1.7                       | 0.4±0.1                     | 0.1±0             |

MOLT4 cells were incubated with 100 µg mL$^{-1}$ bacterial extracts and 5 µg mL$^{-1}$ doxorubicin for 6 h in triplicates, $^a$mean±SD

The Inhibitory Effect of Bacterial Extract on Cell Cycle Progression

Cell cycle progression was determined based on DNA content which proportional with PI fluorescence. The apoptotic cell was represented as sub-G1 population. The cells treated with samples had different DNA content compared with untreated cells. As shown in Fig. 3, there was the significant decrease in the number of DNA content in G1, S and G2-M and
accumulation of DNA content in sub-G1 after extract (100 µg mL\(^{-1}\)) and doxorubicin (5 µg mL\(^{-1}\)) treatments for 24 h. HAA 01 treatment caused significant alteration particularly by arresting the MOLT4 cells cycle at G1 phase, since 38.5% cells were found in sub-G1 phase, 15.2% cells at G1, 4.49% at S, 1.34% at G2-M and more than 40% cells were non-viable after treatment. Meanwhile, in untreated cells, most of the cells were found in G1 phase (58.9%), S phase (24.1%), G1-M phase (13.6%) and only a few cells in Sub-G1 (2.32%).

**NRPS-PKS Gene Expression in Different Carbon Sources**

We studied the effect of different carbon sources in the mRNA levels of PKS and NRPS genes of the most potential isolate, HAA 01. In this analysis, glycerol was employed as a control since this particular carbon source was used in former assays. As shown in Fig. 4, expression of PKS gene was down-regulated in glucose, lactose and amyllum as compared to that glycerol. Interestingly, lactose significantly up-regulated the expression of NRPS genes with 1.2 fold increase compared to glycerol, on the other hand, glucose and amyllum repressed the expression of NRPS genes.

**Anticancer Activity of Bacterial Extract from Different Carbon Sources**

The extract from HAA 01 grown in different carbon sources was further tested for its anticancer activity against MOLT4 cells. The IC\(_{50}\) value among the extracts was various. Bacterial extracts from lactose and glycerol exhibited the best IC\(_{50}\) value, at 112.03±2.15 µg mL\(^{-1}\) and 149.63±43.47 µg mL\(^{-1}\), respectively. However, no differences were found between the IC\(_{50}\) value of extract from glycerol and lactose. In contrast, glucose has the lowest IC\(_{50}\) value than other carbon sources at 525.95±5.74 µg mL\(^{-1}\), which was 3 and 2 times lower than lactose and amyllum (Fig. 5).

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Fig. 3. The effect of bacterial extracts (100 µg mL\(^{-1}\)) on the cell cycle distribution in MOLT4 cells after 24 h treatment. Analysis of cell number was presented as the percentage of each cell cycle phase relative to total phases. Doxorubicin (5 µg mL\(^{-1}\)) was used as positive control while untreated cells and DMSO treatment were employed as the negative control.
Biochemical Identity of the Most Potential Isolate

HAA 01 strain showed some of the biochemical profiles and carbon source utilization in AP01 CHB strips. Based on its characteristics, HAA 01 strain was closely related to *Bacillus subtilis* with 99.8% similarity.

Discussion

Sponge associated bacteria can produce bioactive compounds that expected to have some of the medicinal property, especially anticancer activity. The bacterial crude extract used in this study may contain the variety...
of compounds. In this study, 6 bacterial metabolites showed different anticancer activities, specific for each cancer cell lines. Supporting those results, reduction of cell density, morphological changes including cell shrinkage and formation of the apoptotic body of cancer cells lines, particularly CEM6, were found. As it might be due to the inhibitory effect of bacterial extract treatment.

Apoptosis is an important mechanism to remove unnecessary and unwanted cells (Korbakis and Scorilas, 2012). Induction of apoptosis is a predominant mechanistic approach to target cancer in treatment. To confirm the anticancer activity of four extracts with the best IC50 value, apoptosis analysis was conducted on MOLT4 cells. Indeed, treatment with HAA 01 extract showed the strongest effect in causing apoptosis of MOLT4 cells. We found that MOLT4 cells treated with HAA 01 extract have fewer viable cells (70.5 %) compared than untreated cells (98.7%). Our data indicate the future application of HAA 01 extract in treating MOLT4 cells. MOLT4 cells belong to an acute lymphoblastic leukemia, which is cancer that starts from the early (immature) stage of lymphocytes in the bone marrow. Without treatment, most people with acute leukemia would live only a few months while most of the people with chronic leukemia can live for many years (ACS, 2017). Thus, further analysis regarding the specific target of HAA 01 extract toward MOLT4 cells proliferation should be conducted.

Based on our cell cycle analysis, HAA 01 likely has an anti proliferative property through cell cycle arrest, as the sub-G1 phase of MOLT4 cells was accumulated following extract treatment. It is worth noting that the HAA 01 extract substantially shows the cytotoxic effect against MOLT4 cells via cell cycle arrest as well as apoptosis induction. Those results are in agreement with several previous studies. In instance, bacterial extract from sponge induced the expression of genes, compared to glycerol. On the other hand, lactose repressed the expression of both genes. It is worth noting that the HAA 01 isolate has both higher level compared to that glycerol. The synthesis of secondary metabolites may correlate with the preferential use of medium carbon source. Some of the carbon sources can inhibit the transcription of specific genes encoding bioactive compounds. These mechanisms are renowned as carbon catabolite repression (Ruiz et al., 2010). Our study suggests that glycerol, lactose and amylum could be the preferred carbon source in HAA 01 strain to optimize the expression of NRPS and PKS genes. While glucose repressed the expression of both genes. In addition to that gene expression results, the bacterial extract derived from glucose-containing culture exhibited depletion of its anticancer activity against MOLT4 cells.

The carbon source may activate the anticancer properties or, on the contrary, inhibit the formation of anticancer compounds. Although glucose is a preferable carbon source for primary metabolism (Ilíć et al., 2010), however, the glucose may inhibit secondary metabolites synthetic pathway in microorganisms (Ruiz et al., 2010). In our experiment, lactose and glyceral showed a great potential as a carbon source for the production of anticancer compounds in this experiment, instead. Similarly, previous study reported that lactose, glyceral, amylum were found to be suitable carbon source to produce secondary metabolites, including elaiophylin and hexaene H-85 (Ilíć et al., 2010), AK-111-81 macrolide antibiotic (Gesheva et al., 2005), prodigiosin (Elkenawy et al., 2017), rapamycin (Kim et al., 2014), MSW2000 antibiotic (El-Naggar et al., 2003). On the other side, glucose, which the most simple of medium carbon source, is affecting the synthesis of some bacterial metabolites. Similar to the results in this study, glucose depresses prodigiosin production in Serratia marcescens by influencing the activity of Glucose Dehydrogenase (GDH) activity (Fender et al., 2012) and decreasing the pH (Sole et al., 1997). The presence of glucose also inhibited the synthesis of actinorhodin in Streptomyces lividans. Glucose repressed the specific gene for stimulation of secondary metabolites production, afsR2 (Kim et al., 2001). Therefore, the utilization of medium carbon source is the main factor required for optimizing the production of bacterial bioactive compounds.

Our study indicates a unique correlation between NRPS-PKS gene expression in different carbon sources toward its respective anticancer activity. A high anticancer activity is in line with the high expression of NRPS-PKS genes, exhibited by the extract from glyceral, lactose and amylum-culture. Conversely, the low expression of NRPS-PKS in glucose correlates with the low anticancer activity of the extract. These data strongly suggest that PKS-NRPS genes are likely involved in the synthesis of anticancer compounds, particularly of that HAA 01 isolate. HAA 01 isolate has both PKS and
NRPS genes, thus this isolate may potentially synthesize anticancer compounds from the hybrid NRPS-PKS genes. Some of the bioactive compounds have been reported to be synthesized from such hybrid NRPS-PKS mechanism, including bleomycin (Mizuno et al., 2013), leinamycin (Tang et al., 2006) and curacin A (Chang et al., 2004). Further research is needed to verify the role of these genes in the biosynthesis of anticancer compounds.

Based on the phenotypic identification, HAA 01 strain was identified as Bacillus subtilis with high similarity. This result is consistent with the previous genotypic identification based on 16S rRNA that was conducted by Wahyudi (2016). The previous study reported that marine Bacillus can produce some of the bioactive compounds including bacillistatin, mixirin and halobacillin that potential as anticancer (Mondol et al., 2013). Moreover, these studies suggest the future application of marine bacterial extract as a new anticancer drug.

Conclusion

From our study, it is worth noting that secondary metabolites from sponge associated bacteria could be potential as anticancer based on the inhibition of cancer cell proliferation, initiation of apoptosis and cell cycle arrest of that cancer cell lines. Our study indicates that anticancer activity of HAA 01 extract is highly correlated with NRPS-PKS gene expression which is likely controlled by the type of carbon source.

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Author’s Contributions

Jepri Agung Priyanto: Has contributed in a whole experiment, data analysis and publication.

Rika Indri Astuti: Has assisted in the experimental design, results confirmation, interpretation of data and revising the article critically.

Jun Nomura: Has contributed in providing materials for anticancer analysis, interpretation of data and paper preparation.

Aris Tri Wahyudi: Has lead the project, connected the research outcome and involved in paper preparation.

Ethics

This article is authentic from authors works. The corresponding author ensures that all of the other authors have read and recognized the manuscript.

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