RESEARCH ARTICLE

Pyrophen Produced by Endophytic Fungi Aspergillus sp Isolated from Piper crocatum Ruiz & Pav Exhibits Cytotoxic Activity and Induces S Phase Arrest in T47D Breast Cancer Cells

Puji Astuti1*, Willy Erden2, Wahyono1, Subagus Wahyuono1, Triana Hertiani1

Abstract

Ethyl acetate extracts obtained from culture of endophytic fungi Aspergillus sp isolated from Piper crocatum Ruiz & Pav, have been shown to possess cytotoxic activity against T47D breast cancer cells. Investigations were here conducted to determine bioactive compounds responsible for the activity. Bioassay guided fractionation was employed to obtain active compounds. Structure elucidation was performed based on analysis of LC-MS, 1H-NMR, 13C-NMR, COSY, DEPT, HMQC, HMBC data. Cytotoxicity assays were conducted in 96 well plates against T47D and Vero cell lines. Bioassay guided isolation and chemical investigation led to the isolation of pyrophen, a 4-methoxy-6-(1'-acetamido-2'-phenylethyl)-2H-pyran-2-one. Further analysis of its activity against T47D and Vero cells showed an ability to inhibit the growth of T47D cells with IC50 values of 9.2 µg/mL but less cytotoxicity to Vero cells with an IC50 of 109 µg/mL. This compound at a concentration of 400 ng/mL induced S-phase arrest in T47D cells.

Keywords: Aspergillus sp - cytotoxic - T47D - pyrophen - S-phase arrest

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Introduction

Breast cancer is the major cause of death from cancer in women with about 508000 women was estimated died from this disease in 2011 (IARC, 2012; WHO, 2013). The high incidence in developed countries is also occurring in developing countries and the number is increasing due to in part the increase in life expectancy and changes in lifestyles. In addition, the lack of early detection program and inadequate diagnosis and medical facilities result in low survival rates (IARC, 2008). Despite this huge global health problem, the available conventional chemotherapeutic agents possess side effects to normal cells. Therefore, there is an urgent need to discover and develop new compounds to treat breast cancer.

Exploration of endophytic fungi have gained increased interest among scientist as these microorganisms produce biologically-active compounds with therapeutic values (Zhao et al., 2009; Santiago et al., 2012; do Nascimento et al., 2015; Richardson et al., 2015). Many of the compounds have been reported to produce bioactive compounds against various types of cancer cell lines (Chui et al., 2011; Zheng et al., 2013; Zhu et al., 2013; Chen et al., 2015; Wang et al., 2015). Previous studies in our laboratory demonstrated that ethyl acetate extract obtained from the culture of Aspergillus sp isolated from the stem of Piper crocatum Ruiz. & Pav. exhibited cytotoxic activities against T47D cells (Astuti, et al., 2014). This study aims to explore bioactive compounds responsible for the activities.

Materials and Methods

Materials

Potato Dextrose Agar (PDA). Dextrose were purchased from Oxoid. RPMI 1640, Fetal Bovine Serum, Penicillin - Streptomycin, Fungizon, Sodium bicarbonate, were supplied from Gibco. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Invitrogen), Phosphate-Buffered Saline (PBS), propidium iodide (PI) (Sigma-Aldrich), MTT (Sigma-Aldrich). Silica gel F254, Silica gel 60 PF254 containing gypsum, DMSO, chloroform, n-hexane, ethyl acetate (Merck). Endophytic fungi Aspergillus sp isolated from Piper crocatum Ruiz & Pav (Culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy, UGM).

Semipolar extraction of fermentation cultures

The endophytic fungi grown in PDA was inoculated in 1000 mL potato dextrose broth in Erlenmeyer flasks and incubated at room temperature and 160 rpm for 10 days. The crude fermentation broth were filtered with Whatman filter paper and followed by centrifugation at 4 000 r/min.
for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice. The ethyl acetate fractions were then evaporated under reduced pressure to yield an ethyl acetate extract.

Bioassay guided isolation of cytotoxic compound

Ethyl acetate extract was partitioned using preparative thin layer chromatography (stationary phase=silica gel 60 PF254; mobile phase=chloroform: methanol=9:1) to obtain five fractions to be tested for cytotoxic activities. The most cytotoxic fraction with the highest score of selectivity index was further purified using preparative thin layer chromatography (stationary phase: silica gel 60 PF254; mobile phase: ethyl acetate: n-hexane=9:1) to obtain bioactive compounds.

Cytotoxic activity

T47D (Human ductal breast epithelial tumor cell line) and Vero (cell line) were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 µg/mL fungizone. The cultures were incubated in a humidified incubator at 37°C with 5% CO2. A hundred µl of media containing 5x104 cells was added to 96-well plate and incubated for 48 hours until 70% - 80% confluent. Extracts or isolates were added to the wells at various concentrations followed by 24 hours of incubation. The treated cells were gently washed with pre-warmed 1X PBS, and 100 µl media containing 0.5 mg/mL MTT was added to the well. The cells were incubated for 4 hours at 37°C and the reaction was stopped by adding 100 µL of 10% SDS. The plates were read in microplate reader (Bio-Rad) at 595 nm. Data generated were used to plot a dose-response curve of which the concentration of fractions/isolates required to kill 50% of cell population (IC50) was determined. Selectivity index was calculated by dividing IC50 values of fractions/isolates against T47D cells towards IC50 values of fractions/compounds against normal Vero cells.

Cell cycle analysis

Three mL of media containing 5x104 T47D cells were inoculated in six well plates and incubated for 24 hours prior to treatment with bioactive compound. The cells were treated with various concentrations of bioactive compound and further incubated for 16 hours. The cells were harvested and re-suspended in PBS containing 50 µg/mL propidium iodide and ready for cell cycle distribution analysis using flow cytometer BD FASCALiburTM (BD Biosciences).

Structure elucidation of cytotoxic compound

Structure elucidation was conducted based on analysis of LC-MS, 1H-NMR, 13C-NMR, COSY, DEPT, HMQC, HMBC data. Phyrophen: white crystalline powder; ESI-MS m/z 288.2834 [M+H]+ (calcd for C16H17NO3, 288.2834); 1H and 13C NMR in DMSO-d6, see Table 2.

Results

Bioassay guided isolation and bioactivity testing

Ethyl acetate extract was partitioned by preparative thin layer chromatography to obtain five fractions. These fractions were tested for cytotoxicity against T47D and Vero cells. Among five fractions tested fraction 4 was found to have the highest selectivity index (Table 1). This fraction contains a bioactive compound having 95.97% purity as determined by Liquid Chromatography (Figure 1). This compound exhibited IC50 of 9.2 µg/mL against T47D and IC50 of 109.0 µg/mL against normal Vero cells. Further cell cycle analysis demonstrated that this compound induced S-phase arrest when it was administered at 400 ng/mL (Figure 2).

Structure elucidation of the cytotoxic compound

LC-MS peak of isolate appeared at Tg=3.24 min having a 95.97% of purity. Molecular weight of 287 amu was revealed by analysing a pseudomolecular ion peak at m/z 288.2834 having a 100% abundance referred to [M+H]+, which is confirmed by the presences of peaks of [M+Na]+ at m/z 310.2870; [2M+H]+ at m/z 575.6121 and [2M+Na]+ at 597.6084.

1H-NMR data showed that the isolate contains at least 17 protons distributed from the most upfield region at δ 1.78 (3H, s) referred to a -CH3 having an electron-withdrawing neighbour, up to the most down field proton, at δ 8.49 (1H, d, J=8.4 Hz). Evaluation of splitting pattern and coupling constants suggests two isolated substructures which is confirmed by the 1H-1H COSY measurement, i.e. =:-CH2-CH-NH- and a monosubstituted benzene.

Table 1. IC50 Values and Selectivity Index of Fractions Generated from Ethyl Acetate Extract

| Fractions | F1 | F2 | F3 | F4 | F5 |
|-----------|----|----|----|----|----|
| IC50 (µg/mL) against T47D cells | 142.9 | 82.6 | 6.9 | 9.2 | 48.4 |
| IC50 (µg/mL) against Vero cells | 439.5 | 308.4 | 53.8 | 109 | 338.8 |
| Selectivity Index | 3.1 | 3.7 | 7.8 | 11.8 | 7 |

Figure 1. Liquid Chromatography Profiles of Fraction 4 Containing Bioactive Compound. Volume of injection 20 µl, Flow 1 mL/min, Eluent MeOH + water = 95 + 5
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C-NMR resolved 14 carbon resonance peaks of which DEPT analyses revealed the presence of 1 secondary carbon at δ 37.85; 5 quartenary carbons at δ 170.77; 169.08; 163.78; and 163.21. 1H-13C HMQC analyses distinguished 2 CH3s at δ 22.41 and 56.48, while the rest 6 peaks referred to CHs. Each peaks at δ 129.06 and 128.30 represents the presence of 2 CHs having identical environment. These peaks together with a CH peak at δ 126.58 and δ 137.27 suggested the carbon resonances of a monosubstituted benzene. The presence of -CH2-CH-NH- was confirmed by the 1H-13C HMBC analyses, which at the same time proposed the substructure as shown in Figure 3.

Different effect of the protons environment of the CH2 resulted two adjacent peaks at δ 2.89 (1H, dd, J=13.7, 9.1 Hz) and 3.01 (1H, dd, J=5.8, 13.7 Hz). A coupling constant value of 13.7 Hz corresponds to a geminal coupling of the CH2, while a value of 5.8 Hz corresponds to a 3Jtrans coupling and 9.1 to a 3Jcis coupling with the adjacent neighbour at δ 4.76 (1H, ddd, J=5.8, 9.1, 8.4 Hz) confirmed the proposed relative stereochemistry (Figure 3). The COSY correlate to the next proton at δ 8.49 of which both shares a coupling constant value of 8.4 Hz. This finding is in accordance to a presence of an amide moiety adjacent to a tertiary carbon as is also supported by the C13-NMR data and HMBC correlation. Further analyses of the COSY data reveals a present of two isolated neighboring proton having δ 5.57 (1H, d, J=2.6 Hz) and δ 6.05 (1H, d, J=1.9 Hz). A small coupling constant with the neighboring proton (J=1.9 Hz) suggesting that both protons are not directly attached to each other. The HMBC data correlate the protons with a quartenary carbon at δ 170.77 which is also seen by an isolated proton at δ 3.78 (3H, s). This isolated proton is in accordance to a resonance of a methoxy group by having a deshielded singlet peak having integration of 63. A full structure proposed to the compounds confirmed a molecular formula of C16H17NO4 which is fit to a 4-methoxy-6-(1'-acetamido-2'-phenylethyl)-2H-pyran-2-one, known as...
Discussion

Pyrophen is an amino acid-pyrene derivative firstly isolated from Aspergillus niger on maize with the crystal configuration was reported in 1990 (Barnes et al., 1990). The spectroscopic data of this compound was published in 2000 (Varoglu and Crews, 2000), which is in accordance to the spectroscopic data of the bioactive compound isolated from this study. However a comparation of the spectral data reveal a huge shift of the signal of the NH-amide to the downfielded region. As already accepted for an exchangeable proton such as NH, unpredictable chemical shift could occurs, especially in the case of different solvent used. Pyrophen was also reported from a saltwater culture of A. niger isolated from a sponge Hyrtios proteus (Varoglu and Crews, 2000), the culture extract of A. niger EN-13 isolated from the inner tissue of the marine brown alga Colpomenia sinuosa, (Zhang et al., 2010) as well as the culture of Alternaria alternata strain D2006, isolated from the marine soft coral Dendronephthya hemprichii (Shaaban et al., 2012). It was interesting to note that Zhang and collaborators can separate pyrophen from its stereoisomer, isopyrophen, which was obtained from A. niger cultured on yellow maize. The compounds are different at the configuration of Cα as the chiral centre. Nevertheless, unlike pyrophen, Zhang et al (2010) described that the isopyrophen has planar structure, which was not the case for our compound.

In terms of bioactivities, pyrophen was reported to have no antimicrobial activities against Escherichia coli, Staphlococcus aureus, Candida albicans, and A. niger (Zhang et al., 2010) and no activities against several cancer cell lines (Varoglu and Crews, 2000). However, this compound was found to be active against C. albicans (Shaaban et al., 2012). This discrepancy might be resulted from the difference on the concentration used during their studies. In this study we found that pyrophen exhibited cytotoxicity against T47D cells. Cycle cycle analysis of T47D cells exposed with this compound at 400 ng/mL resulted in increased S phase cell population. Indeed, S phase arrest is one of mechanisms delaying progression of cell cycle in response to homeostatic regulators or DNA damage and this involves the decrease rate in DNA replication (Kaufmann and Kaufman, 1993). Further examination of its potential as anticancer agent is still under investigation.

In conclusion, these data suggest the potential activity of a phenethyl-α-pyrone derivative, pyrophen as anti-cancer agent. Further investigation on the mechanism on how this compound kills the cancer cells need to be explored.

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