Jure Leaf Extract (Nerium indium Mill.) Increased 5-Fluorouracil Sensitivity through Inhibition of NF-κB Activation and Transporter Protein in WiDr Colon Cancer Cell

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Abstract

5-Fluorouracil (5-FU) is the first line chemotherapeutic agents for colon cancer therapy. Long term used of 5-FU caused cancer cell resistency. Thus, co-chemotherapeutics agent should be developed to increase cells sensitivity towards 5-FU. Jure leaf (Nerium indicum Mill) extract (JLE) contains oleandrin which has cytotoxic effect on colon cancer cell. The aim of this study was to investigated the mechanism of JLE to sensitized colon cancer cell toward 5-FU through NF-κB inhibition and MRP protein repression. JLE was extracted by soxhletation method. Based on molecular docking to MRP protein, docking score of oleandrin (-50,496) was higher than native ligand ATP (-125,817). Oleandrin could interfere interaction with MRP. JLE increased 5-FU sensitivity a dose of 2 μg/mL JLE dan 12.5 μM 5-FU with the combination index (CI) of 0.594. Combination of JLE and 5-FU also inhibit p65 protein expression on WiDr cell.

Keywords: cytotoxic, Nerium indicum Mill., oleandrin, immunofluorescence, molecular docking

INTRODUCTION

Colon cancer become the third ranking of the death cause in the world caused by cancer (Li and Lai, 2009). Nowadays, the colon cancer was treated using chemotherapeutical agent. The first line chemotherapeutical agent for colon cancer was 5-Fluorouracil (5-FU). Long term use of 5-FU could decreased cancer cell sensitivity toward chemotherapeutics agent.

MRP is transporter protein which involved in eflux process of many chemotherapeutics agent. Overexpression of MRP in cancer cells cause the lob level of chemotherapy efficacy. P-gp was encoded by MDR1. NF-κB is transcription factor which regulated many gen involved in immunity, inflammation, dan cell survival (Ghosh and Hayden, 2008). Phosporilation of IκB by IKK activated NF-κB and translocated NF-κB in nucleus leading to cell proliferation. The inhibition of NF-κB leading to cancer cell arrest, cells dead, increasing sensitivitiy to chemotherapeutics agent (Escárcega et al., 2007). MDR1 was activated trough NF-κB pathway (Thévenod et al., 2000; Kuo et al., 2002).

Jure leaf (Nerium indicum Mill.) contains oleandrin, a glycosyde that has cytotoxic activity toward cancer cell (Newman et al., 2007). The aims of this study was to investigate the potency of jure leaf extract (JLE) as co-chemotherapeutical agent toward 5-FU resistency in colon cancer. The mechanism was investigated by molecular docking between oleandrin and MRP. Cell viability observation was conducted by MTT assay. The inhibition of protein expression was conducted by immunofluorescence assay.

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MATERIAL AND METHOD

Plant Collection and Extraction

Jure leaf (*Nerium indicum* Mill.) was obtained from Yogyakarta and identified by Pharmaceutical Biology Department of Faculty of Pharmacy, Universitas Gadjah Mada. Jure leaf was extracted by soxhletation method using 96% ethanol and evaporated using Rotary evaporator. Identification of phytochemical compound of the extract was conducted by thin layer chromatography (TLC) with silica gel GF254 as stationary phase and chloroform:aceton (8:2) as mobile phase. The TLC visualization was conducted using p-anisaldehyde and AlCl3 as the spray reagent, then was observed in UV 366 nm.

Cell Culture

WiDr colon cancer cell cells were obtained from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, NAIST, Japan). Cells were cultured at 37°C with a humidified incubator, 5% CO2, in suitable medium RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), 10,000 UI/ml penicillin-streptomycin (Gibco).

Cytotoxic Assay

Cytotoxicity of WiDr cells were determined using MTT assay (Mosmann, 1983) with minor modification. Cells were distributed into 96-well, then incubated in 37°C incubator supplemented with 5% CO2. After 24 hours incubation, culture medium was removed, followed by treatment using JLE and 5-FU. Next day, 0.5 mg/ml of MTT (3-[4,5-dimethyl thiazole-2-yl](-2,5-diphenyltetrazolium-bromide)) in PBS was added, followed by 4 hours incubation in 37°C with 5% CO2. After that 10%/v SDS in HCl 0.01N as stopper reagent was then added. Plate was then kept with protection from light overnight, continued with absorbance determination (λ 595 nm) using ELISA reader (Bio-Rad).

Immunofluorescence Assay

Cells were grown on coverslip in 24-well plate up to 80% confluent. Cell were treated with compounds, single and combination with chemotherapeutic agents and incubated for 24 hours. After 24 hours, cell were fixed by 70% ethanol and incubated for 15 min at room temperature. After rinsed with PBS, cells were incubated with blocking serum 1% BSA for 30 min at room temperature. Then, cells were incubated with primary antibody (p65) for 1 hour at room temperature. After rinsed with PBS, cells were incubated with secondary antibody conjugated by FITC for 1 hour at room temperature in the dark. Then, cell were added with DAPI solution and incubated for 10 min at room temperature in the dark. After rinsed with PBS, cell were added with mounting solution (Fluoromount), put on slide glass, and store at 4°C. The protein expressions were observed under fluorescence microscope.

Molecular Docking

Molecular docking was conducted using PLANTS software. Protein target file (2CBZ) was operated using YASARA software to arrange the environment condition which is appropriate with human physiology. Oleandrin structure was build up using Marvin Sketch software. The docking process was conducted then.

In vitro test consist of media making, cell culture, cytotoxic test, and immunofluorescence test. This method is conducted in Parasitology laboratorium, Faculty of Medicine, Gadjah Mada University.

RESULT AND DISCUSSION

Jure Leaf Extract (JLE) Extraction and Identification

Jure leaf extraction was conducted by soxhletation method using 96% ethanol and obtained extract with the rendement of 82.44% b/v. the TLC profile showed that there was a blue fluorescence that showed cardenolyde steroid group in JLE (Figure 1).
Molecular Docking Result of Oleandrin and MRP Protein

Molecular docking was conducted by PLANTS software. The protein PDB code is 2CBZ. The experimental ligand is ATP. The test compound is oleandrin. Based on molecular docking on MRP protein result, the docking score of oleandrin (-50,496) is higher than native ligand ATP (-125,817) (Table 1). This result is strengthened by the molecular docking visualization using MOE that showed the similar binding site on some amino acid residues of native ligand and oleandrin (Figure 2).

<table>
<thead>
<tr>
<th>Table 1. Docking Score</th>
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<tr>
<td>Docking Score of MRP</td>
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<tr>
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<tr>
<td>RMSD</td>
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<tr>
<td>0.8822</td>
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<tr>
<td>Oleandrin</td>
</tr>
<tr>
<td>-50,496</td>
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<tr>
<td>Ligan native</td>
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<td>-125,817</td>
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The Cytotoxic Test Result of JLE on WiDr Colon Cancer Cell

The cytotoxic test on WiDr cell was conducted using MTT assay. C50 of JLE was 4µg/mL, and IC50 5-FU is 100µM. JLE has potent cytotoxic activity on WiDr cell. Based on combination test, JLE increased 5-FU sensitivity with the optimum dose of 2 µg/mL JLE and 12.5 µM 5-FU with the CI score of 0.594. Based on CI score, it could be concluded that combination of JLE and 5-FU had synergist effect.
Figure 3. Cell viability of WiDr after treated with JLE (a) and 5-FU (b) for 48h. cytotoxic assay was performed using MTT assay. Cells viability of combination of JLE and 5-FU (c).

The p65 Protein Expression by Immunofluorescence Assay

The p65 protein expression on WiDr was detected by immunofluorescence assay. The immunofluorescence result showed that JLE inhibited p65 protein expression at the optimum dose of JLE and 5-FU.

Figure 4. p65 protein expression on WiDr cell observed by immunofluorescence assay
This study can give an alternative therapy which is more effective and targeted in the inhibition of colon cancer cell proliferation, and solved 5-FU resistancy in colon cancer therapy.

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REFERENCES


