STRUCTURE MODIFICATION OF ETHYL p-METHOXYCINNAMATE AND THEIR BIOASSAY AS CHEMOPREVENTIVE AGENT AGAINST MICE’S FIBROSARCOMA

JUNI EKOWATI1*, BIMO A. TEJO2, SHIGERU SASAKI3, KIMIO HIGHASIYAMA3, SUKARDIMAN1, SISWANDONO1, TUTUK BUDIATI1

1Faculty of Pharmacy, Airlangga University, Dharmawangsa Dalam, Surabaya 60286, Indonesia, 2Department of Chemistry, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia, 3Institute of Medicinal Chemistry, Hoshi University, Ebara University, 2-4-41, Shinagawa, Tokyo 1528501, Japan.

Received: 2 Feb 2012, Revised and Accepted: 21 March 2012

Email: j_ekowati@yahoo.com

ABSTRACT

In the present study, ethyl p-methoxycinnamate isolated from Kaempferia galanga was used as starting material to produce thiourea derivatives (4a, 4b, 4c) in a good yield. The synthesis products were confirmed by FTIR, 1H-NMR, 13C-NMR and HRMS spectroscopic methods. Their activities against fibrosarcoma were tested in vivo using mouse model induced by 0.3% benzo(a)pyrene injected subcutaneously, which was given five times, once every two days. Our results showed that fibrosarcoma can be inhibited by all synthesized compounds. In silico analysis predicted that one of mechanism chemopreventive activity of all synthesized compounds against fibrosarcoma through inhibition of COX-2.

INTRODUCTION

Fibrosarcoma is one of malignant tumors of mesenchymal cell arising from fibroblast cell that divides without cellular control. The treatment malignant tumor with chemotherapy agents has many side effects, including hair loss, nausea and vomiting, prostatema area, bone marrow depression, thrombocytopenia and fertility disorders. The appearance of serious side effects of cancer treatment has encouraged continued development of research looking for another way to cure it, like searching the new substances from natural or synthetic materials as cancer chemoprevention.

Therapy for cancer such as fibrosarcoma by chemopreventive agent could have an important effect on cancer morbidity and mortality. Nowadays, chemoprevention by using substances that are capable to prevent cancer progression is gaining more attention. One of the targeted enzymes on cancer chemoprevention is cyclooxygenase-2 (COX-2).

Not only are selective COX-2 inhibitors used to block angiogenesis and tumor proliferation process, but they also have prothrombotic and increase the risk of myocardial infarction. Consequently, synthesis of new compounds that can block angiogenesis and tumor proliferation, but with no adverse effects is highly desired.

Ethyl p-methoxycinnamate (EPMC) from Kaempferia galanga Linn., has been used for the treatment of pain and inflammation. This compound also shows inhibitory activity against proliferation of tumor cell in the specimen of mouse epidermis and extent of papilloma. In effort to increase its affinity on COX-2, we modified EPMC by introducing some pharmacophore of COX-2. The products of modified EPMC (4a, 4b & 4c) were evaluated in terms of their chemoprevention activity against fibrosarcoma in mice. Study of molecular mechanism of their chemoprevention activities were carried out by docking of all compounds into the binding site of COX-2 with Auto Dock Vina program.

MATERIALS AND METHODS

Materials

Starting from ethyl p-methoxycinnamate (1), the p-methoxycinnamoyl thioureas (4) were synthesized through the reaction of p-methoxycinnamoyl chloride (3) with ammonium thiocyanate, and then several primary amines (Fig.1). The structure of the synthesized compounds were confirmed by 1H-NMR, 13C-NMR, IR and HRMS spectral data; meanwhile, purity of the compounds were ascertained by melting point and TLC tests.

General Experimental

Ethyl p-methoxycinnamate was isolated from Kaempferia galanga by percolation with ethanol overnight at room temperature. All reagents and solvents were purchased from standard commercial suppliers. Melting points were measured with an Electrothermal melting point apparatus without correction. IR spectra were recorded in KBr on Jasco FT-IR 5300, and major absorption was listed in cm⁻¹. 1H-NMR and 13C-NMR spectra were taken at BRUKER BioSpin Avance III NMR spectrometer (400 MHz), and chemical shift were reported in ppm on the δ-scale from internal Me₄Si. MS spectra were measured with a JEOL JMS 600 spectrometer by using the EI methods. TLC was carried out on glass plates coated with silica gel F₂₅₄ (Merck). Spot detection was performed with UV 254 nm.
Synthesis Thiourea derivatives of ethyl p-methoxyxinnaminate

Ethyl p-methoxyxinnaminate in 5% KOH/ethanol was heated under reflux then added to produce p-methoxyxinnaminate acid, which was isolated by precipitation with dry benzene and one drop of pyridine, a 5-fold excess of thiouyl chloride was added. The mixture was heated overnight to produce p-methoxyxinnamoyl chloride. After that, ammonium thiocyanate, appropriate p-methoxyxinnamoyl chloride, PBS-400 and dichloromethane were refluxed in waterbath. Then aromatic amines (i.e. p-methylbiline, p-methoxyiline and p-chloro-aniline) were added and the mixture was heated again. The mixture was filtered off to remove inorganic salts and the filtrate was concentrated under reduced pressure. The resulting solid was recrystallised from dichloromethane-ethanol (1:1) to give p-methoxyxinnamoyl thiourea compounds (Fig.1).

Chemopreventive Activity

Thirty six male mice (Mas musculus), 5-6 week old and weighing between 20 to 30 gm were distributed in six groups. They were kept in separate iron cages under controlled condition of 12-h light / 12-h dark cycle at the animal laboratory of Faculty of Pharmacy Airlangga University. Air conditioning was used to keep temperature at 20±2°C. Cages and animals were labeled with water proof marker. The animals were quarantined for a week before the start of the experiment. Commercial mice food and tap water regularly provided to mice were changed with fresh one each day. The particulars of the induction of B[a]P. In this study, celecoxib as positive control was produced by hydrosynthesis of that B[a]P. The carcinogenesis was observed for three consecutive days. That carcinogenesis was observed for three months. This activity assay was approved by the Ethics Commission of Airlangga University Indonesia. The paraffin section of 1-5 mm of fibrosarcoma in mice. White arrows were used to point to that fibrosarcoma.

Molecular Docking

Software and program

PyMol (Delano Scientific LLC, USA) and DS Visualizer (Accelrys, Inc., USA) were employed to envision and modify the receptor and ligand structures. AutoDock Vina was the primary docking program used in this work. The preparation of the COX-2 pdbqt file and determination of the grid box size were carried out using AutoDock Tools version 1.5.4 (The Scripps Research Institute, La Jolla, USA). Post-docking analyses were carried out using the LigPlot12.

Docking Study

Docking study was performed using AutoDock Vina (PDB code 1COX-2). Auto Dock Tools was operated to prepare the input pdbqt file for COX-2 and to put the size and the center of the grid box. The size of COX-2 active site was set to 28 x 20 x 20, in the dimensions of x, y and z using 1.00Å spacing. The center of the grid box was put at 20.8 x 24.9 x 13.1, in the dimensions of x, y and z using 1.00Å spacing. AutoDock Vina requires the pdbqt input files of ligands to be prepared using AutoDock Tools. The predicted binding affinity (Kcal/mol), which specifies how strongly a ligand binds to the receptor, is calculated based on the scoring function used in AutoDock Vina. A more negative binding affinity indicates stronger binding. Docking experiment for COX-2 was performed by AutoDock program based on an assumption : the binding of molecules mimics the binding of SC-558 on the active site of the enzyme. SC-558 is a specific inhibitor against COX-2. The structure of COX-2 complexed with that SC-558. The scoring function in AutoDock Vina is divided into two parts : i) a conformation relatant part that can be performed as a sum of intra molecular and intermolecular contributions, as well as steric, hydrophobic, and hydrogen bonding interactions, and ii) a conformation-independent part that depends on the number of rotatable bonds. Each contribution (steric, hydrophobic, hydrogen bonding and number of rotatable bonds) is given a different weight in the AutoDock Vina scoring function. The best docked structures have to follow these criteria:

1. They have the lowest binding affinity (Kcal/mol).
2. Geometrically, they must occupy the same pocket in the enzymes similar to SC-558. This can be observed visually by comparing the structure of docked molecule with crystal structure of SC-558 (PDB ID 2CO5) inside the active sites.

The validation of docking was carried out by redocking the SC-558 active ligands into their binding sites.

RESULTS AND DISCUSSION

Synthesis of thiourea derivatives of EPMC

Some thiourea derivatives of EPMC were synthesized from p-methoxyxinnamoyl chloride (3) to produce 4a-c (Fig1 in three steps. In the first step, p-methoxyxinnamoyl chloride (2) was produced by hydrosynthesis of that EPMC.

\[ \text{p-methoxyxinnamoyl chloride (2)} \]

The preparation of the COX-2 pdbqt file and determination of the grid box size were carried out using AutoDock Tools version 1.5.4 (The Scripps Research Institute, La Jolla, USA).

\[ \text{pdbqt file and } \]

\[ \text{Chemopreventive Activity} \]

Thirly six male mice (Mas musculus), 5-6 week old and weighing between 20 to 30 gm were distributed in six groups. They were kept in separate iron cages under controlled condition of 12-h light / 12-h dark cycle at the animal laboratory of Faculty of Pharmacy Airlangga University. Air conditioning was used to keep temperature at 20±2°C. Cages and animals were labeled with water proof marker. The animals were quarantined for a week before the start of the experiment. Commercial mice food and tap water regularly provided to mice were changed with fresh one each day. The particulars of the induction of B[a]P. In this study, celecoxib as positive control was produced by hydrosynthesis of that B[a]P. The carcinogenesis was observed for three consecutive days. That carcinogenesis was observed for three months. This activity assay was approved by the Ethics Commission of Airlangga University Indonesia. The paraffin section of 1-5 mm of formalin fixed tissue were prepared by standard histological techniques and stained with Haematoxylin & Eosin (H&E)12. Olympus BX-50 Pentax optio 230 microscope with Camera Digital 2.0 mega-pixel was used to expose microscopic structural characteristics of fibrosarcoma in mice. White arrows were used to point to that fibrosarcoma.

Molecular Docking

Software and program

PyMol (Delano Scientific LLC, USA) and DS Visualizer (Accelrys, Inc., USA) were employed to envision and modify the receptor and ligand structures. AutoDock Vina was the primary docking program used in this work. The preparation of the COX-2 pdbqt file and determination of the grid box size were carried out using AutoDock Tools version 1.5.4 (The Scripps Research Institute, La Jolla, USA). Post-docking analyses were carried out using the LigPlot12.

Docking Study

Docking study was performed using AutoDock Vina (PDB code 1COX-2). Auto Dock Tools was operated to prepare the input pdbqt file for COX-2 and to put the size and the center of the grid box. The size of COX-2 active site was set to 28 x 20 x 20, in the dimensions of x, y and z using 1.00Å spacing. The center of the grid box was put at 20.8 x 24.9 x 13.1, in the dimensions of x, y and z using 1.00Å spacing. AutoDock Vina requires the pdbqt input files of ligands to be prepared using AutoDock Tools. The predicted binding affinity (Kcal/mol), which specifies how strongly a ligand binds to the receptor, is calculated based on the scoring function used in AutoDock Vina. A more negative binding affinity indicates stronger binding. Docking experiment for COX-2 was performed by AutoDock program based on an assumption : the binding of molecules mimics the binding of SC-558 on the active site of the enzyme. SC-558 is a specific inhibitor against COX-2. The structure of COX-2 complexed with that SC-558. The scoring function in AutoDock Vina is divided into two parts : i) a conformation relatant part that can be performed as a sum of intra molecular and intermolecular contributions, as well as steric, hydrophobic, and hydrogen bonding interactions, and ii) a conformation-independent part that depends on the number of rotatable bonds. Each contribution (steric, hydrophobic, hydrogen bonding and number of rotatable bonds) is given a different weight in the AutoDock Vina scoring function. The best docked structures have to follow these criteria:

1. They have the lowest binding affinity (Kcal/mol).
2. Geometrically, they must occupy the same pocket in the enzymes similar to SC-558. This can be observed visually by comparing the structure of docked molecule with crystal structure of SC-558 (PDB ID 2CO5) inside the active sites.

The validation of docking was carried out by redocking the SC-558 active ligands into their binding sites.
\( N\text{-}(p\text{-chlorophenyl})\text{-}N'\text{-}(p\text{-methoxycinnamoyl})\text{thiourea  (4c) } \) [yield 55\%] as pale green amorph (m.p. 188 °C). HRMS m/z EI, 346 (M'). Calculated Mass C16H15NO3 346.0550. Measured Mass 346.0543. \( ^{1}H \) NMR (DMSO-\(d_6\)) 3.87 (3H, s), 6.32 (1H, d, \( J = 4.5 \) Hz), 6.93 (2H, d, \( J = 14.5 \) Hz). 7.52 (2H, d, \( J = 7.74 \) Hz). 7.97 (1H, s). \( ^{13}C \) NMR (DMSO-\(d_6\)) 55.47 (C(4'-OCH3)), 114.58 (C(3'/C5')), 115.19 (C=C=O), 125.38 (C(1)'), 126.28 (C(3/C5)), 128.97 (C(2'/C6')), 130.45 (C(4)), 132.94 (C(4')), 136.20 (C(1)). 146.71 (C(1')=C(C)), 162.89 (C(2)=C(6')), 166.09 (C(2)), 178.78 ppm (C6). IR (KBr) 3455 (N-H), 1643 (C=C), 1662 (C=O), 3015 (C-H). Mass of each thiourea derivatives were confirmed by HRMS spectrometer. All of spectral data 4a-c also fully support the structure assigned to those.

Chemopreventive Activity

In this study, induction of B[a]P 0.3\% in oleum olivarum was given every other day for five times in mice and left for 3 months caused a fibrosarcoma in that mice. There was the decreasing of body weight of mice with fibrosarcoma (CN group) (data not shown), that could be because of lack of appetite and because of cancer-induced could alter metabolism. The cancer could interfere food chewing, swallowing, and digestion process, so decrease the appetite. Microscopically, there was a bump in the neighborhood area of the tumor. When it was stained with Haematoxylin & Eosin, it appeared pleumorfic including an oval in the original fibroblast cells, with rounded edges or uneven irregular core appeared on fibrosarcoma slice. Our previously study showed that ability 4b as antioxidant was 3S (Size, Shape and Stain). Changes of fibroblast morphology occurred on group CN (control negative) including the cell sizes that were larger than normal cells, dominant proliferating fibroblast as a herringbone pattern was different from that of normal (N) mice. Tissue-embedded file looked solid and irregular arrangement. Many forms (pleumorphic) including an oval in the original fibroblast cells, with rounded edges or uneven irregular core appeared on fibrosarcoma slice. When it was stained with Haematoxylin & Eosin, it appeared darker in colors (hyperchromatine) with the nucleus surface looked rough and nucleolus in more than one location is not contiguous. In addition, there was some areas of chronic inflammation and loss of hair restricted to the site of the application (Figure 2).

Docking Results

To predict the inhibition mechanism of fibrosarcoma growth, whether through a COX-2 inhibition, docking study was performed on binding site COX-2. The result of docking study was shown at Table 1.

In Table 1 can be seen inhibition (%) of fibrosarcoma of tested compounds and mean range of histopathology analysis by Kruskal Wallis. Double test Z\textsubscript{Cop} analysis shows that there is a significant difference between CN and CP. 4a-c (p<0.05). In addition, there was a significant difference between EPMC and CP. 4a-c (p<0.05). All of tested com-pounds showed chemoprevention activity on fibrosarcoma in mice.

Microscopically, fibrosarcoma was characterized by cellular changes on 3S (Size, Shape and Stain). Changes of fibroblast morphology occurred on group CN (control negative) including the cell sizes that were larger than normal cells, dominant proliferating fibroblast as a herringbone pattern was different from that of normal (N) mice. Tissue-embedded file looked solid and irregular arrangement. Many forms (pleumorphic) including an oval in the original fibroblast cells, with rounded edges or uneven irregular core appeared on fibrosarcoma slice. When it was stained with Haematoxylin & Eosin, it appeared darker in colors (hyperchromatine) with the nucleus surface looked rough and nucleolus in more than one location is not contiguous. In addition, there was some areas of chronic inflammation and loss of hair restricted to the site of the application (Figure 2).

Docking results showed that affinity on binding site COX-2 of all of compounds and mean range of histopathology analysis by Kruskal Wallis analysis shows that there is a significant difference between CN and CP. 4a-c (p<0.05). All of tested com-pounds showed chemoprevention activity on fibrosarcoma in mice.

Docking results showed that affinity on binding site COX-2 of all of the tested compounds lower than SC550 (mimic with celecoxib), but their activity on chemoprevention of fibrosarcoma in mice were not different to celecoxib. So, it could be another mechanism involved in their activity. The result of tested compounds interaction with residue of amino acid of COX-2 was shown in Figure 3.
Table 1: Structure of Tested Compounds with Their Chemopreventive Activity against fibrosarcoma (macroskopic and microscopic observation) and Docking Score

<table>
<thead>
<tr>
<th>Groups</th>
<th>Structures</th>
<th>Inhibition (%) of Fibrosarcoma</th>
<th>Mean Range Grade of fibrosarcoma*</th>
<th>Docking Score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>-</td>
<td>0</td>
<td>30.8</td>
<td>-11.0</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td>67</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td>67</td>
<td>12.7</td>
<td>-7.4</td>
</tr>
<tr>
<td>4b</td>
<td></td>
<td>67</td>
<td>12.8</td>
<td>-7.4</td>
</tr>
<tr>
<td>4c</td>
<td></td>
<td>67</td>
<td>15.8</td>
<td>-7.4</td>
</tr>
<tr>
<td>EPMC</td>
<td>33</td>
<td>27.0</td>
<td></td>
<td>-6.0</td>
</tr>
</tbody>
</table>

*Histopathology assignment by Kruskal Wallis analysis with parameters pleumorfism & number of mitosis. CN= control negative, CP=control positive, 4a=N-(p-methylphenyl)-N’-(p-methoxy-cinnamoyl)thiourea, 4b=N-(p-methoxyphenyl)-N’-(p-methoxycinnamoyl)thiourea, 4c=N-(p-chloro-phenyl)-N’-(p-methoxycinnamoyl)thiourea, EPMC=ethyl p-methoxy cinnamate.

Fig. 3: Interaction EPMC (A), 4a (B), 4b (C) and 4c (D) on binding site of COX-2.
In Figure 3 (A), it was appeared that the hydrogen bonds between oxygen atoms of methoxy groups of EPMC and residue Arg120 with a distance of 3Å. Besides that, it also observed that there are very weak hydrogen bondings (more than 3Å) among Tyr 385, Ser530 with oxygen atom of the methoxy group. Hydrophobic contacts appeared between C atom of ester group and Tyr 355, Ala531, Leu 527. Docking results of EPMC showed the binding affinity on COX-2 as much as -6.0 kcal/mol. Interaction of EPMC in binding site of COX-2 in line with interaction of Diclofenac had been reported16.

Figure 3 (B) showed the interaction 4a on binding site of COX-2. Interactions occurred as the hydrogen bonds between Leu 352 and the N atom of thiourea, Ser353 with N atom of amide moiety. Hydrophobic bonding of 4a occurred with residues Val523, Val 349, Gly354, and His90. Docking results of 4a showed the binding affinity on COX-2 as much as -7.4 kcal/mol.

Figure 3(C) showed the interaction 4b on binding site COX-2. Interactions occurred as the hydrogen bonds between Leu 352 with the N atom of thiourea, Ser353 with N atom of amide moiety. Hydrophobic bonding occurs between methyl from methoxy moiety, aromatic ring and vinilyc double bond with residues Val523, Val 349, Gly354, and His90. Docking results of 4b showed the binding affinity on COX-2 as much as -7.4 kcal/mol.

The in silico test result showed different interactions between thiourea derivatives of ethyl p-methoxycinnamate and COX-2 specific inhibitors had been reported16. All of test compounds in Figure 3 (A-D) show similarity on affinity at binding side COX-2, that means substitution similarity on affinity at binding side COX-2, that means substitution position have not influence in their affinity.

The docking study result of test compounds on binding site of COX-2 was in line with the in vivo test result, supports the presumption that one of inhibition mechanism of fibrosarcoma was through COX-2 activity inhibition. This supports the report of other researchers who claimed that there was a correlation of COX-2 expression at the level of malignancy in fibrosarcoma17.

From this research was concluded that three thiourea derivatives of EPMC (4a, 4b and 4c) showed activity as chemopreventive agent on fibrosarcoma in mice. There were no significant different activities between these derivatives and celecoxib as positive control group, but their activities were higher than that of EPMC. These compounds also were predicted to bind into COX-2 binding site with higher affinities than EPMC as one of mechanism inhibition of that fibrosarcoma in mice. There could be another mechanism involved on their chemoprevention activity against fibrosarcoma which needed further study.

ACKNOWLEDGEMENT

We are thankful to the technical staff of the Pathology Veterinary Laboratory at Faculty of Veterinary Medicine Airlangga University, Dr. Hani Plumeriastuti, MSc., Vet. for histopa-thology examination of fibrosarcoma. We also thankful to Indonesian Higher Education Directorate by Hibah Doctoral Grant Airlangga University for financial support.

REFERENCES

1. Kumar V, Abbas A K, Fausto N. Pathologic Basis of Disease 7th