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Research Article

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

JUNI EKOWATI[1]*, BIMO A. TEJO[2], SHIGERU SASAKI[3], KIMIO HIGHASIYAMA[3], SUKARDIMAN[1], SISWANDONO[1], TUTUK BUDIATI[1]

[1]Faculty of Pharmacy, Airlangga University, Dharmawangsa Dalam, Surabaya 60286, Indonesi, [2]Department of Chemistry, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysi, [3]Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo 1528501, Japan. Email: j_ekowati@yahoo.com

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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, ^{1}H-NMR, ^{13}C-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.

Keywords: *Kaempferia galanga*, cyclooxygenase-2, Ethyl *p*-methoxycinnamate, Thiourea derivatives, Fibrosarcoma

INTRODUCTION

Fibrosarcoma is one of malignant tumors of mesenchymal cell arising from fibroblast cell that divides without cellular control[1]. The treatment malignant tumor with chemotherapy agents has many side effects, including hair loss, nausea and vomiting, prostrema area, bone marrow depression, thrombocytopenia and fertility disorders[2,3]. 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[4].

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[4,5]. One of the targeted enzymes on cancer chemoprevention is cyclooxygenase-2(COX-2)[5,6].

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[6,7]. 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[8]. This compound also shows inhibitory activity against proliferation of tumor cell in the specimen of mouse epidermis and extent of papilloma[9, 10]. In effort to increase its affinity on COX-2, we modified EPMC by introducing some pharmacopore of COX-2[11]. 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 ^{1}H-NMR, ^{13}C-NMR, IR and 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}. ^{1}H-NMR and ^{13}C-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 Me_4Si. 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 F_{254} (Merck). Spot detection was performed with UV 254 nm.

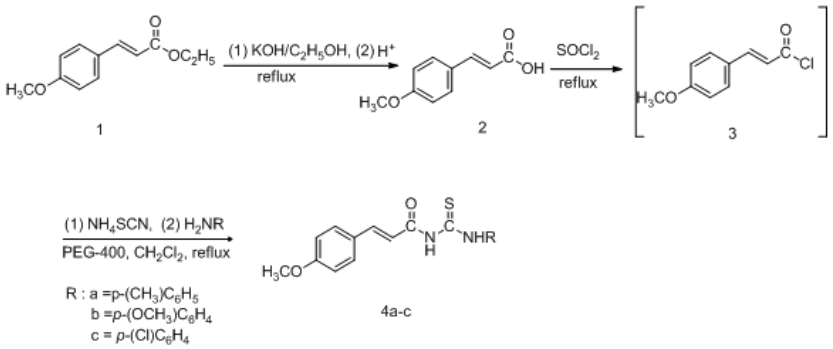

Fig. 1: The schematic representation of synthesize thiourea derivatives of EPMC

Synthesis Thiourea derivates of ethyl *p*-methoxycinnamate

Ethyl *p*-methoxycinnamate in 5% KOH/ethanol was heated under reflux then acidified to produce *p*-methoxycinnammic acid. To *p*-methoxycinnamic acid in dry benzene and one drop of pyridine, a 5-fold excess of thionyl chloride was added. The mixture was heated overnight to produce *p*-methoxycinnamoyl chloride. After that, ammonium thiocyanate, appropriate *p*-methoxycinnamoyl chloride, PEG-400 and dichlorometane were refluxed in waterbath. Then aromatic amines (i.e. *p*-methylaniline, *p*-methoxyaniline 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 recrystalised from dichlorometane-ethanol (1-1) to give *p*-methoxy-cynnamoyl thiourea compounds (Fig.1).

Chemopreventive Activity

Thirthy six male mice (*Mus musculus*), 5-6 week old and weighing between 20 to 30 gm were distributed in six group. 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±2ºC. Cages and animals were labeled with water proof marker. The animals were quarantined for a week before start the experiment. Commercial mice food and tap water regularly provided to mice were changed with fresh one each day. The particulars of each animal such as the name of the group, period for administration of the carcinogen and weight in grams were recorded at start and at the end of experiment. Benzo(a)pyrene (B[a]P) as carcinogen was obtained from Sigma Chemical Company. It was dissolved in oleum olivarum (0.3%) and was prepared just before the use. EPMC and its derivatives were subjected to test against fibrosarcoma's carcinogenesis at dosage 40 mg/kg, orally for thirty days, along with the induction of B[a]P. In this study, celecoxib as positive control (**CP**) and group without treatment only CMC-Na as negative control (**CN**). Fibrosarcoma's carcinogenesis was produced by the induction of 0,3% B[a]P subcutaneously into mice once in every twice concecutive days. That carcino-genesis was observed for three months. This activity assay was approved by the Ethics Commission 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 characterizations 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 LigPlot[12].

Docking Study

Docking study was performed using Auto-Dock Vina (PDB code 1CX-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 at 28 x 20 x 20, in the dimensions of x, y and z using 1.000Å 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 1.000Å 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 reliant part that can be performed as a sum of intra mo-lecular 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 between heavy atoms in the ligand. 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 (for COX-2) 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 derivates of EPMC

Some thiourea derivates of EPMC were synthesized from *p*-methoxycinnamoyl chlo-ride (**3)** to produce 4**a -c** (Fig.1) in three steps. In the first step, *p*-methoxycinnamic acid **(2)** was produced by hydrolysis of that EPMC.

p -methoxycinnamic acid **(2).** (Yield 80%) as white crystal (m.p. 169°C). HR-MS m/z EI, 178 (M^+), Calculated Mass $C_{10}H_{10}O_3$ 178.0630. Measured Mass 178.0617. ^{1}H NMR (DMSO-d_6) 3.78 (3H, s), 6.60 (1H, d, *J* = 16 Hz), 6.96 (2H, d, *J* = 5.0 Hz), 7.53 (1H, d, *J* = 16 Hz), 7.62 ppm (2H, d, *J* = 5.0Hz), 12.23 (1H,s). ^{13}C NMR (DMSO-d6) 55.5 (C4 -OCH_3), 114.54 (C4-OCH_3), 116.71 (C=C-C(O)) 127.02 (C1), 130.13 (C3/C5), 143.92 (C4-C=C-C(O)), 161.12 (C1), 168.03 ppm (C(O). IR (KBr) : $\bar{\upsilon}_{max}$ (cm^{-1}) 2937 ($\bar{\upsilon}$, -C(O)-OH), 1685 ($\bar{\upsilon}$, -C(O)-OH), 1624 ($\bar{\upsilon}$, -C=C (aril), 1444 ($\bar{\upsilon}$, -C-C (aril) sp_2), 1255 ($\bar{\upsilon}$, C-O-C), 975 (-C=C- *trans*), 827 (2H aril) cm^{-1}. Spectral data of **2** fully support the structure assigned to it.

In the second step, *p*-methoxycinnamoyl isothiocyanate was produced by nucleophylic substitution reaction between *p*-methoxycinnamoyl chloride with ammonium thiocyanate. In the third step, thiourea derivatives of EPMC were synthezised (**4a-c**) by an addition reaction of nucleophile to *p*-methoxycinnamoyl isothiocyanate, where the nucleophile are *p*-methyl aniline (R=a), *p*-methoxyaniline (R=b) and *p*-chloroaniline (R=**c).**

N-(*p*-methylphenyl)-*N'*-(*p*-methoxycinnamoyl)thiourea **(4a)**. (yield 70%) as yellow crystal (m.p. 199°C). HR-MS m/z EI, 326 (M^+), Calc. Mass $C_{16}H_{15}NO_3$ 326.1095. Meas. Mass 326.1089. ^{1}H-NMR ($CDCl_3$) 2.37 (3H, s), 3.87 (3H, s), 6.30 (1H, d, *J* = 15.40 Hz), 6.93 (2H, d, *J* = 8.40 Hz), 7.21 (2H, d, *J* = 8.40 Hz), 7.78 (1 H, d, *J* = 15.40 Hz), 7.53 (4H, m), 8.68 (1H, s), 12.51 ppm (1H,s). ^{13}C-NMR (DMSO-d_6) 22.10 (C4-CH_3), 55.43 (C4'-OCH_3), 114.51 (C3'/C5'), 115.53 (C=C-C(O), 124,25 (C1'), 126.42 (C3/C5), 129.44 (C2'/C6'), 130.39 (C2/C6), 135.10 (C4), 136.74 (C1), 146.27 (C1'-C=C), 162.12 (C4'), 166.08 (C=O), 178.78 ppm (C=S). IR (KBr) : $\bar{\upsilon}_{max}$(cm^{-1}) 3224 ($\bar{\upsilon}$, -NH-(C=O), 3025 ($\bar{\upsilon}$, -NH-(C=S), 1672 ($\bar{\upsilon}$, -NH-C=O), 1626 ($\bar{\upsilon}$, -C=C (aril), 1590 ($\bar{\upsilon}$, -N-H), 1423 ($\bar{\upsilon}$, -C-C (aril) sp_2), 1349 ($\bar{\upsilon}$,-O-CH_3), 1248 ($\bar{\upsilon}$, -C-O-C), 1151 ($\bar{\upsilon}$, -C=S), 984 ($\bar{\upsilon}$, -C=C- *trans*), 825 ($\bar{\upsilon}$, 2H aril) cm^{-1}.

N-(*p*-methoxyphenyl)-*N'*-(*p*-methoxycinnamoyl)thiourea **(4b).** (yield 79%) as pale green crystal (m.p. 181°C) MS m/z EI, 342 (M^+). Calculated Mass $C_{16}H_{15}NO_3$ 342.1038. Measured Mass. 342.1031. ^{1}H NMR (DMSO-d_6) 3.76 (3H,s), 3.80 (3H,s), 6.86 (1H, d, *J* = 15.80 Hz), 6.95 (2H, d, *J*= 8.80), 7.05 (2H, d, *J* = 8.80 Hz), 7.51 (2H, d, *J*=), 7.59 (2H, d, *J*= Hz), 7.70 (1H, d, *J* = 15.80 Hz), 11.43 (1H, s), 12.58 ppm (1H,s). ^{13}C NMR (DMSO-d_6) 55.49 (C4'-OCH_3), 55.61 (C4 -OCH_3), 114.01 (C3/C5), 114.84 (C3'/C5'), 117.23 (C=C-C(O)), 125.92 (C2/C6), 126.88 (C1'), 130.33 (C2'/C6'), 130.94 (C1), 144.60 (C1'-C=C), 157.59 (C4), 161.65 (C4'), 166.83 (CO), 179.23 ppm (CS). IR (KBr) : $\bar{\upsilon}_{max}$(cm^{-1}) 3235 ($\bar{\upsilon}$, -NH-(C=O), 3034 ($\bar{\upsilon}$, -NH-(C=S), 1673 ($\bar{\upsilon}$, -NH-C=O), 1593 ($\bar{\upsilon}$, -N-H), 1423 ($\bar{\upsilon}$, -C-C (aril) sp_2), 1252 ($\bar{\upsilon}$, C-O-C), 1150 ($\bar{\upsilon}$, -C=S), 991 ($\bar{\upsilon}$, -C=C- *trans*), 825 ($\bar{\upsilon}$, 2H aril) cm^{-1}.

N-(*p*-chlorophenyl)-*N'*-(*p*-methoxycinnamoyl)thiourea **(4c)** (yield 55%) as pale green amorf (m.p. 188°C). HRMS m/z EI, 346 (M^+). Calculated Mass $C_{16}H_{15}NO_3$ 346.0550. Measured Mass 346.0543. ^1H NMR (DMSO-d_6) 3.87 (3H, s), 6.32 (1H, d, *J* = 14.5 Hz), 6.93 (2H, d, J=), 6.96 (2H,d, J=), 7.52 (2H,d, J=), 7.69 (2H, d, J=), 7.74 (1H, d, *J* = 14.5 Hz), 8.77 (1H, s), 12.6 ppm (1H,s).^{13}C NMR (DMSO-d_6) 55.47 (C4'-OCH_3), 114.58 (C3'/C5'), 115.19 (C=C-C(O), 125.38 (C1'), 126.28 (C3/C5), 128.97 (C2'/C6'), 130.45 (C2/C6), 132.04 (C4), 136.20 (C1), 146.71 (C1'-C=C), 162.29 (C4'), 166.09 (CO), 178.78 ppm (CS). IR (KBr) 3455 (ῡ , -NH-(C=O), 3015 (ῡ, -NH-(C=S)), 1677 (ῡ, -NH-C=O), 1590 (ῡ, -N-H), 1243 (C-O-C), 1168 (ῡ, -C=S), 982 (ῡ, -C=C- *trans*), 821 (ῡ , 2H aril) cm^{-1}.

Unlike the reported condition reaction to prepare some thiourea compounds which in room temperature and microwave irradiation, preparation of thiourea compounds in this study from EPMC was carried out by heated using conventional method[13,14].

The structure transformation of **2** became thiourea derivatives (4**a-c**) could be cha-racterized by the conversion of -C(O)OH moiety to -$C(O)NH_2$. Besides that, there is an additional of -$C(S)NH_2$ and aromatic ring moiety at those thiourea derivatives. All the alterations were confirmed in the IR, ^{1}HNMR and ^{13}CNMR. 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[1].

Macroscopically, there was a bump in the neighborhood area of the mice intra scapular, where the treatment B[a]P occurs. Macroscopic observation of the tumor growth inhibition activity of the sample (CP, EPMC, 4a, 4b and 4c at dose 40 mg/kg) as the amount of mice have fibrosarcoma in those groups compared to normal cell (N) is shown in Table 1. Celecoxib was used as control positif (CP).

In addition to macroscopic observation, there was a microscopic observation on fi-brosarcoma through Haematoxylin & Eosin staining. Parameters observed of fibrosarcoma were pleumorfism and number of mitotic cells (5 hpf in 400x)[15]. Mean Rank Score of that fibrosarcoma through Kruskal Wallis analysis were shown also in Table 1.

Microscopically, fibrosarcoma was characterized by cellular changes on 3S (Size, Shape and Stain). Changes of fibroblast morphology occured 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. Tuesstacked pile looked solid and irregular arrangement. Many forms (pleumorfic) 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)

Treatment with B[a]P caused an increasing in PGE_2 production, affected an increasing number of COX-2 protein. B[a]P also caused an increase in the number of COX-2 mRNA 2-fold in normal cells or cells that have been transformed. This strengthens the suspicion of COX-2 affects carcinogenesis by increasing the production of mutagens and prostaglandins[4].

Different blockade of carcinogenesis in percentage on mice having fibrosarcoma in the group and grade of fibrosarcoma could be based on the ability of thiourea derivatives of EPMC inhibited initiation step of carcinogenesis so B[a]P could not changed to its metabolite[4]. Our previously study showed that ability 4b as antioxidant was better than EPMC. The anti-oxidant activity was mainly at the initiation stage of carcinogenesis that could inhibite the oxidation B[a]P become active metabolites as carcinogens.

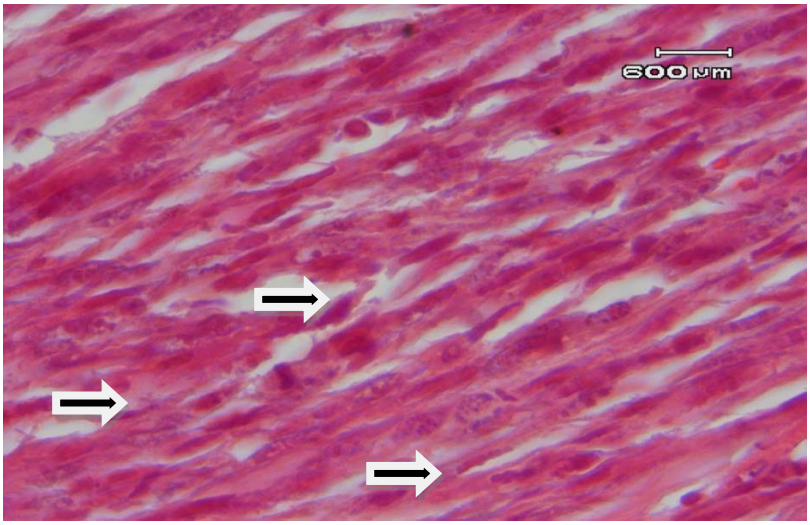

Fig 2: Microscopic structural characterizations fibrosarcoma in mice exposed to B[a]P (CN group) (H & E, 100 x). White Arrows pointing to fibrosarcoma.

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 $Z_{(\square=;0,05)}$ analysis shows that there is a significant difference between **CN** and **CP**, **4a, 4b, 4c** ($p<0.05$). 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.

Docking results showed that affinity on binding site COX-2 of all of the tested compounds lower than SC558 (mimic with celecoxib), but their activity on chemo-prevention 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

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

*Histophatology 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*-methoxycinnamate.

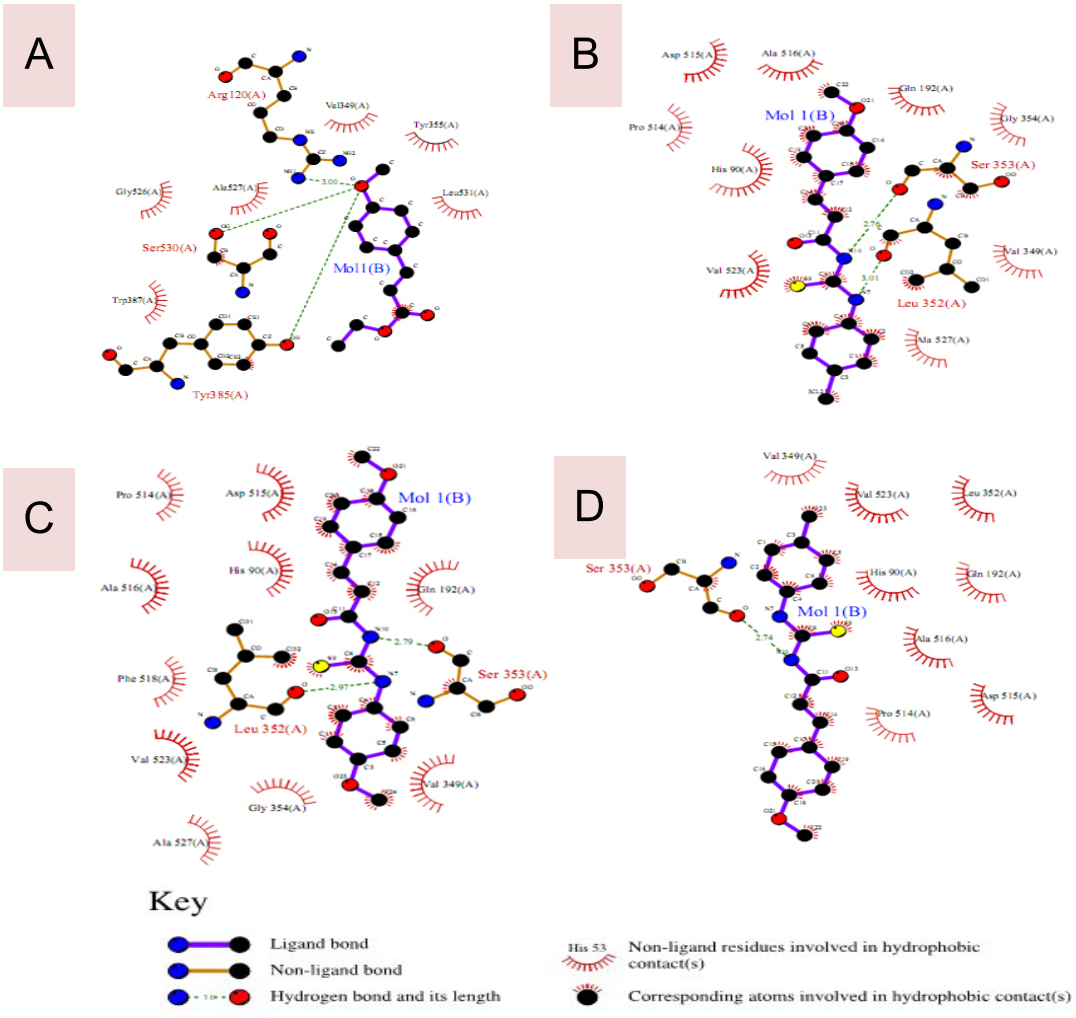

Fig. 3: Interaction EPMC (A), 4a (B), 4b (C) and 4c (D) on binding site of COX-2.

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 3Aº. Besides that, it also observed that there are very weak hydrogen bondings (more than 3Aº) 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 reported[16].

Figure 3 (B) showed the interaction **4a** on binding site of COX-2. Interactions occured 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 occured 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, Val349, Gly354, and His90. Docking results of **4b** showed the binding affinity on COX-2 as much as -7.4 kcal / mol.

Figure 3(D) showed the interaction **4c** on binding site COX-2. Interactions occured as the hydrogen bonds between Ser353 and the N atom of thiourea. Hydrophobic bonding occurs between methyl moiety, aromatic ring, vinilyc double bond and thiocarbonyl with residues of Val523, Val 349, Ala516, Pro514 and His90. Docking results of **4c** 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 reported[7,16]. All of test compounds in Figure 3 (A-D) show similarity on affinity at binding side COX-2, that means substitution moiety in *para*- 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 fibrosarcoma[17].

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 acivities 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.

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REFERENCES

1. Kumar V, Abbas AK, Fausto N. Pathologic Basis of Disease 7th Ed. Noordanesh Medical Publication; 2005. pp. 270-342.
2. Preview of: Feline Fibrosarcoma. South Carolina Veterinary Specialist. www.scvet specialist.com. Cited in December 19, 2011.
3. Medina PJ, Davis L.E. Colorectal Cancer**.** *In* : Dipiro, J.T., Talbert, R.L., Matzke G.R., Wells, B.G., Posey, L.M (Eds). Pharmacotherapy A Pathofisiologic Approach**,** 6th Ed., New York: The McGraw-Hill Co., Inc; 2005. pp. 2383-2415.
4. Sporn MB & Suh N. Chemoprevention of Cancer. Carcinogenesis 2000; 21(3): 525-530.
5. Thornalley PJ. Isothiocyanates: mechanism of cancer chemopreventive action. Anti Cancer Drug 2002; 13 (4): 331-338..
6. Greehough A, Smart HJM, Moore AE, Roberts HR, Williams AC, Paraskeva C, Kaidi A. Review The COX-2/PGE_2 Pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis 2009; 30: 377-386.
7. Little D, Jones SL, Blikslager AT. Cyclooxygenase (COX) Inhibitors and the Intestine. J Vet Intern Med Rev. 2007; 21: 367-377.
8. Kusumawati I & Yusuf H. Phospholipid Complex as a carrier of *Kaempferia galanga* Rhizoma extract to improve its analgesic activity. IJPPS 2011; 3 (Suppl.1): 44-46.
9. Sae-wong C, Ridtitid W, Rean-mongkol W, Wongnawa M. Anti-nociceptive activity of the methanolic extract of *Kaempferia galanga* and its possible mechanisms in experimental animals. Thai J Pharmacol 2008; 30 : 26-34.
10. Vimala S, Norhanom AW, Yadav M. Anti-tumor promoter activity in Malaysian ginger rhizoma used in traditional medicine. Brithis J Cancer 1999; 80(1-2) : 110-116.
11. Michaux C, de Leval X, Fabien J, Dogne JM, Pirotte B, Francois D. Structure based pharmacophore of COX-2, selective inhibitors and identification of original lead compounds from 3D data base searching method. Eur J of Med Chem 2006; 41: 1446-1455.
12. Protocol on line. Histology Special Staining. www.polyscience.com, cited in May 10, 2011.
13. Lim SV, Rahman MBA, Tejo BA. Structure-based and ligand-based virtual screening of novel methyltransferase inhibitors of dengue virus. BMC Bioinformatics 2011; 12 (Suppl 13) : S24. http://www.biomedcentral.com/1471-2105/12/S13/S24.
14. Xiao L, Liu C, Li Y. Ultrasound Promoted of Bis (subs-tituted pyrazol-4-yl-carbonyl)-Subs-tituted Thioureas. Molecules 2009 ; 14: 1423-1428.
15. Xu X, Qian X, Li Z, Huang Q, Chen G. Synthesis & Insecticidal Activity of New Substituted *N*-aryl-*N'*-benzoylthiourea Compounds. J Fluorine Chemistry 2003; 121: 51-54.
16. D'Mello P, Gadhwal MK, Joshi U, Shetgiri P. Modeling of COX-2 Inhibitory Activity of Flavonoids. IJPPS 2011; 3 (4): 33-40.
17. Nowak M, Madez JA, Dziegiel P. Intensity of COX-2 Expression in Cell of Soft Tissue Fibrosarcomas in Dogs as Related to Grade of Tumour Malignancy. Bull Vet Inst Pulawy 2007; 51: 275-279.