Synthesis, anti-angiogenic activity and prediction toxicity of (*E*)-3-(3-methoxyphenyl) propenoic acid

Juni Ekowati,[1] Kholis Amalia Nofianti,[1] Maya Nurwartanti Yunita,[2] Iwan Sahrial Hamid,[3] Fitria Dwiningrum,[4] Darwin Ryan Ramadhan,[4] Ghinalya Chalbi Ananda[5]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; [2]Department of Pathology Veterinary, Faculty of Veterinary Medicine, PSDKU Banyuwangi, Indonesia; [3]Department of Basic Veterinary, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia; [4]Bachelor Program Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; [5]Dentist Professional Program, Faculty of Dentistry Medicine, Universitas Airlangga, Surabaya, Indonesia

Correspondence: Juni Ekowati, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, 60115, Surabaya, Indonesia.
Tel.: +62.81332041503 - Fax: +62.5935249
E-mail: juni-e@ff.unair.ac.id

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Abstract

Background: Anti-angiogenic medications, one of cancer chemo preventive mechanism were permitted for different cancers. Nevertheless, major primary and secondary resistance obstruct efficacy in several tumor types. Moreover, the improvement of safe and effective NSAIDs for angiogenesis inhibition is complicated, because of their serious toxicity. So, we require improving clinically appropriate strategies to boost efficacy of anti-angiogenic drugs with low risk of toxicity.

Objectives: The present study aimed to synthesize the (E)-3-(3-methoxyphenyl)propenoic acid (3MPCA), to determine the anti-angiogenic activity and predict its toxicity.

Methods: 3MPCA was obtained by Knoevenagel reaction using microwave irradiation at 400 Watt. The anti-angiogenesis experimental was performed using chorioallantois membrane of embryonated chicken eggs induced by b-FGF. The potency of 3MPCA was verified at dosage 30 and 60 ng and compared with celecoxib 60 ng. Toxicity prediction of 3MPCA was performed by ProTox II online program.

Results: The results showed that 3MPCA was achieved in good yield (89%). Anti angogenic activity was showed by endothelial cells growth in neovascular capillaries of new blood vessel of chorioallantois membrane of embryonated chicken eggs. The endothelial cells growth decreased until 41.7-83%. The prediction LD50 was 1772mg/kg.

Conclusion: (E)-3-(3-methoxyphenyl)propenoic acid can be obtained through Knovenagel reaction using microwave irradiation and it has potential as anti-angiogenesis inhibitor with low toxicity.

Introduction

Many attempts at cancer therapies, such as surgery, radiotherapy, and chemotherapy, still have limitations including drug resistance or unfavorable side effects.[1,2] The side effects of cancer chemotherapy remain a concern for patient comfort and compliance despite the improved efficacy and survival offered by modern treatments. Drugs available in the market to counteract the side effects of chemotherapy are often not fully effective, or can even cause other side effects that only add to the patient's discomfort.[3] Especially for patients obtaining long-term cures, many late side effects gain more serious consideration.[4]

One of the cinnamic acid derivatives, such as 3-methoxycinnamic acid or (*E*)-3-(3-methoxyphenyl)propenoic acid (3MPCA) prospects as cancer chemoprevention because of it has some similarity functional group with 4-hydroxy-3-methoxycinnamic acid or

ferulic acid that has anti-angiogenic activity.[5] Both compounds have one difference at para position of phenyl ring, which ferulic acid has hydroxy moiety, but compound 3MPCA has not have that moiety. The compound 3MPCA has been reported to have anti-inflammatory analgesic activity with stronger COX-2 inhibitory abilities than aspirin.[6] Ferulic acid has also reported can induce cell cycle arrest and autophagy in cervical cancer cells.[7] Relationship among COX-2 inhibition with angiogenic activity has been testified by Yuan *et al*.[8] Until now, anti-angiogenesis of (*E*)-3-(3-methoxyphenyl) propenoic acid has not been publicized before.

The synthesis of 3MPCA has been reported in previous studies by reacting 3-methoxybenzaldehyde with malonic acid for 5 hours (temperature 80ºC). From this method, the yield was 70%.[6] Therefore, the development of a method using a microwave is carried out to get a faster time. In this experiment, we reported synthesis of 3MPCA using microwave irradiation as source of energy. Antiangiogenic activity will conduct using chorioallantois membrane as reported by Castro (2014)[9] and Ekowati *et al.* (2020).[10] To verify 3MPCA mechanism in inhibition of angiogenesis, we accomplished docking study on FGFR-1 receptors.

Materials and Methods

Synthesis

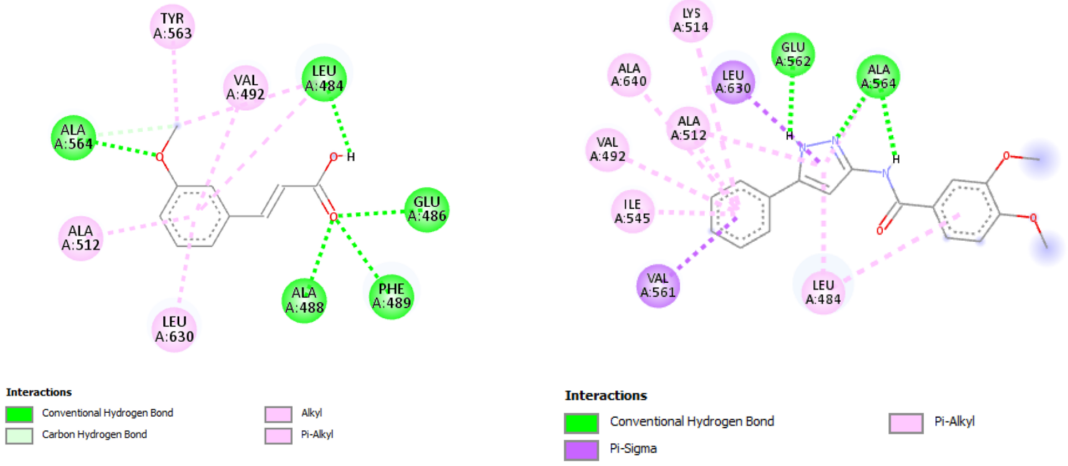
7.5 mmol of malonic acid (7.5 mmol), 5 mmol of *m*-methoxy benzaldehyde, and 0.2 ml of morpholine (2.5 mmol) are mixed until homogenic. Then the microwave oven was set for power 400 Watt. The reaction was conducted every 30 seconds, until the reaction is complete. After that, the mixture was added with 2N HCl solution till it was acidic condition and the precipitate formed. The precipitate was filtered, then tested by TLC using eluents chloroform: ethyl acetate = 4:2, and chloroform-acetone-acetic acid = 7.5:7,5:0.5. The stain was observed in a UV lamp at 254 nm, and its Rf value was calculated. The product was characterized by UV, IR, HNMR and CNMR spectroscopy methods.

Anti-angiogenesis activity using chorioallantoic membrane model

The embryonated chicken eggs of nine days old were incubated at 37°C with 60-70% humidity for one day, then induced with b-FGF (1 ng/µL) in Tris-HCl solution pH 7.5. After that, each of it was given with 3MPCA at doses of 30 and 60 µg; compared to the positive control group containing celecoxib 60 µg and the negative control group containing solvent. Into an egg that had been perforated (in diameter 1 cm^2), a sterile paper disc with a diameter of 5 mm was dripped with the test compound and impregnated in CAM. The holes were closed and incubated for 72 hours at 37°C with 60% humidity. During observation, the top of the eggshell was opened, and neovascularization was seen from the main blood vessels to the paper disc. The microscopic histopathological observation was performed on CAM vessels using hematoxylin and eosin staining. Endothelial cell growth is exposed to neovascular capillaries in CAM sections with a reversed-phase contrast microscope, Nikon H600L. The numeral of endothelial cells in the five-field graph was counted, while each slide was detected at 400x expansion and related with those controls groups.[9]

Docking study

The receptor was FGFR1 (PDB ID: 4UWC) with the native ligand ID: 4Y0 in chain A, which was taken from http://www.rcsb.org/pdb/ in a pdb format. The receptor was removed from its natural ligand and the water using the Auto dock 1.5.7 program. The docking validation was carried out with the same program using RMSD (Root Mean Square of Deviation) parameter value <2.0 Å. The structure of 3MPCA (Figure 1A) as a ligand was drawn in the 2D format using the ChemDraw version 20.1.1 program, then it was changed to a 3D design. The energy minimization of the 3D molecular structure was achieved using the ChemDraw Ultra 3D program with the MMFF94 calculation. The optimized structures were saved the file in pdb format. The prepared ligands were set on 100 independent genetic algorithms (GA Runs). The parameter in the docking simulation of the population dimensions were 150, the maximum number of energy evaluations were 2.500.000 (medium), and the maximum generations were



A B

Figure 1. Interaction tested compounds (3MPCA (A) and native ligand (B)) with amino acids residue of protein FGFR-1.

27.000, the maximum number of active sites was 1, the gene mutation rate was 0.02, and the border rate 0.8 was used in the genetic algorithm method. All interaction visualizations between ligands and proteins were evaluated using the Discovery Studio program.

Prediction toxicity

Toxicity prediction was performed using ProTox II online program. Structure 3MPCA in 2D and smiles format also were drawn using the same program.

Results

Synthesis of 3MPCA compound was carried out through the Knoevenagel reaction, between *m*-methoxybenzaldehyde and malonic acid, with a morpholine catalyst using power microwave irradiation at 400 Watt. After 5x30 seconds, the reaction was complete and the yield of 89%. Organoleptic test results showed that the compound is needle-shaped, white and odorless with a melting point of 118°C. The results of thin layer chromatography test with silica gel GF254 as stationary phase and eluents (ethyl acetate-chloroform, 2: 4; Rf 0.40), (chloroform-acetone-acetic acid, 7.5:7,5:0.5; Rf 0.72). Spectroscopic data showed the following results: IR (KBr; cm^{-1}): 3544 (O-H), 2965 (Csp2-H); 1680.31 (-C = O); 1632 (-C = C-, alkene); 1547 (-C = S); 1279 (-C-O-C); 781 (aromatic ring metha-substituted). UV / Vis λmax (EtOH) nm (log ε): 218 and 274. ^{1}H NMR (400 MHz, CDCl3, ppm): 3.85 (3H, s), 6.45 (1H, d, J = 16Hz), 6.97 (1H, dd, 4Hz, 1.6Hz), 7.07 (1H, *t*, J = 2Hz), 7.16 (1H, d, J = 8 Hz), 7.33 (1H, t), 7.77 (1H, d, J = 16Hz). ^{13}C NMR (100 MHZ, CDCl3, ppm) δ: 50.5 (-OCH3), 108.3, 111.9, 112.7, 116.3, 125.3, 130.5 (aromatic ring), 142.2, 155.1 (alkene), 167.3 (carbonyl). Based on the above data, it is concluded that the target compound has been formed.

Antiangiogenesis activity

Angiogenesis in the CAM model is significantly repressed ($P<0.05$) by 3MPCA at dosages of 30 and 60 ng which are characterized by the embarrassment of endothelial cell growth of blood vessels. After remedy with 3MPCA and celecoxib, a reduction in the numeral endothelial cell of blood vessels were occured. Measurement of endothelial cells' growth of blood vessel inhibitions was performed in Figure 2. Statistical analysis by one-way ANOVA program showed that there is no significant differences in effectiveness of endothelial cells inhibit growth of blood vessels among celecoxib and 3MPCA at dosage 60 µg ($P<0.05$), but potency 3MPCA at 30 ng smaller than 60ng. Celecoxib 60µg inhibits 83% angiogenesis in the CAM model.

Docking study

Validation docking study revealed RMSD was 1.069 Å. Interaction between tested compounds (3MPCA), and native ligand were presented in Figure 1 and Table 1.

Toxicity prediction

Prediction toxicity of 3MPCA was exposed at Figure 3.

Discussion

Structural characterization of 3MPCA (Figure 1A) was strengthened by NMR spectra. By HNMR spectroscopy, the number of protons was calculated. Its characterization gave a chemical shift at 6.45 ppm and 7.77 ppm respectively giving a doublet multiplicity with the integration of each peak is one, indicating the presence of two protons from aliphatic alkenes. Both have a coupling constant of 16 Hz indicating that the proton position in the aromatic alkene is *trans*. Methoxy group revealed as singlet with three integrations of proton (3H, s) at 3.82 ppm,[5] it also reported that the proton of methoxy at chemical shift 3.83 ppm.[11] The ^{13}C-NMR spectrum provides structural information about the carbons in an organic molecule exhibited 10 signals for 10 carbon atoms, consisting of one carbonyl carbon (167.3 ppm), one methoxy carbon (50.5 ppm), six aromatic carbons (C3=130.5 ppm); (C4=111.9 ppm); (C5=155.1 ppm); (C6=108.3 ppm); (C7=125.8 ppm); (C8=116.3 ppm), and two aliphatic alkene carbons (C1=112.7 ppm); (C2=142.2 ppm).

The use of microwave radiation has proven to be very effective as a heat supply in chemical reactions.[12] In addition, microwaves can quicken the reaction rate, provide better and uniform results, selective heating, and achieve greater productivity than conventional heating reactions.[13] During conventional heating methods, the surface temperature of the vessel is increased followed by heating of the internal materials. This process is called wall heating. The wall heating method has the potential to dissipate a certain amount of energy to the environment through convection currents and material conduction. Meanwhile, microwave radiation increases the temperature in all parts of the compartment evenly and simultaneously. In terms of heating and cooling speed, the microwave method has a greater speed of increasing and decreasing temperature than conventional heating.[14]

CAM is a place where high vascularization occurs and is easy to observe in proportion to the growth process of the chicken embryo, therefore an anti-angiogenesis assay is carried out on CAM embryonated chicken eggs.[15,16] CAM was formed after 4 days of incubation, and consisted of a merging of the chorion and allantois of the chicken egg membrane.[15] The development of new blood vessels initiates from capillaries that ascend from blood vessels.

Table 1. Binding energy and types interaction of native ligand and 3MPCA at amino acid residue of protein FGFR-1.

Compounds	Binding energy (kcal/mol)	Interaction
3,4-dimethoxy-N-(5-phenyl-1H-pyrazol-3-yl) benzamide	-8.57	Conventional Hydrogen Bond: Glu A:562; Ala A:564 (native ligand ID: 4Y0) Pi-sigma: Val A:561; Leu A:630 Pi-alkyl: Val A: 492, Ala A:512, Ala A: 640; Ile A:545
(E)-3-(3-methoxyphenyl) prop-2-enoic acid	-6.09	Conventional Hydrogen Bond: Leu A:484, Glu A-486, Ala A: 488, Phe A: 489; Ala A: 564; Carbon Hydrogen Bond: Leu A: 484 Alkyl: Leu A: 484 Pi-alkyl: Tyr A:563; Val A: 492, Ala A:512, Leu A: 630

Neovascularization can't be removed from preceding growth mechanisms and the organization of fresh endothelial cells, while one of the phases of angiogenesis is endothelial cell relocation.[17] Through this interval, the capillary walls of the blood vessels continue their development into the lumen of other blood vessels. Four phases migration endothelial cells are the progression of two reverse capillary walls, the joining of structured endothelial cells and twisting of the bilayer facilitate growth factors so the cells enter the lumen, the center of angiogenesis is designed among the new blood vessels filled by pericytes and myofibroblasts.[17]

The COX-2 inhibitor, Celecoxib,[18] has been described to stop the growth of prostate, breast, lung, and gastric cancer.[19,20] The inflammation associated with COX-2 activity occurred after b-FGF as a pro-angiogenic compound was released.[21,22] Beside celecoxib, 3MPCA also could inhibit activity of COX-2 enzymes,[6] so that it can inhibit b-FGF activity as a pro-angiogenic compound.[20,23]

The mechanism of tumor cell angiogenesis apart from the VEGF mechanism. Several pathways such as fibroblast growth factor (FGF1), and phosphatidyl inositol 3-kinase (PI3K)-protein

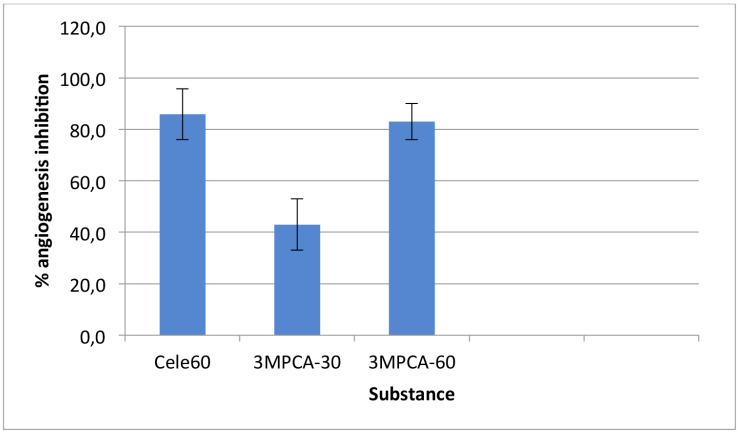


Figure 2. The average angiogenesis inhibition of 3MPCA compare with celecoxib

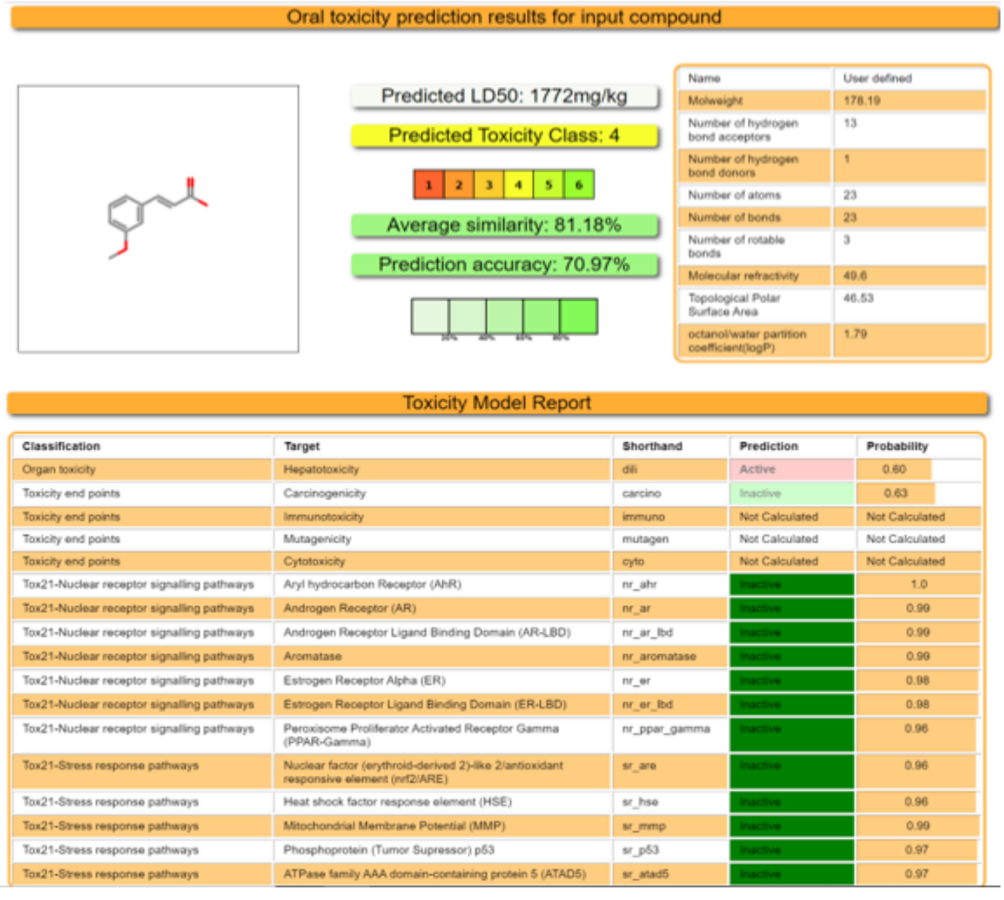


Figure 3. Toxicity prediction of 3MPCA using ProTox II online program.

kinase B (Akt) are the initiation factors or stimuli to initiate endothelial cell migration, invasion, and differentiation.[24] Akt is downstream and also as a PI3K target in the process of angiogenesis. Akt adjusts several cellular processes, including tumor angiogenesis, cell cycle progression, cell growth, cell migration and cell metabolism. Meanwhile, FGF1 is a direct activator of PI3K-Akt. So that if the FGF1 pathway is inhibited, the potential for tumor cell angiogenesis can be inhibited.[25]

The inhibition of angiogenesis consists of several phases, *i.e.*, restraint of b-FGF and VEGF; inhibition the humiliation matrix metalloproteinases, decreasing endothelial cell proliferation; impediment of endothelial cell movement and inhibits endothelial cell activation and differentiation.[26]

Based on molecular docking study, it can be justified the possible relations between each compounds, *i.e.* native ligand and 3MPCA, with amino acid residues in each protein receptor. Figure 2 and Table 1 revealed the likeness interaction at amino acid residues Ala A:564 as conventional hydrogen bond. This is one of key amino acid residue of FGFR-1 protein.[27] Both native ligand and 3MPCA also have match in hydrophobic interaction, namely pi-alkyl or pi-sigma at Val A:492, Ala A:512. Their interactions difference with Ponatinib which interact with Glu520, Asp630 and Met524.[28]

ProTox-II integrates commercial similarity of molecules, fragment properties, most frequent structures and (fragment similarity based CLUSTER cross-validation) machine-learning, based a total of 33 models for the prediction of several toxicity endpoints such as acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes (Tox21) pathways and toxicity targets.[29]

By Protox II online program, LD50 of 3MPCA was predicted 1772mg/kg and its toxicity class was 4. This compound was not causing carcinogenesis and relatively safe but must be careful with oral consumption because it has a potential to cause of hepatotoxicity in probability 0.60. The predictive models are built on data from both *in vitro* assays (*e.g.* Tox21 assays, Ames bacterial mutation assays, hepG2 cytotoxicity assays, Immunotoxicity assays) and *in vivo* cases (*e.g.* carcinogenicity, hepatotoxicity). The models have been validated on independent external sets and have shown strong performance.[30,31]

Angiogenesis plays an important part in the process of tumor development because a blood supply was essential for solid tumors develop to exceed a few millimeters in size. So it becomes one of the vital targets for preventing metastases. However, there was increasing evidence that some solid tumors feat the existing normal blood supply and do not need new vessel formation to grow and metastasize.[32]

Some limitation in this research were 3MPCA contrains angiogenesis on CAM model in the initial process of carcinogenesis, thus it need advance research to study mechanism angiogenesis at other stages.

Conclusions

3MPCA can be synthesized through Knoevenagel reaction using microwave irradiation. This compound is promising as an anti-angiogenic therapeutic agent in earlier stage of carcinogenesis through inhibition FGFR-1 protein and has low toxicity.

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