Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
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Journal of Applied Sciences 10 (14): 1481-1484, 2010
ISSN 1812-5654

Andrographolide Inhibits COX-2 Expression in Human Fibroblast Cells Due to its Interaction with Arginine and Histidine in Cyclooxygenase Site

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Abstract: The aim of this study was to identify and analyze the interaction of andrographolide with COX-2, followed by *in vitro* study of the effect of this compound on COX-2 expression in human fibroblast cells. The molecular modeling study was performed by docking andrographolide to COX-2 enzyme at the site where SC-558, a selective inhibitor of this enzyme, was co-crystallized and compared its interaction to the enzyme with SC-558's. The inhibition of COX-2 expression was determined by measuring PGE2 production in human fibroblast cells stimulated with LPS with and without andrographolide preincubations. Andrographolide interacted with Arg513 and His90 in the cyclooxygenase site of COX-2 and inhibited PGE2 production in human fibroblast cells (IC_{50} = 4 μM). These data confirm that andrographolide's anti-inflammatory activity occurs via inhibition of COX-2 expression.

Key words: Andrographolide, cyclooxygenase enzyme, molecular modeling study, human fibroblast cells, prostaglandin

INTRODUCTION

Andrographolide, an active component of *Andrographis paniculata*, is the major labdane diterpenoidal constituent in this plant, which is used extensively in the traditional medicine in Indonesia to treat inflammations. This compound has an α-alkylidene γ-butyrolactone, two olefin bonds at C8(17) and C12(13) and three hydroxyl groups at C3, C19 and C14 (Nanduri *et al*., 2004). These features make andrographolide able to form hydrogen bonds, both as donors and acceptors and undergo hydrophobic interactions via its methyl group and olefin bonds.

Andrographolide (Fig. 1) has been reported to have anti-inflammatory activity by suppressing inducible nitric oxide synthase expression in RAW 264.7 cells (Chiou *et al*., 2000), prevents oxygen radical production by human neutrophils (Shen *et al*., 2002), inhibits NF-kappaB activation (Xia *et al*., 2004) and reduced COX-2 expression induced by platelet activating factor and *N*-formyl-methionyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo *et al*., 2005). Direct antimicrobial activity of two ethanolic *Andrographis paniculata* extracts was observed for two human pathogens, *Legionella pneumophila* and *Bordetella pertussis* (Xu *et al*., 2006).

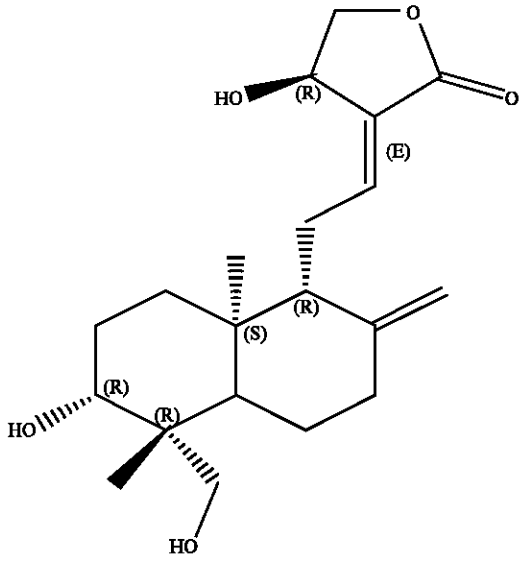

Fig. 1: Andrographolide

The two definitely known isoforms of COX, named COX-1 and COX-2, show distinct expressions patterns

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and distinct biological activities. COX-1 is a constitutively expressed protein that is responsible for the physiological production of prostaglandins. COX-2 is rapidly up-regulated at inflammatory sites and appeared responsible for the formation of prostaglandin. In inflammatory processes COX-2 is over expressed. Non-steroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors and prevent prostaglandin synthesis, thus exhibiting analgesic, antipyretic and anti-inflammatory actions. However, NSAIDs cause gastrointestinal adverse effects, mainly because of their inhibition of the constitutive isoform of COX. Since selective COX-2 inhibitors fail to inhibit constitutive COX-1 isoform, they have no gastrointestinal adverse effects. (Dilber *et al*., 2008). A new class of compounds has recently been developed, named SC-58125, that have a high degree of selectivity for the inducible form of COX-2 over the constitutive form of COX-1. This unique class of compounds exhibits a time dependent irreversible inhibition of COX-2, while reversibly inhibiting COX-1. The molecular basis of this selectivity was probed by site-directed mutagenesis at the active site of COX-2. One difference is observed at the cyclooxygenase active site, where the isoleucine at position 523 in COX-1 is a valine in COX-2 (Gierse *et al*., 1996).

In this study we sought to identify and analyze the interaction of andrographolide with COX-2 in terms of hydrogen bonds, followed by *in vitro* study of the effect of this compound on COX-2 expression in human fibroblast cells.

MATERIALS AND METHODS

Molecular modeling study (December 2008 to March 2009): The X-ray crystallographic 3D structures of COX-2 (PDB code: 1CX2) complexed with its selective inhibitor SC-558 crystallized by Kurumbail *et al*. (1996) was downloaded from online Protein Data Bank. 3D structure of andrographolide was built by using Molecular Operating Environment (MOE-2007.09.02). Energy minimization was carried out by using MMFF94x. Docking of andrographolide to COX-2 enzyme was carried out at the site where SC-558 was co-crystallized.

***In vitro* study (November 2009 to March 2010)**

Chemicals and samples: Andrographolide 98% 500 mg CAS 5508-58-7 for R and D use was purchased from Aldrich. Prior to use the compound was dissolved in 2% dimethylsulfoxide (DMSO) in Phosphate Buffer Saline (PBS). All other chemicals were dissolved in phosphate buffer saline or MilliQ water (Sentra). High glucose Dulbecco's Modified Eagle Medium (DMEM) which contained D-glucose, L-glutamine, sodium pyruvate for culture medium was purchased from Gibco, lipopolysaccharide (LPS) 10 mg derived from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich.

Human fibroblast cell culture and differentiation: Human fibroblast cells were a kind gift from Dr. Endang Winiarti (Research Laboratory, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia). The cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) which contained D-glucose, L-glutamine, sodium pyruvate (Gibco), supplemented with 10% heat-inactivated FBS (fetal bovine serum), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and fungizone, at 37°C under 5% CO_2. The cells were differentiated by incubating them in their culture medium for 48 h and were collected at the third day for further assay.

Cyclooxygenase assay by measuring PGE_2 production: The inhibition of cyclooxygenase was determined based on procedure described by Young *et al*. (1996) with slight modifications. Briefly, fibroblast cells (5.2×10^4 cells) in high glucose DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and fungizone, were placed in a 96-well microplate and were stimulated with LPS (10 μg mL^{-1}) to produce prostaglandin (PGE2), a protein which production was catalyzed by cyclooxygenase enzyme. Various concentrations of andrographolide (2 to 200 μM) were added into the wells and the mixtures were incubated for 18 h at 37°C under 5% CO_2. Acetyl salicylic acid (0.7 to 40 μM) was used as the standard. The production of PGE2 was measured by using microplate reader at 450 nm.

RESULTS AND DISCUSSION

Molecular modeling study: The binding site of COX-2 where its selective inhibitor, SC-558, was bound (Fig. 2a) contained His90, Leu117, Val349, Leu352, Ser353, Tyr355, Trp387, Ala516, Phe518, Met522, Val523, Gly526, Ala527, Ser530, Leu534. This molecular modeling s tudy of COX-2 binding site which contained Valine at position 523 confirmed Gierse *et al*. (1996)'s study.

Based on our study, SC-558 showed only one hydrogen bond with Arg513 (Fig. 2a). The hydrogen bond was formed between SC-558 amine and Arg513 at 3.15 (Table 1), while andrographolide showed interaction with the COX-2 binding pocket via two hydrogen bonds (Fig. 2b) which were formed between oxygen atom of

Table 1: Interaction of Andrographolide with COX-2 binding pocket in comparison with SC-558

Ligand	Energy (kcal mol^{-1})	Close contact amino acids	HB (donor→acceptor)
SC-558	-10.7717	His90, Leu117, Val349, Leu352, Ser353, Tyr355, Trp387, Ala516, Phe518, Met522, **Val523**, Gly526, Ala527, Ser530, Leu534.	Arg513→N
Andrographolide	-11.7963	His 90, Met 113, Val 116, Arg120, Val 349, Leu 352, Ser 353, Tyr 355, Leu 359, Trp 387, His388, Ala 516, Ile517, Phe 518, Met 522, **Val 523**, Gly 526, Ala 527, Ser 530, Leu 534	His90→O Arg513→O

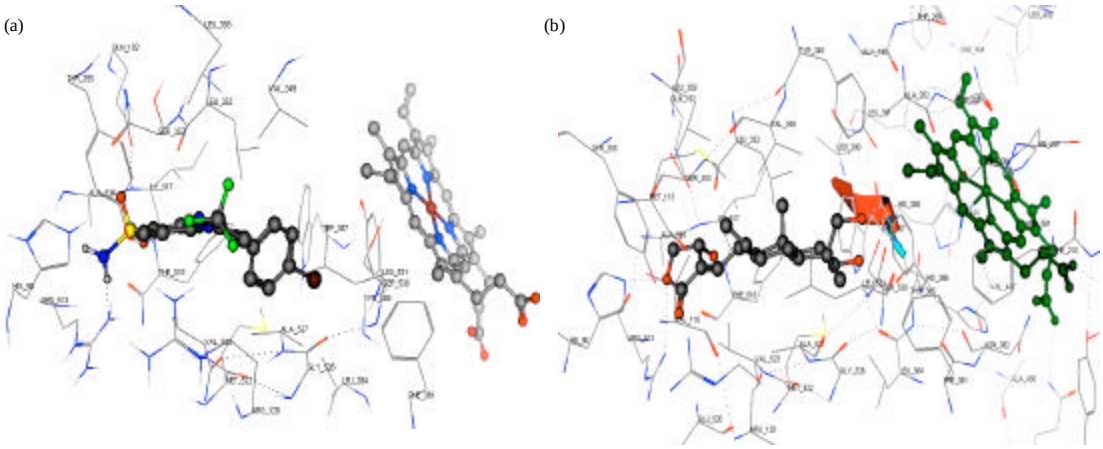

Fig. 2: (a) Interaction of SC-558 and (b) andrographolide in COX-2 binding pocket. Hydrogen bonds were showed by dotted lines

andrographolide's lactone as HB acceptor and hydrogen atom in the Arg513's amine and His90's pyridine (Table 1).

The energy needed for these interactions, either for SC-558 or andrographolide, -10.7717 and -11.7963 kcal mol^{-1}, respectively, were small and not significantly different (Table 1). These data suggested that the interaction of andrographolide with cyclooxygenase site of COX-2 enzyme was favorable.

***In vitro* study:** Andrographolide has been reported to have anti-inflammatory activity by suppressing inducible nitric oxide synthase expression in RAW 264.7 cells (Chiou *et al*., 2000), prevents oxygen radical production by human neutrophils (Shen *et al*., 2002), inhibits NF-kappaB activation (Xia *et al*., 2004) and reduced COX-2 expression induced by platelet activating factor and *N*-formyl-methionyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo *et al*., 2005). The anti-inflammatory activity of this compound on stimulated human fibroblast cells has not been reported.

Fibroblast is a type of cell that synthesizes the extracellular matrix and collagen and plays critical role in wound healing. During inflammation, any damage which occurs in the tissue will stimulate the mitosis of fibroblasts.

In this study andrographolide showed inhibitory activity on COX-2 expression in human fibroblast cells induced by lipopolysaccharide in the concentration range between 2 to 200 μM (IC_{50} = 4 μM), which was 0.7 times than acetosal's (IC_{50} = 2.8 μM) (Fig. 3a, b).

An unexpected result showed from this work. Preincubation of fibroblast cells with low concentrations of acetosal induced the production of prostaglandin, but with the increasing of acetosal concentration, PGE2 level decreased. Andrographolide at increasing concentration (up to 200 μM) did not stimulate PGE2 production. These data confirmed that andrographolide's anti-inflammatory activity was occurred via inhibition of COX-2 expression.

The behavior of acetosal in correlation with PGE2 production was compared with Morgan *et al*. (2009) which concluded that COX-2 appeared to be differentially regulated in aspirin-sensitive patients. Morgan and her colleagues observed that aspirin and LPS increased COX-2 expression on blood monocytes of aspirin-induced asthmatic patients, a finding in contrast with the lack of an effect of the same stimuli on COX-2 expression on monocytes from healthy subjects. They found significantly higher COX-2 expression levels after stimulation with LPS and aspirin (mean 78.8, range 44.9-92.3; p = 0.0002) in comparison to LPS alone (mean 65.9%, range 33.6-82.6) in aspirin-induced asthmatic patients (Morgan *et al*., 2009).

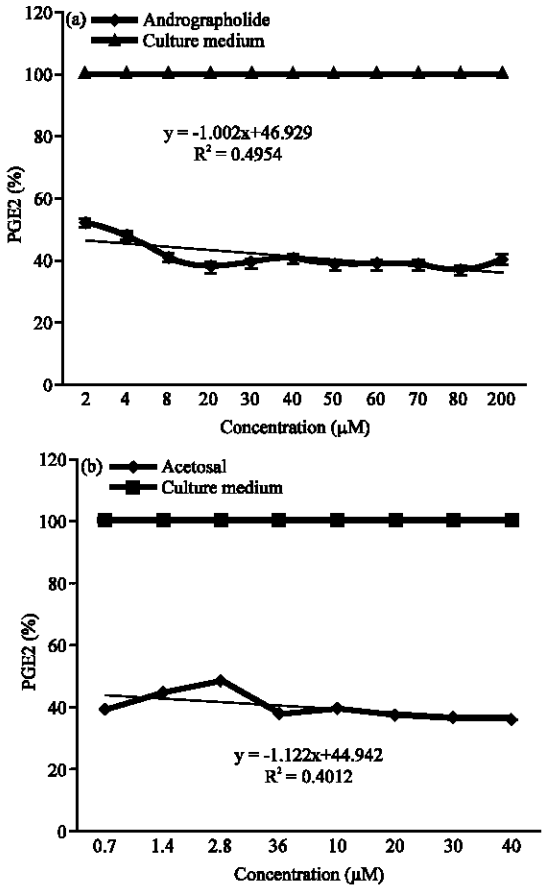

Fig. 3: Spectrometric analysis of PGE2 production in fibroblast cells with and without (a) Andrographolide and (b) Acetosal

CONCLUSIONS

Andrographolide showed interaction with arginine and histidine in the cyclooxygenase site of COX-2 and reduced PGE2 production in human fibroblast cells induced by lipopolysaccharide. Its inhibitory activity to COX-2 enzyme was 0.7 times than acetosal's, but unlike acetosal, andrographolide did not stimulate PGE2 production.

ACKNOWLEDGMENTS

We would like to thank Dr. Endang Winiarti (Research Laboratory, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia) for her kind gift, human fibroblast cells used in this project, the Rector of Universitas Padjadjaran for his continuous supports and Institute Technology Bandung, Indonesia that has partially funded this work via IM HERE grant 2010. The authors have declared no conflict of interest.

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