Dual Inhibition of Cyclooxygenases-2 and 5-Lipoxigenase by Deoxypodophyllotoxin in Mouse Bone Marrow-Derived Mast Cells

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Deoxypodophyllotoxin (Anthricin) is a medicinal herbal product isolated from Anthriscus sylvestris Hoffm. that inhibits cyclooxygenase-2 (COX-2) and COX-1-dependent phases of prostaglandin D2 (PGD2) generation in bone marrow-derived mast cells (BMMC) in a concentration-dependent manner with IC50 values of 1.89 µM and 65.3 µM, respectively. This study also found that this compound inhibited COX-1 and 2-dependent conversion of the exogenous arachidonic acid to PGD2 in a dose-dependent manner with an IC50 value of 0.01 µM and 12.1 µM, respectively using a COX enzyme assay kit. However, this compound did not inhibit COX-2 protein expression up to a concentration of 30 µM in the BMMC, indicating that deoxypodophyllotoxin directly inhibits COX-2 activity. Furthermore, this compound consistently inhibited the production of leukotriene C4 (LTC4) in a dose dependent manner, with an IC50 value of 0.37 µM. These results demonstrate that deoxypodophyllotoxin has a dual cyclooxygenase-2 selective/5-lipoxigenase inhibitory activity, and therefore this compound might provide a basis for novel anti-inflammatory drugs.

Key words Anthriscus sylvestris; deoxypodophyllotoxin; cyclooxygenase-2; 5-lipoxigenase; bone marrow-derived mast cells

Prostaglandins (PGs) elicit a variety of important biological responses. Among these properties are their ability to induce pain, fever and the symptoms associated with inflammatory responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the pain and inflammatory swelling by blocking PG synthesis at the cyclooxygenase (COX) stage. However, most NSAIDs which have been used clinically inhibit the production of the PGs that are not only associated with the inflammatory processes but are also involved in maintaining the normal physiological processes. The main limitation in using NSAIDs are their side effects, including gastrointestinal ulcerogenic activity and kidney dysfunction, which limits their therapeutic value of their safe and long-term use. The enzyme responsible for PG synthesis exists as two isoforms, COX-1 (constitutive isofrom) and COX-2 (inducible form). Arachidonic acid can also be converted to leukotrienes (LTs) by the action of 5-lipoxygenase (5-LOX). Therefore, the development of dual inhibitors that can simultaneously inhibit COX-2 and 5-LOX might enhance their individual anti-inflammatory effects and reduce the undesirable side effects that are associated with NSAIDs. This study describes for the first time a new biological function of deoxypodophyllotoxin for arachidonic cascade metabolism enzymes.

MATERIALS AND METHODS

Materials Dried roots of Anthriscus sylvestris (Umbeliferae, 2 kg) were extracted with MeOH–CH2Cl2 (1 : 1, v/v, 3 l) at room temperature for seven days to obtain an extract (190 g). The extract was resuspended in water (500 ml) and partitioned with the same volume of CH2Cl2. CH2Cl2 soluble fraction (73 g) was applied to a silica gel column (63—200 mesh, 7×100 cm) and eluted with CH2Cl2/MeOH (19 : 1, 9 : 1, 4 : 1, 1 : 1, 0 : 1, each 1.5 l) to give five fractions (Fr 1 to Fr 5). Fraction 2 (between 1.5 and 3.1, 13.2 g) was repeatedly chromatographed over silica gel (5×70 cm, elute: CH2Cl2/EtOAc 9 : 1, 4 : 1, 3 : 2, 0 : 1, each 11) to give four fractions (Fr 21 to Fr 24). Fraction 23 (between 2 to 31, 4.6 g) was crystallized in MeOH to give a white amorphous powder (1.5 g) and its chemical structure was established as deoxypodophyllotoxin by comparison of 1H- and 13C-NMR and optical rotation data with those reported previously (mp 166—167 °C, [α]D23 110° (c=1.0, CHCl3)).5) RPMI 1640 and phosphate-buffer saline were obtained from GIBCO-BRL (Grand Island, NY, U.S.A.). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). Rabbit polyclonal COX-2 antibody and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). LPS (from Escherichia coli 0111:B4, γ-irradiated) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deoxypodophyllotoxin was dissolved in dimethyl sulfoxide (DMSO) before addition to cell cultures: The final concentration of DMSO was 0.05%. Controls with DMSO alone were run in all cases.

Preparation and Activation of Bone Marrow-Derived Mast Cells (BMMC) Bone marrow cells from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mm l-glutamine, 0.1 mm nonessential amino acids, antibiotics and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks >98% of the cells were found to be BMMC when checked by the previously described procedure.13) To measure inhibitory activity on COX-2 by de-
Deoxypodophyllotoxin is a type of lignan which is widely distributed in various plants. It has already been reported that deoxypodophyllotoxin possesses antiproliferative, antitumor and antiviral activity. The dried roots of Anthriscus sylvestris (Umbeliferae) have been used in Korean traditional medicine as an antipyretic, an analgesic and a cough remedy. However, the underlying mechanisms accounting for its anti-inflammatory effects have not yet been reported. In an attempt to develop anti-inflammatory compounds from A. sylvestris Hoffm., deoxypodophyllotoxin, a known lignan compound, was isolated from the MeOH–CH2Cl2 extract of A. sylvestris.

Murakami et al. reported that BMMC exhibit biphasic PGD2 biosynthetic responses over time, in addition to COX-1-dependent immediate and COX-2-dependent delayed responses. The immediate PGD2 generation occurring within 2 h is associated with the coupling of COX-1 and the delayed PGD2 generation, which occurs after several hours of culture (during 2—10 h), is associated with the de novo induction and function of COX-2 after stimulation with particular cytokines and LPS combinations. This cell model also appears to be suitable for assessing the effect of 5-LO inhibitors, since the immediate LTC4 generation elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMC through 5-LO. Therefore, the BMMC system is useful for screening selective COX-1/COX-2 or 5-LO and COX-2/5-LO dual inhibitors from various sources.

In order to determine if deoxypodophyllotoxin inhibits the delayed phase of PGD2 generation via direct inhibition of COX-2 enzyme activity, the COX-1 and COX-2 activities were measured using a colorimetric COX (ovine) inhibitor screening assay kit. Deoxypodophyllotoxin inhibited the COX-2-dependent phase of PGD2 generation in a dose-dependent manner with an IC50 value of approximately 1.89 μM, while that for the COX-1-dependent phase of PGD2 generation was approximately 65.3 μM (Table 1). The relative value for the selectivity was approximately 30 (IC50 for COX-1/IC50 for COX-2).

### Table 1. IC50 Values of Deoxypodophyllotoxin on COX-1, COX-2 and 5-LOX

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<th>COX-1</th>
<th>COX-2</th>
<th>5-LOX</th>
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<td></td>
<td>65.3 μM</td>
<td>1.89 μM</td>
<td>0.37 μM</td>
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BMMC were preincubated for 30 min with the indicated concentrations of deoxypodophyllotoxin A and then stimulated with KL (100 ng/ml), IL-10 (100 U/ml) and LPS (100 ng/ml) at 37°C for 8 h in the presence or absence of deoxypodophyllotoxin. For measuring COX-1 activity, cells without aspirin pretreatment were incubated at 37°C for 2 h with activators. PGD2 released into the supernatant was quantified by EIA kit. All data was the arithmetic mean of triplicate determinations.
It was clearly shown that the inhibition of 
PGD\(_2\) production by deoxypodophyllotoxin in the BMMC 
was not due to the reduction of the COX-2 expression level. 
This inhibition could also be attributed to the step of PLA\(_2\), 
which is the initial enzyme in the arachidonic acid cascade. 
Since the delayed phase of PGD\(_2\) generation by BMMC de-
pends on the coupling between group II-like sPLA\(_2\) and 
COX-2,\(^{13}\) the effect of deoxypodophyllotoxin on sPLA\(_2\)-IIA 
was next examined using the sPLA2-transfected HEK293 cell 
supernatants.\(^{14}\) Deoxypodophyllotoxin failed to inhibit the 
group IIA sPLA\(_2\) activity up to 50 \text{µM} (data not shown). 
Overall, these results suggest that deoxypodophyllotoxin di-
rectly inhibits the COX-2 enzyme activity without altering 
the COX-2 protein level and PLA\(_2\) activity.

Arachidonic acid can also be converted to leukotrienes 
(LTs) by the action of 5-lipoxygenase (5-LOX) in BMMC. 
The inhibition of 5-LOX is believed to be the ideal treatment 
for allergic diseases and asthma.\(^{15}\) Therefore, the inhibitory 
activity of deoxypodophyllotoxin on the generation of LTC\(_4\) 
in the BMMC was examined. Figure 4 shows that the 
BMMC stimulated with SCF for 15 min produced \text{ca}. 
500 pg/ml LTC\(_4\), and preincubation of the BMMC with de-
oxypodophyllotoxin resulted in the dose-dependent suppres-
sion of this LTC\(_4\) biosynthesis with an IC\(_{50}\) value of 0.37 \text{µM}.

In conclusion, antipyretic and an analgesic activity of 
deoxypodophyllotoxin could be attributed at least in part 
to the dual inhibition of COX-2/5-LOX. Therefore, deoxy-
podophyllotoxin may be a useful biochemical and pharmaco-
logical tool for determining the role of COX-2/5-LOX dual 
inhibitors in certain physiological and pathological events.

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REFERENCES