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Alkamides from the fruits of *Piper longum* and *Piper nigrum* displaying potent cell adhesion inhibition

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ABSTRACT

Eight alkamides **1–8** were isolated by bioassay-guided isolation of EtOH extracts of the fruits of *Piper longum* and *Piper nigum* (Piperaceae). Their structures were elucidated by spectroscopic analysis (^{1}H, ^{13}C NMR, and ESI-MS) as follows: guineensine (**1**), retrofracamide C (**2**), (2*E*,4*Z*,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,4,8-nonatrienoyl]piperidine (**3**), pipernonaline (**4**), piperrolein B (**5**), piperchabamide D (**6**), pellitorin (**7**), and dehydropipernonaline (**8**). Their compounds **3–5**, **7**, and **8** inhibited potently the direct binding between sICAM-1 and LFA-1 of THP-1 cells in a dose-dependent manner, with IC_{50} values of 10.7, 8.8, 13.4, 13.5, and 6.0 μg/mL, respectively.

Lymphocyte function-associated antigen-1 (LFA-1) belongs to the β2 integrin subfamily of cell adhesion molecules, exists as a heterodimeric glycoprotein of α and β chain, CD11a and CD18. LFA-1 is expressed on cell membranes of leukocytes, macrophages, neutrophils, and monocytes and it plays a critical role in the migration of these cells into the sites of inflammation.[1] The major ligand for LFA-1 is intercellular cell adhesion molecule-1 (ICAM-1), which is a member of the immunoglobulin superfamily and plays an important role in inflammation and in T-cell activation.[2] Interaction between LFA-1 and its ligand, ICAM-1, participates in cell to cell interaction that is important in the progression of the inflammatory diseases such as rheumatoid arthritis, stroke, psoriasis, allergy, and atherosclerosis.[3] Recently, in vivo studies using anti-LFA-1 antibodies demonstrate that LFA-1 plays a central role in leukocyte extravasation in recruitment model.[4] In addition, clinical trials using efalizumab,[5] the humanized anti-LFA-1 antibody, are approved for the treatment of moderate in psoriasis. Also, numerous small molecule LFA-1/ICAM-1 interaction antagonists, such as antagonists mimicking the ICAM-1 ligand-binding site and targeting LFA-1 I domain, have been developed and applied in several preclinical studies.[6] Therefore, inhibitors of LFA-1/ICAM-1 mediated cell adhesion may be of use as novel therapeutic agents for the treatment of inflammatory diseases.

In our searching for cell adhesion inhibitors from natural sources, some alkamides isolated from the EtOH extracts of Piper species plants (*P. longum* and *P. nigrum*) showed inhibitory activity on cell adhesion assay. *P. longum* and *P. nigrum* belong to the family of Piperaceae; they are very important oriental medicinal plants and their fruits had been used for the treatment of cholera, dyspepsia, various gastric ailments, and arthritic disorders.[7] Particularly, various biological activities of an alkamide from these plants, including insecticidal, anti-bacterial, and anti-inflammatory properties, have been reported.[8] We also reported that the alkamides inhibited acyl-CoA:cholesterol acyltransferase (ACAT)[9] and diacylglycerol aclytransferase (DGAT).[10]

The seeds of *P. longum* and *P. nigrum* were extracted with EtOH, and the EtOH extract was partitioned with $CHCl_3$. Chloroform-soluble materials inhibiting the cell adhesion were subjected to a silica gel, C-18 open-column chromatography and followed by semi-preparative HPLC to afford eight alkamides **1**–**8** (Fig. 1). Four known compounds **1**–**4**, isolated from the *P. longum* fruits, were identified as guineensine (**1**), retrofracamide C (**2**), (2*E*,4*Z*,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,4,8-nonatrienoyl]piperidine (**3**), and pipernonaline (**4**) in comparisons with previously published data.[9,11] Also, the other compounds isolated from *P. nigrum* fruits

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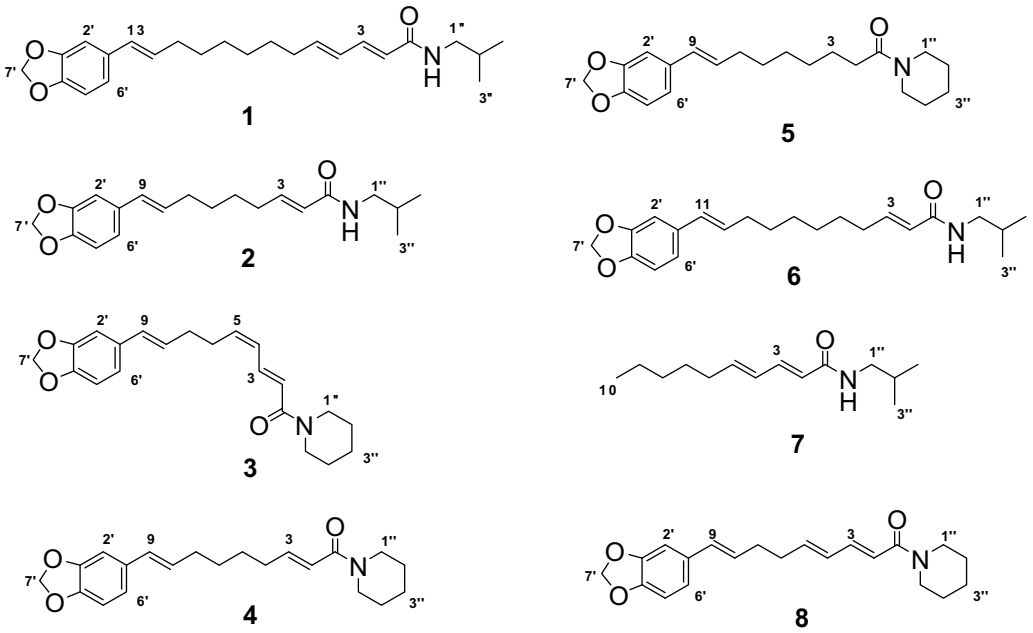

Figure 1. Structures of alkamides **1**–**8** isolated from *P. longum* and *P. nigrum*.

were identified as piperrolein B (**5**), piperchabamide D (**6**), pellitorin (**7**), and dehydropipernonaline (**8**).[8,12] The alkamides can be divided into two groups: those with isobutyl (**1**, **2**, **6**, and **7**) and piperidine moieties (**3–5** and **8**). All of these compounds possess identical 3,4-methylenedioxyphenyl and carbonyl groups except compound **7** which possesses an aliphatic acyl group instead of a 3,4-methylenedioxyphenyl group. Whereas compounds **1**, **3**, **7**, and **8** possess conjugated dienamide groups, compounds **2**, **4**, and **6** possess conjugated monoenamide groups, and compound **5** has non-conjugated amide systems. Herein, we describe the structure–activity relationship (SAR) of the alkamides **1**–**8** isolated from piper species plants as inhibitors of cell adhesion.

We investigated whether these compounds (**1**–**8**) affected the cell adhesion by interfering with contact between LFA-1 of THP-1 cells and sICAM-1 using modified ELISA method.[13] 96-Well plates were coated with 100 μL of sICAM-1 (R&D systems) at a concentration of 5 μg/mL in PBS overnight at 4 °C, and BCECF-AM-labeled THP-1 cells were stimulated for 20 min at 37 °C by LFA-1/2 mAb.[14] And then, THP-1 cells and test compounds were added to the wells. After incubation for 1 h at 37 °C, the plates were washed twice with PBS and the remaining cells were dissolved with 1% Triton X-100 in PBS. Fluorescent intensity was measured in a multi-detection microplate reader (FLx800™, BioTek® Instruments, Inc.) with an excitation wavelength at 485 nm and an emission wavelength at 530 nm. This activity was verified using lovastatin[15] as a positive control, which inhibited cell adhesion with an IC_{50} value of 8.0 μg/mL in the assay system. Because cytotoxicity can influence the activity, THP-1 cell viability was measured by the MTT-based cytotoxicity assay.[16] These compounds **1–8** were not observed to be toxic at the concentration employed in this study (data not shown). The biological data for alkamide derivatives **1–8** have been shown in Table 1. Compounds **1**, **2**, and **6** having isobutyl amide moiety were less potent in the inhibition of the direct binding between sICAM-1 and LFA-1 of THP-1 cells (36.8 ± 6.9% for **1**, 36.0 ± 6.5% for **2**, and 42.6 ± 8.3% for **6** at 25 μg/mL). However, compounds **3–5**, and **8** substituted with piperidine amide group showed potent inhibitory activity for the direct binding between sICAM-1 and LFA-1 of THP-1 cells in a dose-dependent manner. Compound **7** also showed moderate activity. Namely, compound **8** having one trans double bond and trans conjugated double bonds between 3,4-methylenedioxyphenyl and piperidine amide exhibited higher activity with IC_{50} value of 6.0 μg/mL, whereas the corresponding analogues **4** having two trans double bond moieties at C-3 and 9 showed slightly attenuated potency with IC_{50} value of 8.8 μg/mL. Compound **5** has one trans double bond at C-9 position compared with compound **8**, and its inhibitory activity of cell adhesion was twofold less than that of compound **8**. The compound **3** involving cis and trans conjugated double bond at C-3 and 5 and one trans double bond at C-9 showed less potent inhibitory activity with IC_{50} value of 10.7 g/mL. Compound **7** substituted

Table 1
Inhibitory effect of compounds **1**–**8** on the direct binding between sICAM-1 and LFA-1 of THP-1 cells[a]

Compounds (μg/mL)	1	2	3	4	5	6	7	8	Lovastatin*
25.00	36.8 ± 6.9[b]	36.0 ± 6.5	92.1 ± 0.3	93.8 ± 4.7	67.6 ± 5.6	42.6 ± 8.3	80.6 ± 4.6	73.4 ± 8.9	—
12.50	31.7 ± 3.3	35.9 ± 3.3	58.4 ± 7.1	70.2 ± 6.0	46.5 ± 6.4	26.9 ± 5.2	46.4 ± 5.9	57.7 ± 7.4	61.4 ± 6.9
6.25	14.9 ± 4.5	29.9 ± 6.2	13.1 ± 3.4	36.8 ± 2.5	28.5 ± 1.0	20.9 ± 4.9	16.5 ± 4.0	52.5 ± 4.7	37.7 ± 3.3
3.13	—[c]	—	—	12.5 ± 1.1	16.2 ± 1.3	15.6 ± 1.3	11.3 ± 6.6	30.1 ± 2.9	—
IC_{50}	50>	50>	10.7	8.8	13.4	30	13.5	6.0	8.0

[a] BCECF-AM-labeled THP-1 cells and test compounds (**1**–**8**) were added to 96-well plates were coated with sICAM-1. After incubation for 1 h at 37 °C, cells were dissolved with 1% Triton X-100 in PBS, and fluorescent intensity was measured in a multi-detection microplate reader at an excitation of 485 nm and an emission of 530 nm.
[b] The data are presented as the means of three independent experiments performed in duplicate.
[c] There is no inhibitory activity.
* The lovastatin was used as a positive control.

with aliphatic acyl group possessing trans conjugated bond showed moderate cell adhesion inhibitory activity with IC_{50} value of 13.5 μg/mL. These results demonstrated that the inhibitory activity may be affected by conjugated dienamide system. In addition, 'trans' configuration of these compounds might exert some influence on cell adhesion inhibitory activity because compound **8** had the more potent activity than 'cis' configuration of compound **3**. As a result, cell adhesion inhibitory activities of alkamides were more positively influenced by the presence of piperidine moiety than isobutyl moiety, and the activities were also influenced by the number of conjugated double bond.

In conclusion, we have proven that various alkamides **1**–**8** isolated from *P. longum* and *P. nigrum* inhibited the direct binding between LFA-1 and its ligands, ICAM-1, and showed anti-inflammatory activity in vivo mouse model (data not shown). These results will be useful for the design of new cell adhesion inhibitors leading to anti-inflammatory agents, and present the first natural alkamide-derived cell adhesion inhibitors documented.

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