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# CUCURBITACINS, CELL ADHESION INHIBITORS FROM *CONOBEA SCOPARIOIDES*

LÁSZLÓ L. MUSZA,* PHYLLIS SPEIGHT, SUSAN MCELHINEY, COLIN J. BARROW, AMANDA M. GILLUM, RAYMOND COOPER, and LORAN M. KILLAR

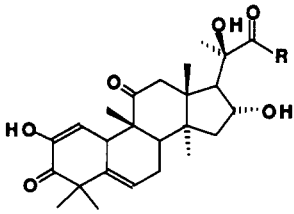
*Sterling Winthrop Pharmaceuticals Research Division, 9 Great Valley Parkway, Malvern, Pennsylvania 19355*

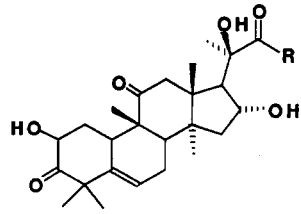
ABSTRACT.—In the course of screening natural products for antagonists of CD18-mediated cell adhesion, an extract with inhibitory activity was identified from the stem and leaves of *Conobea scoparioides*. Bioassay-guided fractionation led to a pure compound, identified by spectroscopy as cucurbitacin E [**1**]. Although many biological activities have been reported for the cucurbitacins, this is the first report of cell adhesion inhibition. Furthermore, closely related cucurbitacin analogues had different potencies, pointing to substructural features that are important for the activity.

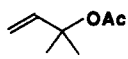
Cell adhesion is a multistage process mediated by various adhesion molecules. For example, when leukocytes are migrating towards inflamed tissue, they are slowed down by interactions with the endothelium. These interactions, which lead to the characteristic rolling motion of the leukocytes, are governed by the binding of selectins to their ligands. The latter stages in the process, for example, the actual adhesion and extravasation through the vascular endothelium, are mediated by the binding of the activated lymphocyte function associated antigen (LFA-1, CD11a/CD18), to intercellular adhesion molecule-1 (ICAM-1, CD54) (1). Antibodies against LFA-1 and other leukocyte integrins have been shown to attenuate inflammation-associated tissue damage in animal models (2,3). Inhibitors of integrin-mediated cell adhesion may therefore have therapeutic potential as anti-inflammatory agents.

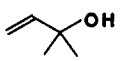
In the course of screening natural products for antagonists of leukocyte integrin-mediated cell adhesion in a whole cell assay, a plant extract obtained from the stem and leaves of *Conobea scoparioides* Benth. (Scrophulariaceae) was found to inhibit cell adhesion but was not overtly toxic to the cells. Using a three-step bioassay-guided isolation procedure, a pure compound was obtained and identified by spectroscopy as cucurbitacin E [**1**].

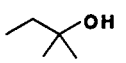
Compound **1** is a highly oxygenated member of tetracyclic triterpenoids present in such various plant families as the Cucurbitaceae, Scrophulariaceae, Euphorbiaceae, Liliaceae, and Elaeocarpaceae (4–9). A wide range of biological activities has been

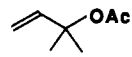


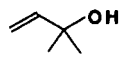



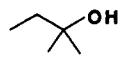

1 R= 


3 R= 


5 R= 


2 R= 


4 R= 


6 R= 

reported for the cucurbitacins; however, to the best of our knowledge, cell adhesion inhibition has not been described (10–13). Several related cucurbitacins were tested to determine if different substructural regions of the molecule were responsible for cell adhesion inhibition. Studies to determine if **1** was directly inhibiting leukocyte integrin-mediated cell adhesion or acting through a nonspecific mechanism were performed.

## RESULTS AND DISCUSSION

*Conobea scoparioides* is a tropical tree indigeneous to South America. The plant was previously investigated for its essential oil content by Alpande de Morais *et al.* (14), but no other studies have been reported on its chemical constituents.

In the course of high-volume screening with the JY/HeLa cell adhesion assay, a $CH_2Cl_2$ plant extract of *C. scoparioides* was found to be significantly active. The extract was further fractionated using a bioassay-guided procedure, and, after three chromatographic steps, a single active compound was isolated. One- and two-dimensional $^1H$- and $^{13}C$-nmr spectroscopy suggested the presence of a highly oxygenated and methylated tetracyclic triterpenoid structure with an unsaturated side-chain, and the compound was identified as cucurbitacin E [**1**]. Spectroscopic data were identical to those obtained for an authentic sample of **1**.

The biological activity of **1** in the cell adhesion and cytotoxicity assays is shown in Table 1. Compound **1** was not toxic upon exposure to JY cells for 5 h in a tritiated thymidine uptake assay. No toxicity was observed for 45 min (the length of the adhesion assay), followed by washing, then testing in the 48-h MTT assay. Significant toxicity was seen, however, when the JY cells were exposed to **1** for longer periods in the tritiated thymidine uptake assay (24 h) and in the 48-h MTT assay (data not shown).

To determine if **1** was interacting with ICAM-1, plates coated with immunoaffinity-purified native ICAM-1 were preincubated with the compound. Preincubating ICAM-

TABLE 1. Activity of Cucurbitacins **1–6** in the Cell Adhesion and Cytotoxicity Assays.

| Compound | Dose (μM) | Thymidine Uptake (24 h) % Inhibition | Cell Adhesion Inhibition[a] $IC_{50}$ (μM) |
|---|---|---|---|
| **1** | 0.3 | 19 | |
| | 1.0 | 60 | 0.18±0.03 |
| | 3.3 | 68 | |
| **2** | 0.3 | 39 | |
| | 1.0 | 63 | 0.30±0.07 |
| | 3.3 | 71 | |
| **3** | 0.3 | 34 | |
| | 1.0 | 63 | 0.95±0.02 |
| | 3.3 | 58 | |
| **4** | 0.3 | 12 | |
| | 1.0 | 45 | 1.36±0.39 |
| | 3.3 | 58 | |
| **5** | 0.3 | — | |
| | 1.0 | — | >50 |
| | 3.3 | — | |
| | 50.0 | 69 | |
| **6** | 0.3 | 20 | |
| | 1.0 | 22 | >50 |
| | 3.3 | 7 | |
| | 50.0 | 74 | |

[a]Triplicate samples were run in three separate experiments.

1 with **1** had no effect on the adhesion of JY cells. Preincubating the JY cells with the compound, however, resulted in inhibition of adhesion to HeLa cells. These results suggest that **1** inhibited cell adhesion by interfering with LFA-1 and not ICAM-1.

Six related cucurbitacins, including **1**, were obtained from Dr. David Lavie. Cucurbitacin B [**2**] differs from **1** in the absence of the A-ring double bond. Cucurbitacins I [**3**] and D [**4**] correspond structurally to **1** and **2**, respectively, but lack a side-chain acyl group. Cucurbitacins L [**5**] and R [**6**] are the side-chain saturated analogues of **3** and **4**, respectively.

Cell adhesion and toxicity data for the six cucurbitacin analogues showed a considerable range (Table 1). Compounds **1** and **2** had submicromolar potencies, while **3** and **4** were approximately fivefold less potent than **1** and **2**, respectively. Compounds **5** and **6** were inactive at doses as high as 50 μM and were also less toxic than the other analogues. The data indicate that minor structural changes in the side-chain, but not in the A ring, markedly affect potency levels for cell adhesion inhibition.

Cell adhesion may be disrupted non-specifically by inhibiting cell activation by PMA (15). To analyze the effect of cucurbitacins on cell activation, **1**, **3**, and **6** were tested for their effects on $Ca^{2+}$ flux in activated T cells. None of the compounds inhibited $Ca^{2+}$ flux, nor did any inhibit protein kinase C in JY cells.

Another mechanism that would result in cell-adhesion inhibition is disruption of the cytoskeleton. The six cucurbitacins were tested for effects on actin polymerization in fMLP-stimulated neutrophils. As shown in Table 2, inhibition of actin polymeriza-

TABLE 2. Effect of Curcurbitacins **1–6** on Actin Polymerization.

| Pretreatment | Dose (μM) | % Positive[a] | MCF Shift[b] |
|---|---|---|---|
| Experiment 1[c] | | | |
| Control ..... | — | 85.8 | 71.7 |
| Anti-CD18 MAb .. | 20 μg/ml | 85.6 | 76.0 |
| Cytochalasin B .... | 10 | 4.2 | −5.8 |
| | 1 | 45.5 | 30.9 |
| **1** ..... | 3.3 | 3.8 | −1.5 |
| | 1.0 | 24.7 | 3.8 |
| | 0.33 | 21.5 | 3.6 |
| **2** ..... | 3.3 | 9.6 | 2.6 |
| | 1.0 | 19.5 | 3.4 |
| | 0.33 | 29.8 | 7.0 |
| **6** ..... | 3.3 | 86.0 | 69.1 |
| | 1.0 | 82.8 | 57.7 |
| | 0.33 | 79.2 | 62.3 |
| Experiment 2 | | | |
| Control ..... | — | 76.7 | 39.3 |
| Anti-CD18 MAb .. | 20 μg/ml | 80.1 | 38.8 |
| Cytochalsin B..... | 10 | 16.4 | 3.8 |
| | 1 | 30.4 | 11.8 |
| **3** ..... | 3.3 | 20.5 | 12.5 |
| | 1.0 | 15.3 | 3.7 |
| | 0.33 | 60.5 | 27.7 |
| **4** ..... | 3.3 | 24.3 | 10.9 |
| | 1.0 | 64.6 | 31.8 |
| | 0.33 | 75.2 | 36.0 |
| **5** ..... | 3.3 | 77.7 | 38.0 |
| | 1.0 | 79.2 | 41.8 |
| | 0.33 | 86.2 | 62.8 |

[a]Percent of total cells that had a positive fluorescence signal over the background.

[b]Mean channel fluorescence for stimulated cells minus non-stimulated cells.

[c]Due to the size, two separate experiments were performed. Controls are shown for both assays. There is day-to-day variability in the magnitude of the MCF shift.

tion by the compounds paralleled adhesion inhibition, suggesting that the mechanism through which the cucurbitacins inhibit cell adhesion is by disrupting the cytoskeleton.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Fabms was performed on a Finnigan MAT TSQ 70 mass spectrometer. Nmr spectra were recorded on a Varian Gemini 300 spectrometer at 25° in $CDCl_3$. Photodiode-array-detected hplc separations were performed using a YMC ODS A-323 semi-prep. column on a Waters system. Tlc was carried out on Si gel 60F254 and RP-18 hptlc plates (Merck). Fluorescence quantitation was done on a Fluoroskan II microplate reader (Lab Systems Instruments, Raleigh, NC) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm, or on a Coulter Flow Cytometer (Coulter, Hialeah, FL) with an argon laser at an excitation wavelength of 488 nm and an emission wavelength of 530±10 nm. Color quantitation on an ELISA plate reader (Bio-Tek Instruments, Winooski, VT) was carried out at 570 nm with a reference wavelength of 630 nm. A Biomek 1000 Automated Laboratory Workstation (Beckman Instruments) was used for assay automation.

PLANT MATERIAL.—The stem and leaves of *Conobea scoparioides* were collected in Peru and taxonomically identified by Professor Sidney McDaniel. Voucher specimens are deposited in the herbarium of Mississippi State University.

ISOLATION AND IDENTIFICATION OF **1**.—The $CH_2Cl_2$ extract (700 mg) of the dried stem and leaves (200 g) was first fractionated on a Sephadex LH-20 gel permeation column eluting with a 1:1 mixture of MeOH and $CH_2Cl_2$. Fractions were pooled based on normal- and reversed-phase tlc. The cell-adhesion activity was concentrated in a single pooled fraction ($IC_{50}$ of 10 μg/ml). Reversed-phase flash cc of this fraction with a MeOH/$H_2O$ gradient gave six further fractions, one of which showed a tenfold increase in potency. Further purification using reversed-phase hplc gave 2 mg of a pure compound with an $IC_{50}$ value of 180±30 nM, which was identified as cucurbitacin E [**1**], by direct comparison with a reference sample.

CELL ADHESION ASSAY.—JY cells, an Epstein-Barr virus transformed B cell line which expresses LFA-1, were stained with a fluorescent, lipophilic membrane dye (PKH26, Zynaxis Cell Sciences). Approximately $2\times10^5$ stained JY cells and potential inhibitors were added to the wells of 96-well microtiter plates containing confluent monolayers of HeLa cells, a carcinoma line that expresses ICAM-1. Phorbol myristate acetate (PMA) 50 ng/ml, was then added to activate JY cells. The cultures were incubated for 45 min at 37°. Non-adherent JY cells were washed off, the remaining cells were solubilized, and fluorescence quantitated. Anti-CD18 monoclonal antibody was used as a positive control. Percent inhibition was calculated. The assay was automated for high-volume screening.

In some experiments, the wells were coated with immunoaffinity enriched ICAM-1 fractions from detergent solubilized Jijoye cells instead of live HeLa cells.

CYTOTOXICITY ASSAYS.—Tritiated thymidine uptake/proliferation assays were performed with PKH26 stained JY cells. The cells were incubated at 37° with the test compounds for either 5 or 24 h, and pulsed with tritiated thymidine during the last 5 h. In some assays, the cells were treated with test compounds for 45 min (the length of the adhesion assay), washed extensively, then incubated for 24 h and pulsed as described. Thymidine uptake was quantitated by scintillation counting.

The MTT assay was performed with stained JY cells. The cells were incubated at 37° with test compounds for 48 h. The tetrazolium dye, MTT, which is cleaved metabolically by active mitochondria, was added during the last 4 h. The cells and the crystals formed by cleavage of the dye were solubilized, and the color reaction quantitated on an ELISA plate reader. The 45-min long washout experiments described above were also performed.

ACTIN POLYMERIZATION ASSAY.—Human peripheral blood neutrophils were isolated according to the method of Eggleton *et al.* (16) and incubated with the test compounds for 45 min at 37° in assay medium (PBS with $Ca^{2+}/Mg^{2+}$, 10% fetal calf serum). The tripeptide, *N*-formyl-methionine-leucine-phenylalanine (fMLP, Sigma), which is chemotactic for, and activates neutrophils, was added at a final concentration of $1\times10^{-8}$ M. After a 1-min incubation at 37°, the cells were fixed and permeabilized by incubating them for at least 30 min at room temperature in a 3% paraformaldehyde solution in PBS with $Ca^{2+}/Mg^{2+}$, pH 7.6, containing lysophosphatidylcholine (12.5 μg/ml). The cells were washed in PBS, then stained with NBD-phallacidin (Molecular Probes, Eugene, OR; concentration per manufacturer's instructions), which binds to polymerized actin (F-actin), but not to nonpolymerized actin (G-actin). The cells were incubated with the dye for 15 min at 37° then washed three times with PBS and fluorescence quantitated.

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