Costunolide and Dehydrocostus Lactone as Inhibitors of Killing Function of Cytotoxic T Lymphocytes

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Costunolide and dehydrocostus lactone were isolated from an extract of mokko (Saussurea lappa Clarke) as inhibitors of killing activity of cytotoxic T lymphocytes (CTL). Mokko lactone was also isolated as an inactive compound from the extract. The structure–activity relationship indicated that 3-methylene-γ-butyrolactone is required for the inhibitory effect. Costunolide markedly inhibited the granule exocytosis and the production of inositol phosphates in response to anti-CD3 monoclonal antibody (mAb) stimulation at a concentration that did not affect the binding of anti-CD3 mAb. Tyrosine phosphorylation induced by crosslinking of CD3 molecules was significantly inhibited by costunolide in a dose-dependent manner. These results suggest that costunolide inhibits the killing activity of CTL through preventing the increase in tyrosine phosphorylation in response to the crosslinking of T-cell receptors.

Cytotoxic T lymphocytes (CTL) are important in the elimination of virus-infected cells and tumors and in graft rejection. Although recent reports have clearly shown that CTL have perforin-based and Fas-based cytotoxic pathways to destroy these target cells, the molecular mechanisms of the cascade from the recognition of the target cells to the expression of effector functions such as granule exocytosis are not known. Because low molecular weight probes are useful tools for studying complex biochemical pathways, we have been looking for specific inhibitors of CTL-mediated cytotoxicity. During the course of our study, we found that a herbal medicine, mokko (Saussurea lappa Clarke), strongly inhibited the killing activity of a CTL clone. Here we report the isolation of costunolide, dehydrocostus lactone, and mokko lactone from the extract of mokko and the characterization of the mechanism of action of these compounds on CTL-mediated cytotoxicity.

Materials and Methods

Cell cultures. RPMI 1640 medium (GIBCO laboratories, Grand Island, NY) with 10% (v/v) fetal calf serum (FCS, Bioserum, Victoria, Australia), 50 μg/ml 2-mercaptoethanol (2-ME), 50 μg/ml kanamycin, and 8 μg/ml tylosin tartrate was used for cell cultures otherwise specified.

Cells. An H-2a-specific CD8+ CTL clone, OE4, was maintained in RPMI 1640 medium with 10% (v/v) FCS and 5% (v/v) rat-conditioned medium (RCM); culture supernatant of rat splenocytes stimulated with 5 μg/ml of concanavalin A for 24 h. OE4 was stimulated with mitomycin C-treated splenocytes from BALB/c mice (female, 6 to 8 wk old, Charles River Company Ltd., Yokohama, Japan) every two weeks. P815 mastocytoma (H-2b) was maintained in RPMI 1640 medium plus 10% (v/v) FCS.

Reagents. 145-2Ci¹¹¹ (hamster anti-CD3 monoclonal antibody; mAb) was prepared as culture supernatants of hybridoma cells, collected by ammonium sulfate precipitation, and dialyzed four times against phosphate-buffered saline (PBS). 4G10 (mouse anti-phosphotyrosine mAb) was purchased from Upstate Biotec Technology Inc. (Lake Placid, NY). Herbinycin A was a commercial product of Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Assay for cytotoxic activity. P815 was labeled with 1850kBq of ¹³¹I-sodium chromate (Amersham International, Buckinghamshire, UK) in 100 μl of 50% (v/v) FCS for 1 h and washed three times with the medium. Otherwise, P815 was incubated in the presence of 37 kBq/ml of ¹³¹I-thymidine (Tdr; ICR Biomedical Inc., Costa Mesa, CA) for 18 h and washed three times with the medium. OE4 (2 × 10⁴ cells/well in 100 μl) was incubated with inhibitors for 2 h in U-bottom microtiter plates and then mixed with 100 μl of P815 (1 × 10⁵ cells/ml). The plates were centrifuged (300 × g) for 3 min and incubated for 4 h. To assess ¹³¹I release, 100 μl of supernatant was removed and measured for radioactivity. To assess ¹³¹I/Tdr release, 10 μl of 2% (v/v) Triton X-100 was added to each well, and the cells were solubilized by pectolyzing and then centrifuged (600 × g) for 5 min. One hundred μl of supernatant was removed and measured for radioactivity. The experiments were done as triplicate cultures. The percentage of specific lysis was calculated by using the following formula: % of specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Assay for granule exocytosis. OE4 (1 × 10⁵ cells/well) in anti-CD3 mAb-coated microtiter plates was centrifuged (300 × g) for 3 min and incubated for 4 h. At the end of the culture, 5 μl of the culture supernatant was removed and incubated in 200 μl of 200 μM N-α-benzoylxy-carbonyl-L-lysine thiobenzoic acid (BLT) and 220 μM L-2-dithio-bis (L-nitrobenzoic acid) (DTNB) in PBS at 37°C for 15 min. The reaction was stopped by addition of PMSF at the final concentration of 1 mM and A₄₁₅ was measured.

Assay for binding of anti-CD3 mAb. OE4 (1 × 10⁵ cells/well) in anti-CD3 mAb-coated plates was centrifuged (300 × g) for 3 min and incubated for 20 min. After the plates were washed with PBS, the cells were fixed and stained with 0.2% crystal violet in methanol for 10 min. After the plates were washed thoroughly with H₂O and dried, the dye was extracted with 50% methanol and A₅₉₅ was measured.

Assay for production of inositol phosphates. OE4 (2 × 10⁴ cells/ml) was labeled for 16 to 24 h with 370 kBq/ml of myo-¹³¹Iinositol (Amersham)
in inositol-free RPMI 1640 medium with 10% (v/v) FCS and 5% (v/v) RCM. After it was washed, [3H]inositol-labeled OE4 was incubated in inositol-free RPMI 1640 plus 10mM LiCl for 10 min, and then cultured in anti-CD3 mAb-coated plates for 20 min. Cells were extracted with 1 ml of chloroform–methanol (1:2, v/v) and 0.1 ml of 0.22 M HCl. The extract was mixed with H2O (0.25 ml) plus chloroform (0.25 ml) and centrifuged (3000 g) for 5 min. The aqueous phase was removed and neutralized to pH 7, diluted to 5 ml with H2O, and put on Dowex 1 x-8 columns (bed volume, 1 ml) in the formate form. The columns were washed with 10 ml of H2O followed by 10 ml of 60mM sodium formate plus 5mM sodium tetraborate. [3H]inositol phosphates were then eluted with 5 ml of 1M ammonium formate plus 0.1M formic acid, and measured for radioactivity.

Immunoblotting of phosphotyrosine. OE4 (2 x 10^6 cells) was incubated with anti-CD3 mAb for 10 min on ice. Cells were washed to remove unbound anti-CD3 mAb and then incubated in the RPMI 1640 medium containing anti-hamster IgG mAb for 2 min at 37°C. The reaction was immediately stopped by addition of the lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 250 μM PMSF, 20 μg/ml leupeptin, 1 mM DTT, 1 mM sodium orthovanadate, and 10% Triton X-100). Nuclei were removed by centrifugation (10,000 x g, 15 min), and proteins in the supernatants were separated in 7.5% SDS-polyacrylamide gel electrophoresis under the reducing condition. The proteins were transferred to a nitrocellulose membrane (Hybond-C extra, Amersham). The blot was treated with 5% bovine serum albumin (BSA) and 0.1% Tween 20 in Tris-buffered saline (TBS, 20 mM Tris HCl (pH 7.6), 137 mM NaCl) and then incubated for 2 h with 4G10 in TBS containing 1% BSA and 0.1% Tween 20. After washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG mAb in TBS containing 2% BSA and 0.1% Tween 20. After washing, the protein bands were made visible by using ECL Western blotting detection reagent (Amersham).

Properties of compounds 1, 2, and 3. Costunolide (1): mp 106 C, [α]D25 = 128° (c 0.45, CHCl3); δ2 (400 MHz, CDCl3) 1.42 (3H, br s), 1.68 (1H, m), 1.71 (3H, d, J = 1.5 Hz), 2.03 (1H, dd, J = 4.9, 10.5 Hz), 2.10 (1H, m), 2.15 (1H, br d, J = ca. 11 Hz), 2.18 (1H, br r), 2.25 (1H, dd, J = 4.3, 11.8 Hz), 2.32 (1H, m), 2.45 (1H, dd, J = 5.4, 12.7 Hz), 2.57 (1H, m), 4.57 (1H, dd, J = 9.0, 10.0 Hz), 4.74 (1H, br d), J = ca. 10 Hz), 4.85 (1H, br d, J = 11.5 Hz), 5.33 (1H, d, J = 3.3 Hz), 6.27 (1H, d, J = 3.3 Hz); δ2 (67.5 MHz, CDCl3) 16.1 (q), 17.4 (q), 26.2 (t), 28.1 (t), 39.5 (t), 41.0 (t), 50.4 (d), 81.9 (d), 119.7 (t), 127.1 (d), 127.3 (d), 137.0 (s), 140.1 (s), 141.5 (s), 170.5 (s).

Dehydrocostus lactone (2): mp 61 C, [α]D25 = −9.0° (c 0.42, CHCl3); δ2 (200 MHz, CDCl3) 3.97 (1H, dd, J = 9.0, 9.0 Hz), 4.82 (1H, s), 4.90 (1H, s), 5.07 (1H, m), 5.28 (1H, m), 5.49 (1H, d, J = 31.2 Hz), 6.22 (1H, d, J = 3.6 Hz); δ2 (22.5 MHz, CDCl3) 30.2 (t), 30.9 (t), 32.5 (t), 36.2 (t), 45.0 (d), 47.5 (d), 51.9 (d), 83.3 (d), 109.4 (d), 112.4 (t), 119.9 (t), 139.5 (t), 142.9 (d), 150.9 (s), 170.0 (s). Mokko lactone (3): mp 35 C; [α]D25 = +8.1° (c 0.16, CHCl3); δ2 (200 MHz, CDCl3) 1.24 (3H, d, J = 6.8 Hz), 3.92 (1H, dd, J = 9.0, 9.0 Hz), 4.78 (1H, s), 4.88 (1H, s), 5.05 (1H, m), 5.20 (1H, m).

Results and Discussion

Isolation of costunolide, dehydrocostus lactone, and mokko lactone from the extract of mokko

About one hundred extracts of herbal medicines were screened for inhibitors of cytotoxic activity of CD8+ CTL clone OE4. Among these, only the extract of mokko showed a strong inhibitory effect. The active principles as guided by biological activity were purified as follows: active materials were extracted with ethyl acetate from roots of Saussurea lappa Clarke (100 g) and the extract was concentrated in vacuo. The residue, then extracted, was put on a silica gel column and eluted successively with n-hexane, toluene, ethyl acetate, acetone, and methanol. The toluene-ethyl acetate fraction (1:1) was concentrated in vacuo and then fractionated by using a silica gel column eluted with n-hexane–ethyl acetate (1:0, 3:1, 1:1, 1:3, and 0:1). A fraction eluted with n-hexane–ethyl acetate (3:1) was concentrated in vacuo, put on an HPLC column (Capcell Pak C18; 250 x 20 mm i.d.; flow rate 12 ml/min) and eluted with 60% methanol. Two active fractions were evaporated to dryness to yield a white powder 1 (tR 26.3 min; 157.3 mg) and an oily material (tR 30.8 min; 91.0 mg). A part of the oily material (15 mg) was purified by HPLC (10 μm silica gel: 250 x 4 mm i.d.; flow rate 3.1 ml/min) with a solvent system of n-hexane–ethyl acetate (95:5) and 9.17 mg of 2 (tR 10.8 min) and 1.69 mg of 3 (tR 9.7 min) were obtained. From the spectra of 1H and/or 13C NMR and optical rotation, the compounds 1, 2, and 3 were identified with costunolide, dehydrocostus lactone, and mokko lactone, respectively (Fig. 1). So far, costunolide(9,10) and related compounds have been found to have antitumor effects(11,12) antitumor effects,13 bio-mutagenic effects,14 inhibitory effects on the movement of Antisakisis,15 and allergic activity.16,17 However, it has not been reported that these compounds inhibit the cytotoxic activity of CTL. Therefore, their inhibitory mechanism on CTL-mediated cytotoxicity was further studied.

Requirement of α-methylene-γ-butyrolactone for the inhibitory effect of CTL-mediated killing

The effects of costunolide, dehydrocostus lactone, and mokko lactone were examined on the cytotoxic activity of OE4 (Fig. 2). Costunolide and dehydrocostus lactone inhibited the activities of cytosis (Fig. 2A) and DNA degradation (Fig. 2B) at ID50 from 3.6 μM to 10 μM, but mokko lactone showed no effect on the cytotoxic activity of OE4 (Fig. 2C). Therefore, the results demonstrated that α-methylene-γ-butyrolactone is the active principle in mokko that inhibits the cytotoxic activity of CTL.
lactone did not affect the activities up to 43 μM. Thus, \( \alpha \)-methylene-\( \gamma \)-butyrolactone seems to be required for the inhibitory effect on the cytotoxic activity of OE4. \( \alpha \)-Methylene-\( \gamma \)-butyrolactones are known to be alkylating agents and undergo a Michael reaction with biological nucleophiles such as t-cysteine or SH-containing enzymes.\(^{19}\)

Thus, we tested whether the inhibitory effect of costunolide can be neutralized or enhanced by addition or depletion of SH-containing components in the medium such as glutathione and 2-ME (Fig. 3). High concentration of glutathione completely abrogated the inhibitory effect of costunolide. Therefore, costunolide seems to inhibit CTL-mediated killing by its binding to SH-containing proteins.

Characterization of targets of costunolide on CTL-mediated cytotoxicity

To find whether costunolide affects OE4 or P815, both cells were treated with or without costunolide for 2 h and then incubated in the presence or absence of costunolide during a 4-h killing assay. Specific \(^{3}H\)TdR release was strongly inhibited only when OE4 but not P815 was treated with costunolide (Fig. 4). Therefore, the direct target of costunolide is thought to be the effector cells rather than the target cells.

Lytic granules are released from OE4 upon stimulation such as crosslinking of T-cell receptors. To examine the effects of costunolide on granule exocytosis, the release of BLT esterase activity into the culture medium in response to anti-CD3 stimulation was measured (Fig. 5). Costunolide inhibited the granule exocytosis in a dose-dependent manner, completely at 13.6 μM. However, under these conditions costunolide did not prevent the binding of anti-CD3 mAb to OE4 (Fig. 6). At higher concentrations (e.g., 43 μM) costunolide may inhibit the expression of CD3 molecules on OE4.

Stimulation of T-cell receptors induces rapid tyrosine phosphorylation followed by the activation of phospholipase C (PLC). PLC hydrolyzes PIP\(_2\) yielding the second messengers IP\(_3\) and diacylglycerol, which induce an increase in cytoplasmic free calcium and activation of protein kinase C (PKC), respectively. To examine the effects of costunolide on the activation of PLC, the amount of inositol phosphates was measured after anti-CD3 stimulation (Fig. 7). Costunolide strongly inhibited the production of inositol phosphates at an ID\(_{50}\) of 2.8 μM. Finally, the effects of costunolide on tyrosine phosphorylation were examined (Fig. 8). Crosslinking of CD3 molecules immediately induced an increase in tyrosine phosphorylation of proteins.

![Fig. 3. Abrogation of the Inhibitory Effect of Costunolide by Glutathione.](image)

OE4 was incubated in conventional RPMI 1640 medium (filled circles), 2-ME-depleted RPMI 1640 medium (open squares), and RPMI 1640 medium containing 32.5 μM glutathione (filled triangles) throughout the experiments. OE4 was incubated with costunolide for 2 h and then incubated with \(^{51}Cr\) sodium chromate-labeled P815 for 4 h. Spontaneous release was less than 25%. Results shown are one of two separate experiments.

![Fig. 4. Effects of Treatment with Costunolide on the Cytotoxic Activity of OE4.](image)

P815 which was labeled with \(^{3}H\)TdR and/or OE4 were incubated in the presence (+) or absence (−) of 13.6 μM costunolide for 2 h. Both kinds of cells were washed to remove costunolide, then mixed and cultured with (+) or without (−) 6.8 μM costunolide for 4 h. Spontaneous release was 3.1%. Results shown are one of two separate experiments.

![Fig. 5. Effects of Costunolide on the Granule Exocytosis of OE4.](image)

OE4 was incubated with costunolide for 2 h and then cultured in anti-CD3 mAb-coated microtiter plates for 4 h in the presence or absence of costunolide. The means ± SD values of \(A_{450}\) in triplicate cultures are shown. Maximum release and spontaneous release were 2.50 ± 0.10 and 0.46 ± 0.004, respectively. Specific BLT esterase release (3%) was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Results shown are one of two separate experiments.

![Fig. 6. Effects of Costunolide on the Binding of Anti-CD3 mAb to OE4.](image)

OE4 was treated with or without (−) costunolide for 2 h, and then incubated in anti-CD3-coated plates (+) or uncoated plates (−) for 20 min. Cells attached to the plates were fixed and stained with 0.2% crystal violet in methanol for 10 min. The dye was extracted with methanol and \(A_{412}\) was measured. The experiments were done as duplicate cultures. Results shown are one of separate experiments.
that migrated near 45 and 95 kDa, as judged by immunoblotting using anti-phosphotyrosine mAb. Costunolide but not mokkalo lactone markedly inhibited the increase in the tyrosine phosphorylation in a dose-dependent manner, which suggests that costunolide inhibits tyrosine kinases. However, it is not obvious why 44 kDa protein remained tyrosine-phosphorylated in the presence of 43 μM costunolide (lane 3), because under this concentration the binding of anti-CD3 mAb to OE4 was inhibited (Fig. 6). An inhibitor of tyrosine kinase, herbimycin A also inhibited the increase in tyrosine phosphorylation (lane 8). Herbimycin A is thought to inhibit the kinase activity by direct binding to the two cysteine residues in the kinase domain and thereby inhibiting access to ATP. 20 In T-cells, herbimycin A is reported to inactivate tyrosine kinases including p59Fyn and p56^lck. 21 Thus, similarly to herbimycin A, costunolide seems to bind to cysteine residues of tyrosine kinases and inhibit the kinase activity in T-cells.

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References