EI-1511-3, -5 and EI-1625-2, Novel Interleukin-1β Converting Enzyme Inhibitors Produced by *Streptomyces* sp. E-1511 and E-1625

III. Biochemical Properties of EI-1511-3, -5 and EI-1625-2

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EI-1511-3, -5 and EI-1625-2, novel interleukin-1β converting enzyme (ICE) inhibitors from the culture broths of *Streptomyces* sp. selectively inhibited the recombinant human ICE activity with IC_{50} values of 0.09, 0.38 and 0.2 μM, respectively, without inhibiting elastase and cathepsin B. Manumycin G, *ent*-alisamycin, U-56,407, and manumycin A and B isolated simultaneously from the same strains also inhibited ICE. EI-1511-3, -5 and EI-1625-2 also inhibited mature interleukin-1β secretion from THP-1 cells with IC_{50} values of 5.4, 3.6 and 2.2 μM, respectively. In this article, biological properties of EI-1511-3, -5 and EI-1625-2 and, in addition, properties of manumycin-related compound are described.

Interleukin-1 (IL-1), which is primarily secreted by activated monocytes or macrophages, has been implicated in the pathogenesis of acute and chronic inflammation[1]. Interleukin-1β converting enzyme (ICE) which cleaves the inactive precursor of IL-1β into the biologically active IL-1β is involved in the secretory mechanism of IL-1β, one of two forms of IL-1 (α and β)[2,3], and has been purified and cloned[4,5]. The structure of ICE has been shown by crystal analysis and found to be a homodimer of $(p10/p20)_2$[6,7]. Participation of ICE in inflammation has been suggested by experiment using cowpox virus, which produces ICE inhibitory protein, crmA[8]. Thus ICE inhibitors might be useful as anti-inflammatory agents[9].

As described in an accompanying paper, we isolated novel ICE inhibitory compounds, EI-1511-3, -5 and EI-1625-2, from culture broths of *Streptomyces* sp. strain E-1511 and E-1625. *ent*-Alisamycin[10], U-56,407[11], and manumycin A, B[12] and G[13] were also purified from these strains. In this article, we describe the biological properties of EI-1511-3, -5, EI-1625-2 and other manumycin-related compounds. The taxonomy, fermentation of the producing strains, the isolation and studies on structural determination are described in previous papers.

Materials and Methods

Materials

Recombinant human ICE was prepared as described in an accompanying paper. Intact Human ICE was extracted from cultured THP-1 (ATCC TIB 202) cells and purified by ion exchange chromatography as described[4]. All other chemicals were of analytical grade. Synthetic method of derivatives was described in accompanying paper.

Assay of ICE Activity

ICE activities were measured as described in an accompanying paper.

Assay of Cathepsin B and Elastase Activities

The enzymatic activities of cathepsin B and elastase were assayed according to the methods of BARRETT & KIRSCHKE[14] and MUMFORD *et al.*[15], respectively.

Measurement of Interleukin-1β Secretion

THP-1 cells were suspended in RPMI1640 medium supplemented with 10% fetal bovine serum and were distributed into 24-well plates as inocula of 1×10^5 cells/well. The cells were differentiated with phorbol 12-myristate 13-acetate (PMA: 30 nM) for 72 hours in a humidified atmosphere of 5% CO_2 in air at 37°C. After the cells were rinsed with serum-free RPMI1640 medium to remove unadherent cells, adherent cells were stimulated for 4 hours with lipopolysaccharide (LPS: 25 μg/ml) containing various concentrations of each test

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compound. The culture media were harvested and mature IL-1β was measured by an ELISA method using IL-1β assay kit (Amersham).

Detection of Cell Survival

The cytotoxicities of the test compound against THP-1 cells were examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method[16]. MTT solution (10 μl/well; final concentration 1 mg/ml) was added at the time of EI-1511s or EI-1625-2 application. The culture medium was removed and dimethyl sulfoxide (50 μl/well) was added to dissolve formazan. The absorbance of soluble formazan was then measured.

Synthesis of Derivatives

The synthesis of derivatives of manumycin-related compounds were described in an accompanying paper.

Results

Inhibition of ICE

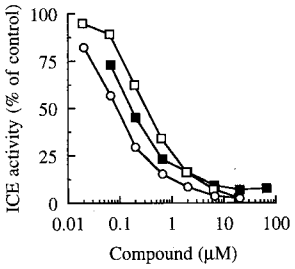
EI-1511-3, -5 and EI-1625-2 inhibited the enzymatic activity of recombinant human ICE in a dose-dependent manner (Fig. 1); IC_{50} values were calculated to be 0.09, 0.38 and 0.2 μM, respectively. EI-1511-3 and -5 also inhibited the enzymatic activity of ICE extracted from cultured THP-1 cells with similar potencies. The specificity of EI-1511-3, -5 and EI-1625-2 was examined by testing them against cathepsin B (another thiol-containing protease) and elastase. EI-1511-3, -5 and EI-1625-2 were inactive against these two enzymes at concentrations up to 20 μM. These data indicate the specificity of EI-1511-3, -5 and EI-1625-2 against ICE and were summarized together with EI-1507s which were isolated in our laboratory in Table 1.

Effects of DTT and Heat-inactivated Fetal Calf Serum (FCS) on ICE Inhibitory Activity

The addition of DTT to the reaction mixture dose-dependently shifted inhibition curve of EI-1511-3 to right (Fig. 2A). In the presence of 2 mM DTT, the potency of EI-1511-3 was weakened to one hundredth. EI-1511-5, EI-1625-2, and other manumycin-related compounds were also less-potent in the presence of DTT (Data not shown). On the other hand, addition of heat-inactivated FCS did not influence to the potency of EI-1511-3 at concentrations up to 10% (Fig. 2B). The inhibitory activities of EI-1511-5, EI-1625-2, U-56,407, manumycin G and *ent*-alisamycin also were not attenuated by heat-

Fig. 1. Inhibition of ICE by the EI-1511-3, -5 and EI-1625-2.

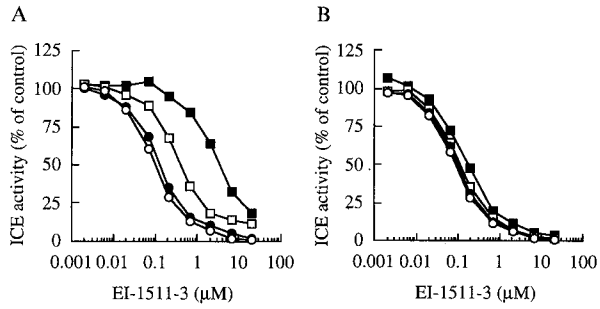
Symbols indicate EI-1511-3 (○), EI-1511-5 (□) and EI-1625-2 (■).



The assay mixture was incubated for 2 hours at room temperature. Then the fluorescent intensity (excitation-wave length: 370 nm, emission-wave length: 440 nm) of the assay mixtures was measured.

Fig. 2. Effects of DTT (A) and heat-inactivated FCS (B) on ICE inhibitory activity of EI-1511-3.

Symbols indicate (A) control (○), presence of 0.02 mM (●), 0.2 mM (□) and 2 mM (■) DTT and (B) control (○), presence of 1% (●), 3.3% (□) and 10% (■) heat-inactivated FCS.



The assay mixture was incubated for 2 hours at room temperature in the presence of various concentrations of EI-1511-3 and DTT or heat-inactivated FCS. Then the fluorescent intensity of the assay mixtures was measured.

Table 1. Effects of ICE inhibitors on various enzyme activities.

Enzyme	IC_{50} value (μM)				
	EI-1511-3	EI-1511-5	EI-1625-2	EI-1507-1	EI-1507-2
Recombinant ICE	0.09	0.38	0.20	0.23	0.42
THP-1 ICE	0.16	0.17	NT	NT	NT
Elastase	>20	>20	>20	>28	>28
Cathepsin B	>20	>20	>20	>28	>28

NT: Not tested.

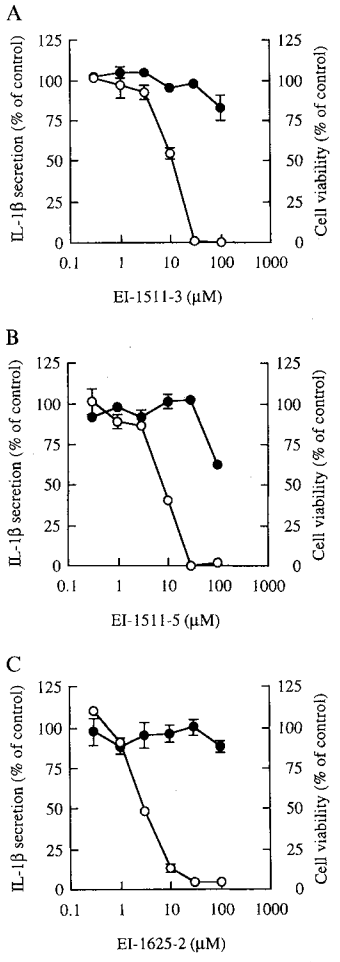
inactivated FCS (data not shown).

Biological Properties

In order to determine whether EI-1511-3, -5 and EI-1625-2 were efficacious even in intact cells, we investigated the effects of EI-1511-3, -5 and EI-1625-2 on the extracellular release of IL-1β from THP-1 cells. EI-1511-3, -5 and EI-1625-2 inhibited the IL-1β secretion in a dose-dependent manner (Fig. 3); IC_{50} values were calculated to be 11, 6.9 and 4.4 μM, respectively. On the other hand, 100 μM of EI-1511-3, -5 or EI-1625-2 did not significantly reduced cell survival (as shown in Fig. 3). Cell viabilities at a concentration of 33 μM, at which EI-1511-3, -5 and EI-1625-2 completely inhibited IL-1β secretion from THP-1 cells were more than 90%. These data indicate that EI-1511-3, -5 and EI-1625-2 inhibited mature IL-1β secretion from THP-1 cells without showing cell toxicity at concentrations lower than 33 μM.

Fig. 3. Effects of the EI-1511-3 (A), -5 (B) and EI-1625-2 (C) on IL-1β secretion from LPS-stimulated THP-1 cells and on cell viabilities of THP-1 cells.

Symbols indicate IL-1β secretion (○) and percentage of cell viability (●).



EI-1511-3, EI-1511-5 and EI-1625-2 were applied to the LPS-stimulated THP-1 cells.

EI-1511-3, -5 and EI-1625-2 showed weak antimicrobial activity against *Enterococcus faecium*, *Staphylococcus aureus* and *Bacillus subtilis*; MIC value were 40, 40 and 20 μM for EI-1511-3, 40, 40 and 20 μM for EI-1511-5 and 20, 40 and 10 μM, for EI-1625-2, respectively, as shown in Table 2.

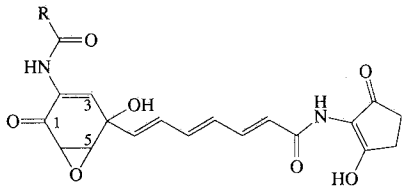
Inhibition of ICE and IL-1β Secretion by Manumycin-related Compound

Various manumycin-related compounds which have different acylamino side chain at C-2 of the cyclohexenone epoxide (Table 3) and oxidated derivatives (Table 4) were tested for their abilities to inhibit ICE, IL-1β secretion and cell viability. All of the compounds tested inhibited ICE in a dose-dependent manner with IC_{50} values of 0.07 to 11 μM. C-4 side chain-deleted derivatives showed inhibitory potencies to ICE with IC_{50} values similar to their original compounds, although potencies to IL-1β secretion were decreased from 11 μM to 91 μM in KT-8110, from 6.9 μM to 83 μM in KT-8112 and from 4.4 μM to more than 170 μM in KT-8108.

Table 2. The antibiotic activities of EI-1511-3, -5 and EI-1625-2.

Test microorganisms	MIC (μM)		
	EI-1511-3	EI-1511-5	EI-1625-2
Staphylococcus aureus subsp. *aureus* ATCC6538P	40	40	40
Enterococcus faecium ATCC10541	40	40	20
Bacillus subtilis No. 10707	20	20	10
Escherichia coli ATCC26	>40	>40	>40
Klebsiella pneumoniae supsp. *pneumoniae* ATCC10031	>40	>40	>40
Proteus vulgaris ATCC6897	>40	>40	>40
Shigella sonnei ATCC9290	>40	>40	>40
Salmonella typhosa ATCC9992	>40	>40	>40
Pseudomonas aeruginosa BMH No. 1	>40	>40	>40
Candida albicans ATCC10231	>40	>40	>40

Table 3. ICE and IL-1β secretion inhibitory activities of manumycin-related compounds.



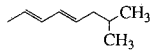
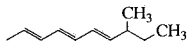
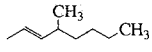
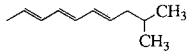
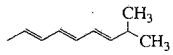
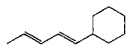
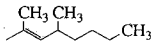
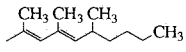
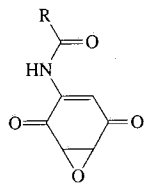
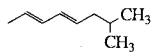
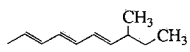
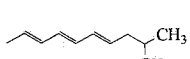
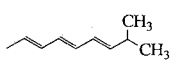
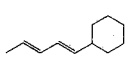
Compound	R	IC_{50} value (μM)		
		ICE	IL-1β secretion	Necrosis
EI-1511-3		0.09	11	>100
EI-1511-5		0.38	6.9	>100
EI-1625-2		0.20	4.4	>100
U-56,407		0.63	11	>100
Manumycin G		0.20	13	>100
ent-Alisamycin		0.38	3.5	>100
Manumycin B		0.65	4.1	>100
Manumycin A		11	6.7	100

Table 4. ICE and IL-1β secretion inhibitory activities of derivatives prepared by chromate oxidation.



Compound	R	IC_{50} value (μM)		
		ICE	IL-1β secretion	Necrosis
KT-8110		0.07	91	>180
KT-8112		1.0	83	>170
KT-8108		3.3	>170	>170
KT-8109		0.24	NT	NT
KT-8111		1.7	NT	NT

NT: Not tested.

Discussion

EI-1511-3, -5, EI-1625-2, *ent*-alisamycin, U-56,407 and manumycin A, B and G inhibited ICE in a dose-dependent manner. The ICE inhibitory properties of EI-1511s and EI-1625-2 were potent and selective as shown in Table 1.

EI-1511s failed to inhibite ICE in the buffer descibed in THORNBERRY *et al.*[4]. The evaluation of each components in the buffer revealed DTT dose-dependently inactivate EI-1511-3. HPLC analysis of EI-1511-3 preincubated with DTT suggested degradation. Similar reduction of potencies by DTT were also observed in other manumycin-related compounds so that inhibitory activities of these compounds were hardly observed under the reaction conditions containing 2 mM of DTT in reaction mixture. Although manumycin-related compounds are easily degraded in the presence of DTT, results that inhibitory potencies against IL-1β secretion were not decreased in the presence of heat-inactivated FCS at concentrations up to 10% for 2 hours suggested stability of these compounds during culture conditions of THP-1 cells and *in vivo*. Indeed, IL-1β secretion from LPS-stimulated THP-1 cells were inhibited by the manumycin-related compounds, and the inhibitions of IL-1β secretion were not due to toxic effects of the compounds as shown in Fig. 3. These results suggest that EI-1511s and EI-1625-2 would be effective even *in vivo*.

The structure-activity relationship of acylamino side chains at C-2 of the cyclohexenone epoxide against ICE was not clear, alternatively, preservation of inhibitory potencies to ICE and reduction of potencies to IL-1β secretion in C-4 side chain-deleted derivatives suggested the C-4 side chain does not contribute to the ICE inhibition, but, does contribute to cellular permeation of the compounds.

In this paper, we showed that EI-1511-3, -5 and EI-1625-2 inhibited ICE together with IL-1β secretion from LPS-stimulated THP-1 cells. Since the discovery of ICE, structural and functional homology of ICE to the gene ced-3 responsible for cell death of *Caenorhabditis elegans*[17], identification of ICE homologs, and participation of ICE and its homologs in apoptosis[18] of various types of cells have been examined. EI-1511s and EI-1625-2 would be useful for clarifying the true pathophysiological and physiological roles of ICE in inflammation and could be for apoptosis, though selectivity of EI-1511s and EI-1625-2 to ICE homologs have not been determined yet.

Acknowledgment

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