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Novel Non-peptide Inhibitors Targeting Death Receptor-Mediated Apoptosis

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Abstract—We have previously reported that ECH, (2*R*, 3*R*, 4*S*)-2,3-epoxy-4-hydroxy-5-hydroxymethyl-6-(1*E*)-propenyl-cyclohex-5-en-1-one inhibits Fas-mediated apoptosis by blocking self-activation of pro-caspase-8 in the death-inducing signaling complex (DISC). A series of ECH derivatives were asymmetrically synthesized via key synthetic intermediates obtained from lipase-catalyzed kinetic resolution. Inhibitory activities of the derivatives towards death receptor-mediated apoptosis both in type I and type II cells were investigated, revealing that novel non-peptide inhibitors, RKTS-33 and RKTS-34, are effective as ECH.

Introduction

Apoptosis, or programmed cell death, plays a vital role in the development and homeostasis of multicellular organisms.[1–3] Depending on death stimuli, one of two signaling pathways is activated: a death receptor-dependent pathway and a death receptor-independent pathway via the mitochondria.[2,3] In both cases, stimulation of the death pathway leads to processing and activation of initiator caspases, that is pro-caspase-8 or -9, which subsequently transmit the signal to downstream effector caspases, for example caspase-3, -6, or-7.[4,5]

Fas antigen (CD95/Apo-1) is a cell surface death receptor molecule, a member of the tumor necrosis factor (TNF), introducing apoptosis-inducing signals into Fas-bearing cells by stimulation with Fas ligand (FasL) or agonistic anti-Fas monoclonal antibodies.[2,3] Following stimulation by FasL, the cytosolic tail of the Fas receptor associates with the death domain (DD) of the adaptor molecule FADD,[6] which then recruits caspase-8 via a homophilic death effector domain (DED) interaction. The resulting assemblage of proteins is known as the death-inducing signaling complex (DISC), formation of which is a trigger for downstream activation.[7,8] In Fas-mediated apoptosis, two types of cells are proposed to transmit distinct death signals.[9,10] In type I cells, such as human Burkitt's lymphoma SKW6.4 cells, sufficient activation of pro-caspase-8 is initiated at the DISC followed by direct activation of pro-caspase-3. However, in type II cells, such as human T lymphoma Jurkat cells, pro-caspase-8 is inadequate for direct activation of pro-caspase-3, and cleaves the Bcl-2 family member Bid instead, activating the mitochondrial pathway with the release of cytochrome *c*, which leads to the activation of caspase-9/-3 cascade.

Since some pathological conditions such as rheumatoid arthritis, liver inflammation, autoimmune diseases, and neurodegenerative disorders are characterized by excess receptor-mediated apoptosis, we screened anti-apoptosis agents among microbial metabolites, and reported identification of an inhibitor of Fas-mediated apoptosis, ECH [(2*R*, 3*R*, 4*S*)-2,3-epoxy-4-hydroxy-5-hydroxymethyl-6-(1*E*)-propenyl-cyclohex-5-en-1-one], which is produced by a fungus.[11–14] ECH blocks the self-activation of pro-caspase-8 in the DISC and selectively inhibits death receptor-mediated apoptosis.[14] Herein, we

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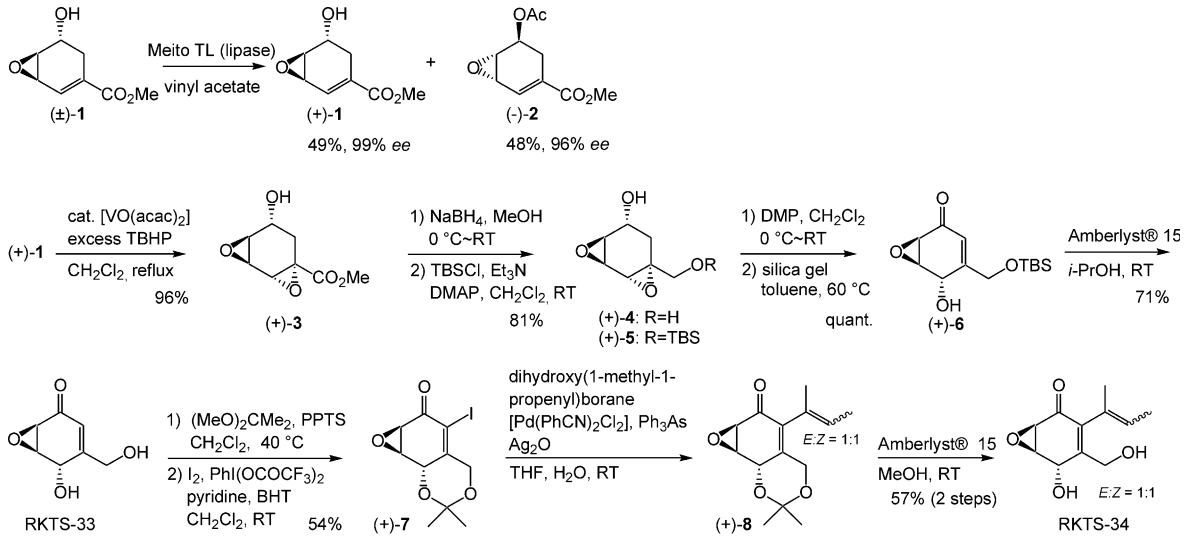

Scheme 1. Synthesis of RKTS-33 and RKTS-34.

describe design, synthesis, and structure–activity relationships (SAR) of ECH-related molecules, shown in Figure 1, in receptor-mediated apoptosis.

Design and Synthesis of ECH-related Molecules

In order to prepare several C-6 side-chain derivatives of ECH efficiently, we designed 6-iodocyclohexenone derivative (+)-**7** as a key synthetic intermediate, because the Suzuki coupling reaction of (+)-**7** with several alkenylboranes would afford the corresponding side-chain analogues (Scheme 1).

The synthesis starts from the Diels–Alder reaction between furan and an acrylate derivative. Though we have already reported the $HfCl_4$-mediated highly diastereoselective Diels–Alder reaction of furan and chiral acrylate with Corey's auxiliary,[15,16] a more practical method for the synthesis of the key intermediate has been developed as follows. The Diels–Alder reaction of furan and acryloylchloride proceeded smoothly without a Lewis acid catalyst, affording the addition product. Hydrolysis, followed by the iodolactonization reaction, provided iodolactone, (±)-2-iodo-4,8-dioxa-tricyclo [4.2.1.$0^{3,7}$]nonan-5-one, in large quantity without column chromatography or distillation. After conversion of the iodolactone to cyclohexenol (±)-**1**, the kinetic resolution by *Pseudomonas stutzeri* lipase (Meito TL) proceeded with high efficiency, affording (+)-**1** and acetate (−)-**2** in 49% with 99% ee and in 48% with 96% ee, respectively.[17] Hydroxy group-directed Sharpless epoxidation[18] of (+)-**1** proceeded diastereoselectively to provide (+)-**3**. The reduction of ester (+)-**3** with $NaBH_4$ and protection of the generating primary alcohol with *tert*-butyldimethylsilylchloride (TBSCl) afforded silyl ether (+)-**5** in good yield. Oxidation of (+)-**5** with Dess–Martin periodinane (DMP),[19] followed by isomerization with silica gel, produced (+)-**6**. Deprotection of the alcohol gave RKTS-33 in 71% yield.[20] Protection of the diol RKTS-33 with acetonide and iodonation afforded the key intermediate (+)-**7**. As the key intermediate (+)-**7** was in hand, the next task was the introduction of the side chain. The Suzuki coupling reaction with dihydroxy(1-methyl-1-propenyl)borane was examined in detail and it was found that coupling reaction proceeded smoothly under Johnson's conditions,[21] affording (+)-**8**. Final deprotection under acidic conditions gave RKTS-34 in 57% yield over two steps.[22]

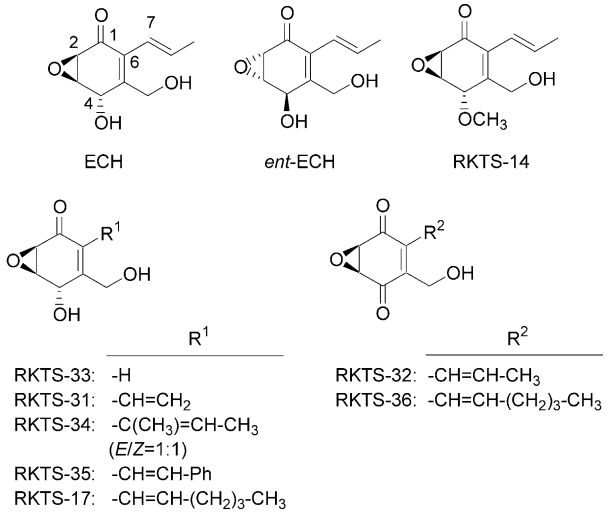

Figure 1. Structures of ECH and ECH-related compounds. The geometry of the double bond at the C-7 position is an *E* configuration.

RKTS-31, RKTS-35, and RKTS-17 were also synthesized from the key intermediate (+)-**7** by the same Suzuki coupling reaction with dihydroxyvinylborane, dihydroxystyrylborane, and dihydroxy(1-heptenyl)borane, respectively, followed by deprotection of the silyl group. RKTS-14 was prepared from ECH in three steps: The protection of the primary alcohol with TBSCl, protection of the secondary alcohol with methyl ether (MeI, Ag_2O),[23] and deprotection of the silyl ether with tetrabutylammonium fluoride (TBAF) afforded RKTS-14 in 54% yield. The enantiomer of ECH, *ent*-ECH, was prepared from (−)-**1**, which was synthesized

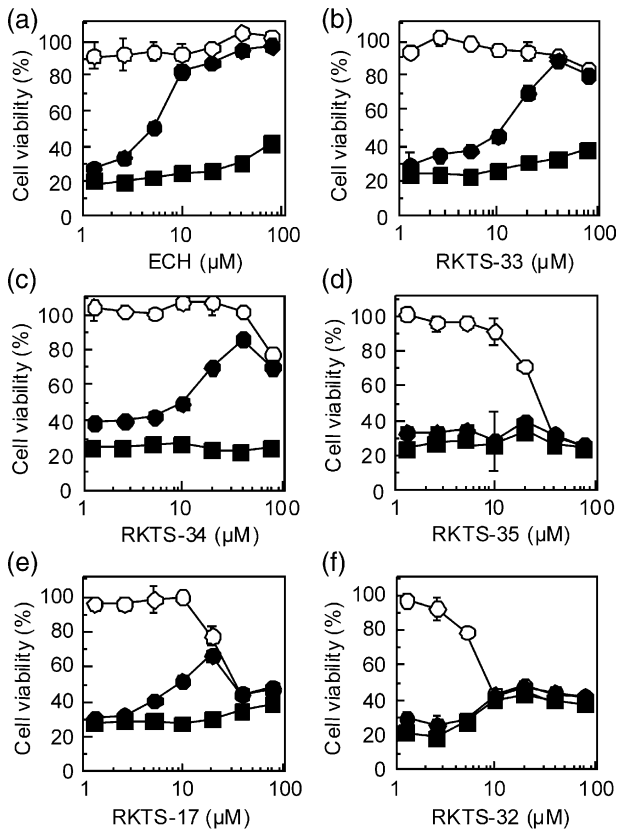

Figure 2. Effect of novel receptor-mediated apoptosis inhibitors on FasL-induced apoptosis in SKW6.4 cells. ○: none, ●: FasL, ■: staurosporine.

by the hydrolysis of ester (−)-**2** in good yield.[17] Epoxyquinone derivatives RKTS-32 and RKTS-36 were synthesized in three steps from ECH and RKTS-17, respectively: Protection of the primary alcohol with TBSCl, followed by oxidation with DMP and deprotection of the silyl ether, gave epoxyquinone derivatives.

Structure–Activity Relationships (SAR) in Blocking FasL-induced Apoptosis

In both type I and type II cells, once pro-caspase-3 is efficiently activated, it cleaves a variety of cellular substrates including DNA repair enzymes, structural proteins, and endonucleases to produce the changes associated with apoptosis. We therefore investigated the biological activities of the above synthetic compounds both in SKW6.4 (type I) and Jurkat (type II) cells.

Apoptosis-inhibitory activities of test compounds were assessed by the measurement of cell viability.[24] Cross-linked FasL and staurosporine (a broad-specificity protein kinase inhibitor) are able to induce apoptosis in both types of cells primarily via the Fas/FasL-system or the mitochondria pathway, respectively.[14] Figure 2 shows the dose-dependent effect of selected compounds on FasL- and staurosporine-induced apoptosis in SKW6.4 cells. ECH inhibited FasL-induced apoptosis without decreasing cell viability at the ED_{50} value of 5 μM (Fig. 2a). We found that RKTS-33 and RKTS-34 with the ED_{50} value of 10 μM selectively inhibited apoptosis induced by FasL as almost effectively as ECH, while RKTS-17 exhibited cytotoxicity at concentrations over 20 μM (Figure 2b, c and e). Moreover, RKTS-35, which possesses a bulky phenyl group, did not inhibit FasL-induced apoptosis but rather showed cytotoxicity at an IC_{50} value of 30 μM (Fig. 2d). In striking contrast to RKTS-33 and RKTS-34, RKTS-32 exhibited cell toxicity without any inhibitory action on FasL-induced apoptosis (Fig. 2f), suggesting that, intriguingly, the apoptosis-inhibitory activity is due to the length of the side chain at C-6 as well as the secondary hydroxy group at C-4. A summary of all the compounds tested in type I and type II cells is shown in Table 1. Compounds *ent*-ECH, the enantiomer of ECH, and RKTS-14, with a methoxy group at C-4 of ECH, inhibited Fas-induced apoptosis with ED_{50} values of 20∼25 μM in both types of cells, indicating that the nature of stereochemistry in addition to the secondary hydroxy group of ECH is of importance. RKTS-31, RKTS-33, and RKTS-34 selectively blocked Fas-induced, but not staurosporine-induced apoptosis, without remarkable cell toxicity, suggesting that these inhibitors are effective in blocking death receptor-mediated apoptosis in both types of cells. Moreover, these three inhibitors also inhibited apoptosis induced by agonistic anti-Fas antibody CH-11 (data not shown). On the contrary, an epoxyquinoid derivative, RKTS-36, with a ketone group at C-4, did not inhibit FasL-induced apoptosis at all; that is consistent with the case of RKTS-32.

Table 1. Effect of novel death receptor-mediated apoptosis inhibitors on FasL- and staurosporine-induced apoptosis in SKW6.4 and Jurkat cells

	SKW 6.4 cells (Type I)[a]			Jurkat cells (Type II)[a]		
	ED_{50}[b]	ED_{50}[c]	IC_{50}[d]	ED_{50}[b]	ED_{50}[c]	IC_{50}[d]
ECH	5	>80	>80	6	>80	>80
ent-ECH	20	>80	80	25	>80	60
RKTS-14	20	>80	>80	25	>80	>80
RKTS-33	10	>80	>80	18	>80	>80
RKTS-31	4	>80	>80	6	>80	>80
RKTS-34	10	>80	>80	10	>80	>80
RKTS-35	ne[e]	ne	30	ne	ne	20
RKTS-17	10	ne	35	ne	ne	15
RKTS-32	ne	ne	9	ne	ne	8
RKTS-36	ne	ne	7	ne	ne	10

[a]See text.
[b]50% effective dose (μM) of FasL-induced apoptosis inhibition.
[c]50% effective dose (μM) of staurosporine-induced apoptosis inhibition.
[d]Concentration (μM) of 50% inhibitory activity on cell growth.
[e]No effect due to its cytotoxicity.

Conclusions

Novel apoptosis inhibitors, RKTS-33 and RKTS-34, which were asymmetrically synthesized via a key synthetic intermediate resulting from lipase-catalyzed kinetic resolution, exhibited selective inhibitory activity toward Fas-mediated, but not staurosporine-induced, apoptosis. Although the class of substrate-mimicking

peptide inhibitors has been previously reported, the important aspect of ECH derivatives described here is that these are membrane-permeable inhibitors as well as non-peptide inhibitors.

Acknowledgements

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20. ^{1}H NMR (400 MHz, CD_3OD) δ 3.39–3.42 (1H, m), 3.75–3.77 (1H, m), 4.18 (1H, d, $J=17.2$ Hz), 4.42 (1H, d, $J=17.2$ Hz), 4.49 (1H, s), 6.03 (1H, brs); ^{13}C NMR (100 MHz, $CDCl_3$) δ 53.89, 58.19, 62.85, 63.85, 120.52, 161.94, 195.76; IR (neat) ν 3352, 2920, 2852, 1676, 1132, 1041, 856, 806 cm^{-1}; MS (FAB-MS) m/z 155 $(M-H)^-$.
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22. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (3H, d, $J=6.4$ Hz), 1.43 (3H, d, $J=6.4$ Hz), 1.69 (3H, s), 1.88 (3H, s), 2.53 (1H, brs), 2.60 (1H, brs), 3.49–3.55 (2H, m), 3.59 (1H, brs), 3.66 (1H, brs), 3.78–3.82 (2H, m), 4.25–4.53 (4H, m), 4.93–5.02 (2H, m), 5.53 (1H, q, $J=6.4$ Hz), 5.64 (1H, q, $J=6.4$ Hz); IR (neat) ν 3400, 2916, 2858, 1668, 1435, 1373, 1300, 1173, 1051, 1026, 866, 823, 783 cm^{-1}; MS (FAB-MS) m/z 211 $(M+H)^+$.
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24. SKW6.4 or Jurkat cells (5×10^4 cells, 100 μL) were cultured with apoptosis inducers (300 ng/mL of cross-linked FasL or 1 μM staurosporine) for 8 h in the presence of test compounds in 96-well microtiter plates prior to treatment with 500 μg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; SIGMA, St. Louis, MO, USA) for 2 h. MTT-formazan was solubilized in 5% SDS overnight, and the absorbance at 595 nm was measured using a plate reader (Wallac 1420 ARVOsx; Amersham Biosciences, Piscataway, NJ, USA). Cell viability (%) was calculated as (experimental absorbance−background absorbance)/(control absorbance −background absorbance)×100.