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# Unnatural enantiomer of chaetocin shows strong apoptosis-inducing activity through caspase-8/caspase-3 activation

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ABSTRACT

Chaetocin, a natural product isolated from *Chaetomium* species fungi, was reported to have various biological activities, including antitumor and antifungal activities. Recently, we reported the first total synthesis of chaetocin and its derivatives. Here, we examined the cell-death-inducing activity of these compounds in human leukemia HL-60 cells. The unnatural enantiomer of chaetocin (*ent*-chaetocin) was more potent than chaetocin, and was found to induce apoptosis through the caspase-8/caspase-3 activation pathway.

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Chaetocin (**1**), a natural product isolated from *Chaetomium* species fungi, exhibits antitumor and antifungal activities.[1,2] Further, Imhof and co-workers reported that it shows inhibitory activity against lysine-specific histone methyltransferases (HMTs), which are key enzymes in the epigenetic control of gene expression.[3] Inspired by the complex chemical structure and interesting biological activities, we began a synthetic study of chaetocin (**1**), and recently reported the first total synthesis of chaetocin and its analogs (Fig. 1). We then examined the inhibitory activity against H3K9 HMT G9a,[4] and found that sulfur-deficient chaetocin (**2**) and its enantiomer (*ent*-**2**) lacked activity, whereas chaetocin (**1**) and its enantiomer (*ent*-**1**) showed comparable activities. These results indicate that the disulfide structure is important for the HMT-inhibitory activity, and also that the chirality of chaeotocin (**1**) is not strictly recognized by HMT.

In addition to HMT inhibition, chaetocin (**1**) and related epidithiodiketopiperazine (ETP) compounds were reported to induce oxidative stress and to be cytotoxic to various cell lines.[5–8] The internal disulfide bridge of ETP is reduced to dithiol by cellular reductants, and redox cycling between dithiol and disulfide is thought to mediate the generation of reactive oxygen species (ROS), which induce cell death.[9,10] On the other hand, several ETP-binding proteins have been identified and suggested to mediate various biological activities.[11,12] Several mechanisms, including a ROS-mediated mechanism and a binding-protein-mediated mechanism, have been proposed, but the nature of ETP-induced cytotoxicity has not yet been fully clarified. In this study, in order to clarify the structure–activity relationship (SAR) and the mechanism of the cytotoxicity, we examined the cell-death-inducing activity of chaetocin derivatives. Interestingly, the unnatural enantiomer of chaetocin (*ent*-**1**) showed more potent activity than

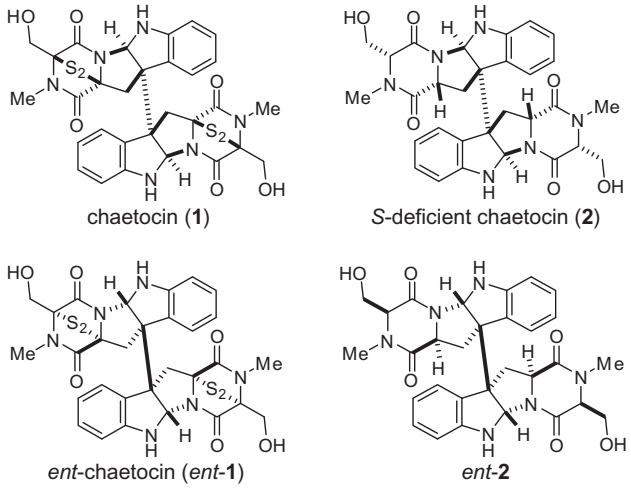


**Figure 1.** Structures of chaetocin derivatives.

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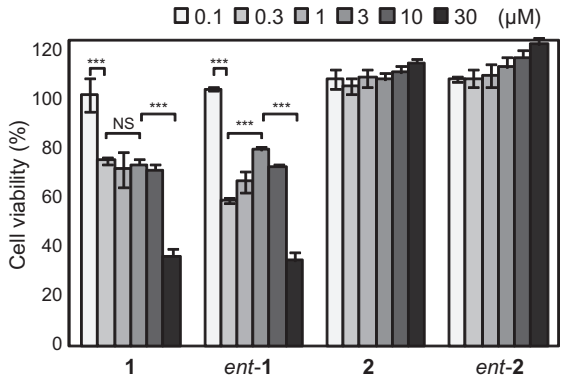


**Figure 2.** Cell-death-inducing activities of chaetocin derivatives in HL-60 cells. HL-60 cells were treated with chaetocin derivatives for 4 h, and cell viability was determined by alamarBlue assay. Statistical differences were determined by Student's *t*-test (N.S., nonsignificant; ***$P$ <0.001).

natural chaetocin (**1**) at low concentration. Moreover, *ent*-**1** was found to induce apoptosis through the caspase-8/caspase-3 activation pathway.

First, we tested the cell-death-inducing activity of chaetocin derivatives using human leukemia HL-60 cells (Fig. 2). Cell viability was determined by alamarBlue assay as described elsewhere.[13,14] Chaetocin (**1**) dose-dependently reduced cell viability, but its sulfur-deficient derivative **2** was inactive, indicating a correlation between HMT inhibition and anticancer activity. The importance of disulfide structure for anticancer activity was already reported by Isham et al.[7] The same structure–activity relationship (SAR) was observed for *ent*-**1** and its disulfide-deficient derivative (*ent*-**2**). Interestingly, though, we found that *ent*-**1**, but not **1**, showed a bell-shaped dose–response curve, that is, recovery of cell viability was seen at moderate concentration. The difference between **1** and *ent*-**1** at low concentration implies the existence of a novel target that recognizes the chirality of chaetocin. The morphology of cells treated at low (0.3 μM) and high (30 μM) concentrations was also distinct (Fig. 3a). Typical apoptotic morphology (cell shrinkage and/or blebbing) was observed in the former case. In contrast, cell swelling (typical necrotic morphology) was observed in the latter case.

To examine more precisely the effects of **1** and *ent*-**1** on HL-60 cells, DAPI/PI staining was performed at low (0.3 μM) and high (30 μM) concentrations. DAPI stains nuclear DNA of normal/dead cells, and PI stains nuclear DNA of dead cells whose cellular membrane has become permeabilized. As shown in Figure 3a, **1** and *ent*-**1** induced apoptotic cell death characterized by nuclear fragmentation (a typical marker of apoptosis; DAPI stain) at low concentration. On the other hand, at high concentration, **1** and *ent*-**1** induced necrotic cell death characterized by cellular membrane permeabilization (a typical marker of necrosis; PI stain). These results indicate that **1** and *ent*-**1** induce apoptosis and necrosis through independent mechanisms.

Compared to natural chaetocin (**1**), *ent*-**1** showed similar necrosis-inducing activity, but its apoptosis-inducing activity seemed to be stronger. To confirm the difference of apoptosis-inducing activity between **1** and *ent*-**1**, we examined DNA ladder formation by DNA electrophoresis.[15] As shown in Figure 3b, a typical DNA ladder was detected in the case of *ent*-**1** treatment at low concentration. In contrast, there was little or no cleaved DNA in the case of treatment with **1** or with *ent*-**1** at a high concentration. These results suggest that a low concentration of *ent*-**1** induces apoptosis through a mechanism that is able to recognize the chirality of chaetocin. Next, to examine the involvement of oxidative stress, we monitored cellular ROS by flow-cytometric analysis. However, neither **1** nor *ent*-**1** increased cellular ROS (data not shown). These results strongly imply that ROS are not involved in the *ent*-**1**-induced apoptosis.

Moreover, since not much difference was observed in HMT-inhibitory activity between **1** and *ent*-**1**, a target protein distinct from HMT is expected to be involved in apoptosis induction by *ent*-**1**.

In order to investigate the action mechanism of ETP compounds, we planned to investigate the signaling pathway of apoptosis induced by *ent*-**1**. Typical apoptosis is mediated by a cascade of proteases, called caspases,[16,17] but recent studies indicate the existence of a caspase-independent pathway for some types of apoptosis.[18–20] To examine the involvement of caspases in apoptosis induced by *ent*-**1**, we monitored the activation of caspase-3, an effector caspase. By means of Western blot analysis, cleavage of caspase-3 was detected in *ent*-**1**-treated HL-60 cells (Fig. 4a). Moreover, the quantification of caspase activation[21] indicated that *ent*-**1** showed much more potent activation of caspase-3 than did **1** (Fig. 4b). Moreover, Z-VAD (a general caspase inhibitor) inhibited apoptosis induced by *ent*-**1** (Fig. 4c). These results taken together indicate that caspases are involved in apoptosis induced by *ent*-**1**.

To identify the major pathway activated by *ent*-**1**, we next examined the effects of subtype-specific caspase inhibitors. Up-

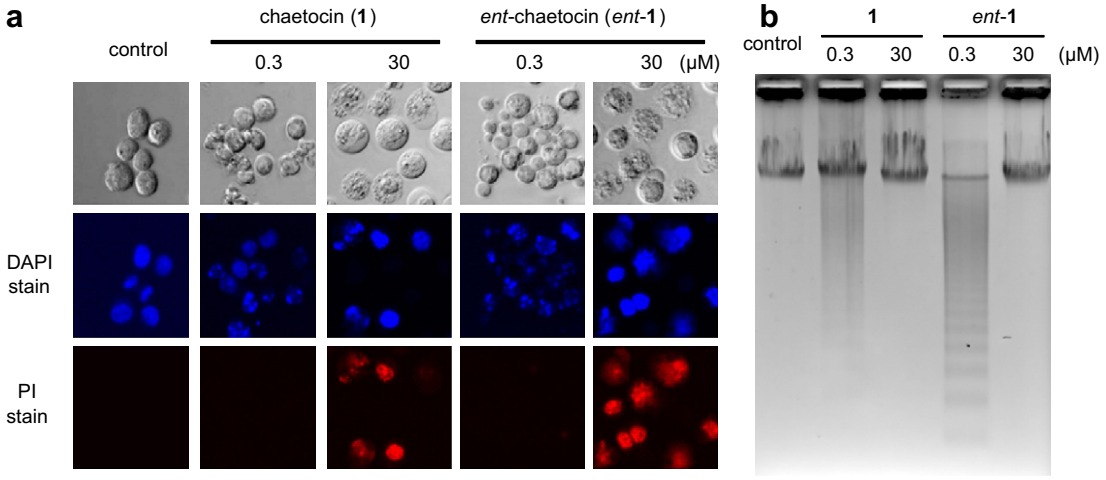


**Figure 3.** (a) Typical apoptotic and necrotic morphological changes induced by chaetocin (**1**) or *ent*-**1** in HL-60 cells. HL-60 cells treated with chaetocin derivatives for 4 h were stained with DAPI/PI. Nuclear fragmentation (DAPI, blue) and PI-stained nuclei (PI, red) were observed using confocal laser microscopy. (b) DNA ladder formation induced by chaetocin (**1**) or *ent*-**1** in HL-60 cells.

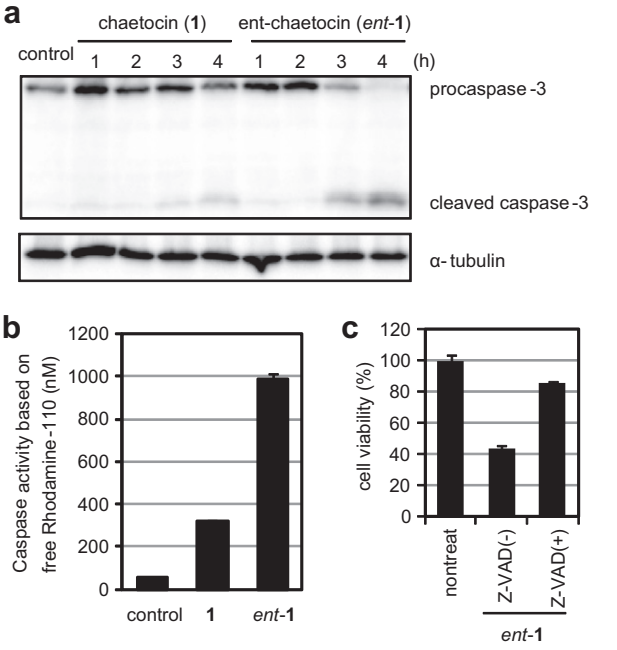


**Figure 4.** Involvement of caspase-3 in apoptosis of HL-60 cells treated with **1** or *ent*-**1** (0.3 μM). (a) Cleavage of procaspase-3 was detected by Western blot analysis. (b) Activation of caspase-3 was quantified by means of a fluorescence-based method. (c) Inhibition of *ent*-**1** (0.3 μM)-induced cell death by a general caspase inhibitor, Z-VAD (50 μM).

stream of caspase-3, there are two distinct pathways: (i) the death-ligand-induced pathway mediated by caspase-8, and (ii) the mitochondrial pathway mediated by caspase-9.[22] Therefore, Z-IETD (caspase-8 inhibitor) and Z-LEHD (caspase-9 inhibitor) were tested for the inhibitory activity against apoptosis (Fig. 5a) and caspase-3 activation (Fig. 5b) induced by *ent*-**1**. We found that the caspase-8 inhibitor, but not the caspase-9 inhibitor, reduced both apoptosis and caspases-3 activation. Moreover, cleavage of caspases-8 was detected in HL-60 cells treated with *ent*-**1** (Fig. 5c). These results suggest that *ent*-**1** induces apoptosis via the caspase-8/caspase-3 pathway.

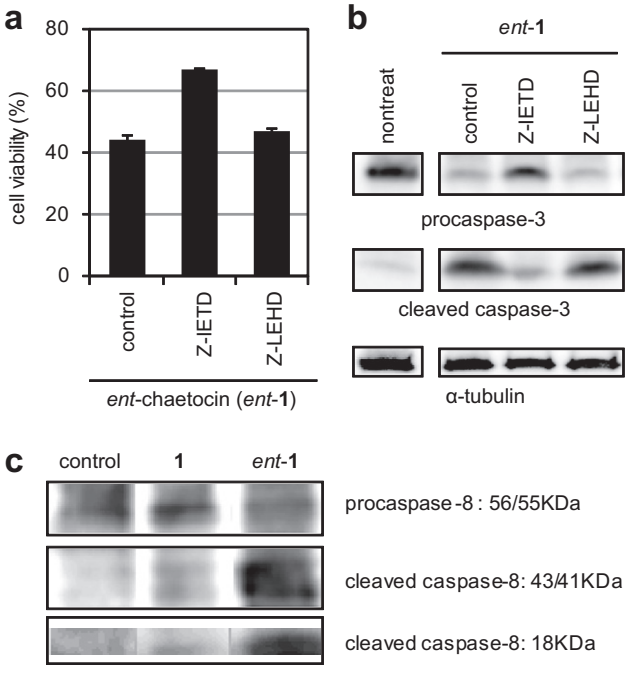


**Figure 5.** Effects of caspase inhibitors (50 μM) on *ent*-**1** (0.3 μM)-induced cell death and activation of caspase-3 and caspase-8. (a) Cell viability was determined by alamarBlue assay. (b) Cleavage of caspase-3 was detected by Western blot analysis. (c) Cleavage of caspase-8 was detected by Western blot analysis.

Caspase-8 activation is consistent with the involvement of death-ligand-induced apoptosis, which is the main pathway of caspase-8 activation. To test this hypothesis, we examined the cooperative effects of *ent*-**1** and physiological death ligand, such as Fas ligand or TNF-α. If the death-ligand-related pathway is involved, *ent*-**1** would enhance the apoptosis-inducing activity. But, interestingly, *ent*-**1** had no effect on apoptosis induced by death ligand at 30 nM, at which *ent*-**1** itself did not induce apoptosis (data not shown). Considering the difference of time courses of apoptosis induction (4 h for *ent*-**1** and 24 h for death ligand), *ent*-**1** might activate caspase-8 through a novel mechanism in the caspase cascade. In most current chemo- or radiotherapy for cancer, apoptosis is induced through the caspase-9 activation pathway. Apoptosis resistance of tumor cells to anticancer drugs is often a result of inactivation of the caspase-9-mediated pathway. Therefore, caspase-8-dependent apoptosis induction might overcome this resistance.[23] Thus, *ent*-**1** might be a candidate as a novel lead compound for development of anticancer drugs. Although the target molecule of *ent*-**1** for apoptosis induction has not yet been identified, the difference between the activities of the enantiomers (**1** and *ent*-**1**) tends to exclude nonspecific physical effects.[24]

In conclusion, the enantiomer of natural chaetocin, *ent*-**1**, was found to show strong apoptosis-inducing activity at low concentration through the caspase-8/caspase-3 activation pathway. Work to clarify the action mechanism and the target molecule of *ent*-**1** is in progress.

## Acknowledgments

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15. *Detection of DNA fragmentation:* HL-60 cells were treated with chaetocin or *ent*-chaetocin for 4 h. Chromosomal DNA was prepared using the Quick Apoptotic DNA Ladder Detection Kit (BioVision) according to supplier's protocol. Each sample was electrophoresed on a 1.5% agarose gel and the gel was stained with EtBr.
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21. *Measurement of caspase-3 activation:* Caspase-3 activation was measured by using a homogeneous caspase assay kit (Roche Ltd) according to supplier's protocol. Briefly, compound-treated cells (4 h) and serial dilutions of standards

were plated in a 96-well plate. The same amount of substrate working solution was then added to each well, followed by 2 h incubation. Substrate (DEVD-R110) was cleaved by activated caspase-3 to release Rhodamine-110 (excitation 485 nm, emission 530 nm). Activated caspase-3 was quantified based on the fluorescence of free rhodamine-110.

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