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Demonstration of direct binding of cIAP1 degradation-promoting bestatin analogs to BIR3 domain: Synthesis and application of fluorescent bestatin ester analogs

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Abstract—Overexpression of cIAP1 correlates with resistance to radiotherapy and chemotherapy in various cancers. Recently, we reported that a class of bestatin ester analogs represented by MeBS (**2**) destabilized and promoted the degradation of cIAP1 through auto-ubiquitination, and thereby sensitized cancer cells to apoptosis. Herein, we present chemical evidence that bestatin ester analogs directly interact with the cIAP1-BIR3 domain by means of fluorescence polarization assay and photoaffinity labeling assay using fluorescent probes.

Programmed cell death, called apoptosis, is required for normal embryonic development, growth, differentiation, and homeostasis of multicellular organisms.[1–4] Apoptosis can be triggered by distinct extracellular and intracellular stimuli, and it can involve the activation of a unique class of cysteine proteases known as caspases.[5–8] The functions of caspases are regulated by another set of proteins called inhibitor of apoptosis proteins (IAPs).[9–11]

IAP proteins (IAPs) interact with multiple cellular partners and inhibit apoptosis induced by a variety of stimuli.[12] This places IAPs in a central position as inhibitors of death signals that proceed through a number of different pathways.[13] The IAPs have one to three zinc-binding baculovirus IAP repeat (BIR) domains that are required for anti-apoptotic activity.[12,14] In addition, some of IAPs also possess carboxy-terminal RING domains that function as ubiquitin ligases.[12,15]

Among human IAPs, cIAP1 and cIAP2 were originally identified through their ability to interact directly with TNF receptor associated factor-1 and -2 (TRAF-1, TRAF-2) in the signaling pathway mediated by tumor necrosis factor 2 (TNFR2).[16] cIAP1 and cIAP2 are known to inhibit directly the activity of caspase-3, caspase-7 and caspase-9.[17,18] There are also RING domain-containing ubiquitin ligases capable of promoting ubiquitination and proteasomal degradation of several of their binding partners and themselves.[17,19–22] cIAP1 is highly expressed in various organs such as kidney, small intestine and lung, and one of the factors causing treatment-resistance of cancer is considered to be the apoptosis-inhibiting activity of cIAP1 in these organs. Thus, the inhibition of cIAP1 function is regarded as an attractive target for the treatment of cancer.

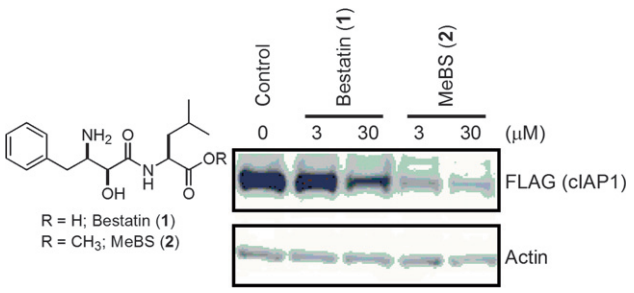

Figure 1. FLAG-cIAP1 stable transfectant HT1080 cells were treated with or without 3 and 30 μM bestatin (**1**) and MeBS (**2**) for 3 h. Cell lysates were analyzed by Western blotting with the indicated antibodies.

Keywords: Bestatin; cIAP1; BIR3 domain; Fluorescence polarization; Photoaffinity labeling.
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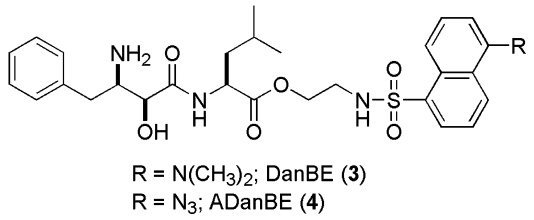


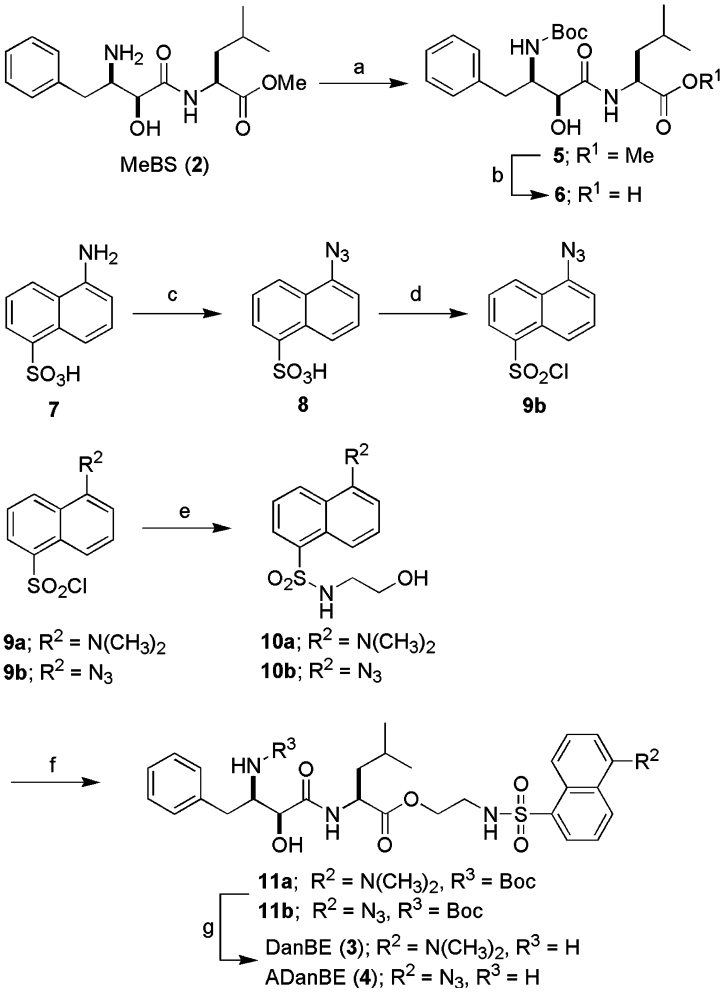
Figure 2. Design of bestatin ester derivatives for fluorescence polarization assay and photoaffinity labeling assay.

Bestatin (**1**, Fig. 1), *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, was isolated from *Streptomyces olivoreticulithe* in 1976,[23] and is a potent, competitive inhibitor of aminopeptidase B and leucine aminopeptidase.[24] Bestatin (**1**) also possesses immunomodulatory effects through the stimulation of humoral and cell-mediated immune responses and the inhibition of aminopeptidases.[25] We recently found that bestatin alkyl ester derivatives, such as MeBS (**2**), possess potent cIAP1 degradation-promoting activity as a novel biological function.[26]

MeBS (**2**) destabilizes cIAP1 but not cIAP2, a close homolog of cIAP1 sharing 73% amino acid identity. Based on this observation, we constructed a series of chimeric molecules containing portions of cIAP1 and cIAP2 to identify the indispensable part of cIAP1 at which MeBS (**2**) elicits the degradation-promoting activity. The result of cIAP1 degradation assay using these chimeric mutant proteins suggested a crucial role of the BIR3 domain in MeBS-induced destabilization of cIAP1.[26] The results led us speculate that direct binding of MeBS (**2**) to the BIR3 domain of cIAP1 might occur, as has been suggested by SPR analysis.[26]

To develop a useful probe to examine the direct binding of bestatin esters and cIAP1-BIR3 domain, we planned to synthesize fluorescence-labeled bestatin esters and apply them to fluorescence polarization and photoaffinity labeling experiments.

Our previous studies showed that various derivatizations of the ester moiety of MeBS (**2**) could be done with the retention of the cIAP1 degradation-promoting activity. So, we first synthesized MeBS (**2**) analogs with



Scheme 1. Reagents and conditions: Synthesis of fluorescence-labeled bestatin analogs. (a) Boc_2O, TEA, CH_2Cl_2, rt, 150 min; (b) NaOH, H_2O/Acetone/MeOH (5:5:1), rt, 5 min, 91% (2 steps); (c) $NaNO_2$, H_2SO_4, 0 °C, 45 min, then NaN_3, H_2O, 4 °C, 8 h, 68%; (d) PCl_5, 75 °C, 1 h; (e) Ethanolamine, TEA, CH_2Cl_2, rt, 49% for **10a**, 39% for **10b** (2 steps); (f) **6**, EDCI, HOBt, DIPEA, rt; (g) TFA, CH_2Cl_2, rt, 24% for **3** (2 steps), 13% for **4** (2 steps).

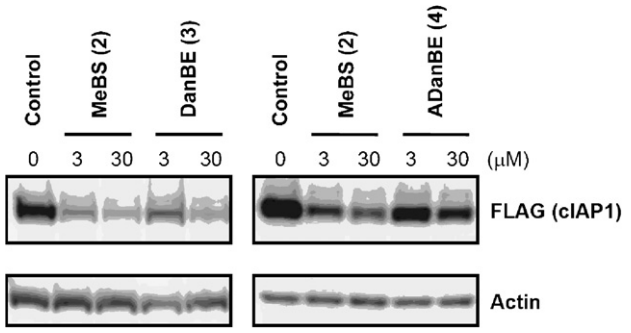


Figure 3. FLAG-cIAP1 stable transfectant HT1080 cells were treated with or without 3 and 30 μM of MeBS (**2**), DanBE (**3**) and ADanBE (**4**) for 3 h. Cell lysates were analyzed by Western blotting with the indicated antibodies.

anthracene or BODIPY as a fluorophore, but these analogs were not suitable for fluorescence polarization assay because of their very low solubility in water. Thus, we newly designed DanBE (**3**, Fig. 2) which has a dansyl group as a fluorophore in the ester part. Moreover, we designed ADanBE (**4**, Fig. 2) which has an azidodansyl group as a fluorophore/photoaffinity labeling moiety in the ester part.[27,28]

The synthesis of fluorescence-labeled bestatin esters (DanBE; **3** and ADanBE; **4**) is summarized in Scheme 1. Briefly, *N*-Boc-bestatin (**6**) was synthesized by the protection of the amino group in MeBS (**2**) with a Boc group, followed by hydrolysis at the methyl ester part to afford **6**. Azidodansyl chloride (**9b**) was obtained from Laurent's acid (**7**) by azidation of the amino group and then acid chlorination of the carboxylic acid part gave **9b**. Condensation of ethanolamine and dansyl chloride (**9a**) or **9b**, followed by condensation with *N*-Boc-bestatin (**6**) and alcohol (**10a** or **10b**) afforded intermediates **11a** and **11b**, respectively. Finally, deprotection of the Boc group in **11a** and **11b** under acidic conditions gave DanBE (**3**)[29] and ADanBE (**4**),[30] respectively.

The cIAP1 degradation-promoting activity of **3** and **4** was measured by the use of FLAG-cIAP1 stably transfected HT1080 cells. The transfectant cells were treated

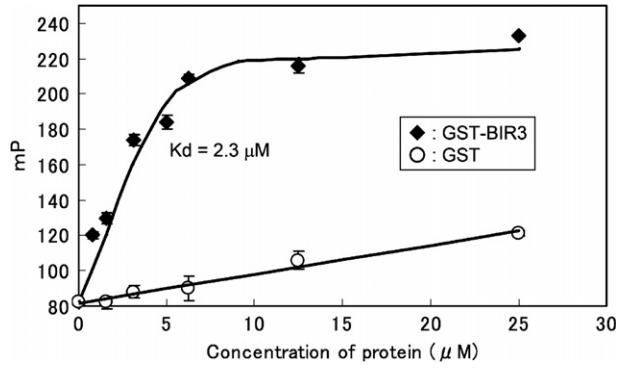


Figure 4. Serial dilutions of GST-BIR3 or GST were incubated in the presence of DanBE (**3**) (1 μM) for 5 min, 25 °C in phosphate-buffered saline (pH 7.4). After incubation, the fluorescence polarization of **3** was measured (Ex: 335 nm, Em: 550 nm).

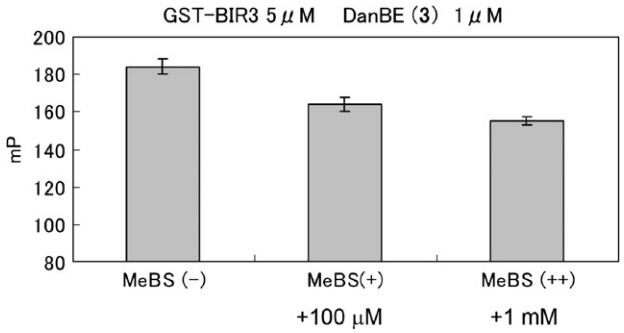


Figure 5. Competitive displacement studies were performed with MeBS (**2**). To a solution of GST-BIR3 5 μM in phosphate-buffered saline (pH 7.4), DanBE (**3**) was added at concentrations of 100 μM or 1 mM. After preincubation (5 min, 25 °C), **2** was serially diluted in the assay buffer to 1 μM, and the solution was incubated (5 min, 25 °C). The fluorescence polarization of **3** was measured (Ex: 335 nm, Em: 550 nm).

with 3 or 30 μM compound **3** or **4** for 3 h (Fig. 3). Compounds **3** and **4** showed moderate cIAP1 degradation-promoting activity. The IC_{50} values of MeBS (**2**), DanBE (**3**), and ADanBE (**4**) were evaluated by a separate set of Western blotting analysis (calculated from the detected amounts of cIAP1 protein) to be 0.69, 1.86, and 18.9 μM, respectively. Though these IC_{50} values of compounds **3** and **4** were larger than that of MeBS (**2**), we expected that they act as fluorescent/photoaffinity probes because (i) our previous studies[22] indicated that the structural requirements for cIAP1 degradation-promoting activity are quite critical, and (ii) both **3** and **4** apparently possess the specific cIAP1 degradation-promoting activity.

Compound **3** was used as a fluorescence polarization (FP) probe to examine its binding to cIAP1-BIR3 domain protein (Fig. 4). A fixed concentration of probe (1 μM) was mixed with different concentrations of GST-BIR3 or GST proteins (*X*-axis) and the FP value (*Y*-axis) was measured. The FP signal rose from approximately 80 mP (background mP value due to intrinsic polarization of the probe under the experimental conditions) to about 230 mP when the concentration of GST-BIR3 was increased from 0 to 25 μM. Although the addition of GST caused a slight increase in the FP value (possibly because of non-specific binding), the result clearly indicates the direct binding of compound **3** to the BIR3 domain.

The addition of MeBS (**2**) to the mixture of GST-BIR3 and compound **3** caused a dose-dependent decrease of the FP values (Fig. 5). Although the competition seems not to be complete, this result suggests that MeBS (**2**) and compound **3** bind cIAP1-BIR3 domain in a mutually competitive manner, at least in part. The incomplete competition and the necessity of extremely high concentration of MeBS (**2**) to compete out DanBE (**3**) from GST-BIR3 might be interpreted by (i) high non-specific binding of **3** to GST-BIR3 and/or (ii) the nature of DanBE (**3**) which binds GST-BIR3 with higher affinity than MeBS (**2**) in spite of its lower cIAP1 degradation-promoting activity than that of **2**. The detailed nature of

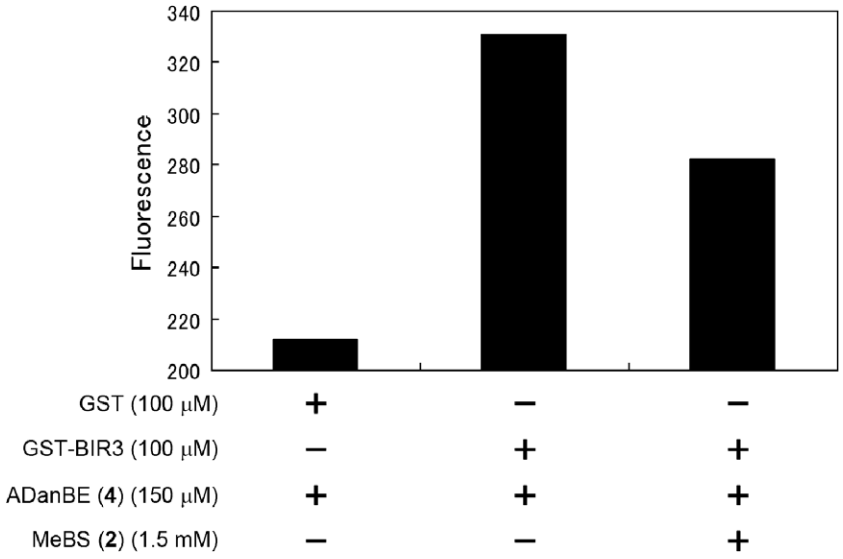


Figure 6. GST-BIR3 and GST (100 μM) were incubated in the presence of ADanBE (**4**) (150 μM) for 30 min at 4 °C in phosphate-buffered saline (pH 7.4). Then the mixture was irradiated with a UV lamp (365 nm) at a distance of 5 cm for 5 min at 0 °C. To the reaction mixture was added phosphate-buffered saline (pH 7.4). Non-binding **4** was removed by ultrafiltration (10000 MWCO × 3000*g*), and the solution was concentrated to 250 μL. The amount of labeled GST-BIR3 or GST was measured with fluorescence counter (Ex: 345 nm, Em: 465 nm). The competitive assay was carried out similarly, in the presence of MeBS (**2**) (1.5 mM).

the binding of compounds **2** and **3** to GST-BIR3 remained to be investigated, but the result shown in Figure 5 afforded the evidence of the direct binding of MeBS (**2**) to GST-BIR3.

Based on the above-mentioned result, we examined the interaction of MeBS (**2**) analogs with cIAP1-BIR3 domain protein using ADanBE (**4**) as a photoaffinity labeling probe. GST-BIR3 and GST (negative control) were incubated with **4** in the absence or presence of the potent cIAP1 degradation promoter MeBS (**2**), and the mixtures were irradiated with UV 365 nm. The protein fractions were separated and washed to remove noncovalently bound ligands, and then the amount of bound fluorescent probe to GST-BIR3 or GST was measured with a fluorescence counter (Ex: 345 nm, Em: 465 nm). As shown in Figure 6, GST-BIR3 was efficiently labeled with compound **4** (the middle bar), though slight non-specific labeling to GST was observed (the left bar). The amount of covalently bound fluorescent probe was decreased by the addition of MeBS (**2**) (the right bar, Fig. 6). Although the decrease was not complete possibly by non-specific labeling reaction, the results indicate that labeling occurred at the specific MeBS (**2**)-binding site(s) in the BIR3 domain, at least in part. The non-specific binding of ADanBE (**4**) seems to be rather high, as well as that of DanBE (**3**), which might be improved by changing the fluorescent/photoreactive moiety to more hydrophilic substitute(s). In spite of this disadvantage of the probes, we have obtained chemical evidence that bestatin ester analogs directly interact with the cIAP1-BIR3 domain based on fluorescence polarization assay and photoaffinity labeling assay. Further analyses of binding of these compounds at the cIAP1-BIR3 domain, for example, identification of the amino acid residues involved, and the mode of binding, are under way.

Acknowledgments

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29. DanBE (**3**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (d, J =8.5 Hz, 1H), 8.30 (d, J = 8.5 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 7.71 (d, J = 7.3 Hz, 1H), 7.35–7.17 (m, 5H), 4.40 (m, 1H), 4.19–4.07 (m, 2H), 4.02 (m, 1H), 3.61 (m, 1H), 3.23–3.18 (m, 2H), 3.00 (dd, J = 14.1, 5.1 Hz, 1H), 2.88 (s, 6H), 2.61 (dd, J = 14.1, 9.0 Hz, 1H), 1.62–1.55 (m, 3H), 0.92–0.87 (m, 6H); HRMS (FAB) calcd for $C_{30}H_{41}N_4O_6$ 585.2747; found: 585.2728 $(M+H)^+$.
30. ADanBE (**4**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37–7.21 (m, 11H), 4.84 (m, 1H), 4.03 (m, 1H), 3.68–3.55 (m, 2H), 3.09 (dd, J = 13.3, 1.3 Hz, 2H), 2.43 (dd, J = 12.8, 11.5 Hz, 2H), 1.92–1.83 (m, 3H), 1.01–0.97 (m, 6H); HRMS (FAB) calcd for $C_{28}H_{35}N_6O_6S$ 583.2339; found: 583.2334 $(M+H)^+$; IR 2120 cm^{-1} (N_3 asymmetric stretch).