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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.

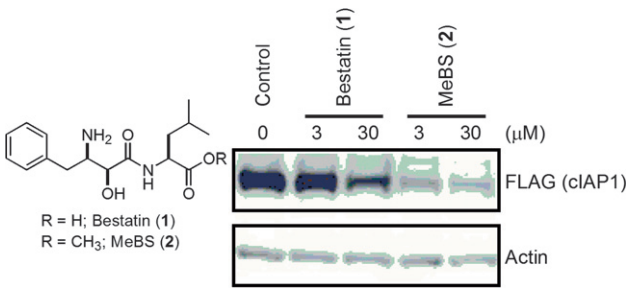

Figure 1. FLAG-cIAP1 stable transfectant HT1080 cells were treated with or without 3 and 30 μM bestatin (**1**) and MeBS (**2**) for 3 h. Cell lysates were analyzed by Western blotting with the indicated antibodies.

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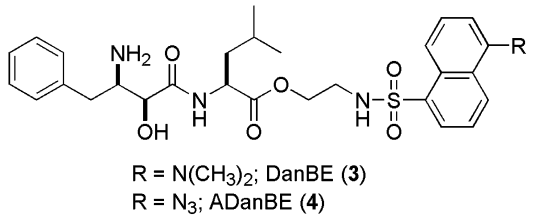


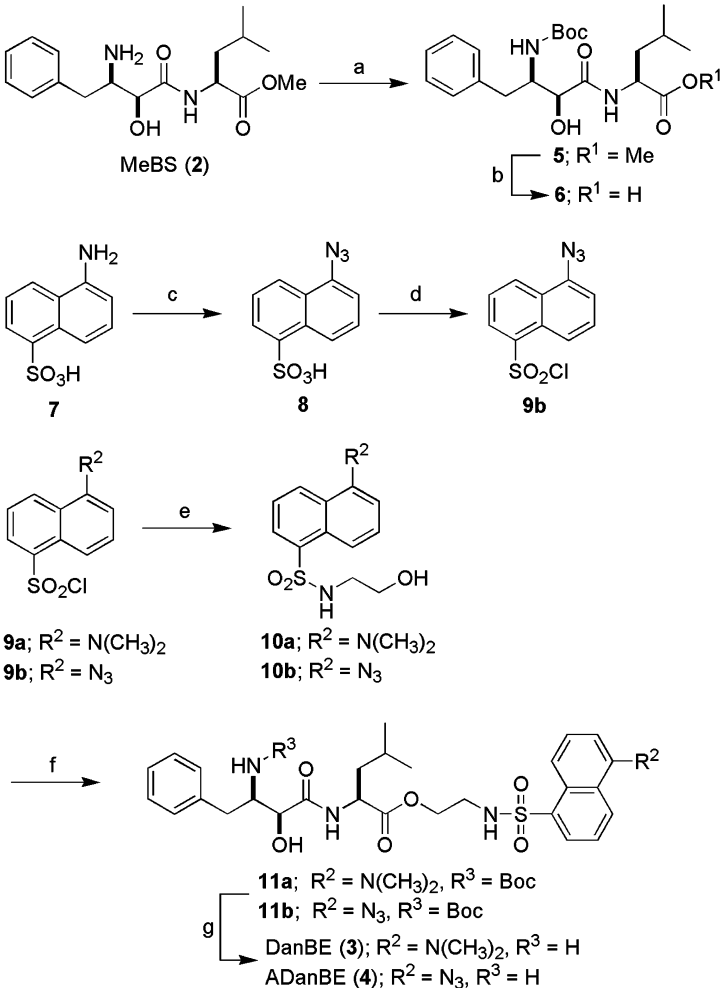
Figure 2. Design of bestatin ester derivatives for fluorescence polarization assay and photoaffinity labeling assay.

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).