Small Molecules Destabilize cIAP1 by Activating Auto-ubiquitylation

Received for publication, November 20, 2007, and in revised form, January 29, 2008. Published, JBC Papers in Press, January 29, 2008. Published, JBC Papers in Press, January 29, 2008. DOI 10.1074/jbc.M709525200

Keiko Sekine, Kohei Takubo, Ryo Kikuchi, Michie Nishimoto, Masayuki Kitagawa, Fuminori Abe, Kiyohiro Nishikawa, Takashi Tsuruo, and Mikihioko Naito

From the Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyoku, Tokyo 113-0032, Pharmaceutical Research Laboratories, Research & Development Group, Nippon Kayaku Co., Ltd, 3-31-12 Shimo, Kita-ku, Tokyo 115-8588, and Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan

Overexpression of an anti-apoptotic protein cIAP1 caused by its genetic amplification was reported in certain cancers, such as hepatocellular carcinoma, esophageal squamous cell carcinoma, cervical cancer, and lung cancer, which confers resistance to chemotherapy and radiotherapy. Here we report cIAP1 to be selectively down-regulated by a class of small molecules (ME-BS), resulting in a sensitization of cancer cells to apoptosis. ME-BS directly interacts with the BIR3 domain of cIAP1, promotes auto-ubiquitylation dependent on its RING domain, and facilitates proteasomal degradation of cIAP1. Other IAPs such as XIAP and cIAP2 were not affected by ME-BS. These results suggest targeted destabilization of cIAP1 by small molecules as a novel method to treat cancers expressing cIAP1, which interferes with treatment. Manipulation of the intrinsic ubiquitin-ligase activity could be a novel strategy to develop small molecules for therapeutic purposes.

IAPs (inhibitor of apoptosis proteins) are a family of anti-apoptotic proteins containing one to three baculoviral IAP repeat (BIR) domains (1–3), some of which are frequently overexpressed in malignant cells. Certain IAPs such as XIAP/hILP/BIRC4, cIAP1/MIHB/hiap-2/BIRC2, cIAP2/MIHC/hiap-1/BIRC3, ML-IAP/Livin/BIRC7 and Apollon/BRUCE/BIRC6 directly interact with and regulate caspases (4–9). The BIR domain plays an important role in the interaction with caspases (8, 10, 11). These IAPs also contain a domain involved in ubiquitin conjugation (RING finger domain or UBC domain), and facilitate proteasomal degradation of caspases and IAPs (8, 12, 13).

cIAP1 (cellular inhibitor of apoptosis protein 1) is overexpressed in human cancers such as esophageal squamous cell carcinoma, hepatocellular carcinoma, cervical cancer, and lung cancer, because of its genetic amplification and is regarded as an oncogene (14–17). cIAP1 overexpression in cervical cancers correlates with resistance to radiotherapy. In addition, a comparative study of the expression of IAP family proteins and the sensitivity to chemotherapeutic drugs in 60 cell lines revealed the level of cIAP1 significantly correlates with resistance against anti-cancer drugs such as carboplatin, cisplatin, etoposide, and cytosine arabinoside (18). This evidence suggests cIAP1 to be a promising target for cancer therapy.

Bestatin, an inhibitor of aminopeptidase N, has an immunomodulatory activity and is approved in Japan to treat patients with adult acute nonlymphatic leukemia (19–21). In a clinical trial with stage I squamous cell lung carcinoma patients, bestatin significantly prolonged survival of these patients (22). Bestatin induces apoptosis in human leukemia cells (23) and augments death ligand-induced apoptosis in human solid tumor cell lines (24). In this paper, we describe selective down-regulation of cIAP1 by esterified analogs of bestatin represented by bestatin-methyl ester (ME-BS) (see Fig. 1A), resulting in a sensitization of cancer cells to apoptosis. Mechanistic analysis showed that ME-BS directly interacts with cIAP1, promotes auto-ubiquitylation dependent on its RING domain, and facilitates proteasomal degradation of cIAP1. Destabilization of a particular anti-apoptosis protein by small molecules is a novel strategy for counteracting the pathological over-expression of such proteins known to interfere with cancer treatment.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—ME-BS and analogs of bestatin were synthesized by Nippon Kayaku Co. Ltd. (Tokyo, Japan) as described previously (25). Anti-Fas (CH11, MBL), MG132, and Z-LLH (Peptide Inst.), as well as TRAIL (Chemicon) were purchased commercially. cDNAs encoding human cIAP1, cIAP2, and XIAP (X chromosome-linked inhibitor of apoptosis protein) were amplified by PCR from a Jurkat cDNA library and cloned into a p3xFLAG-CMV-10 expression vector (Sigma). Human Bax cDNA was PCR-amplified and cloned into pcDNA3 vector (Invitrogen). Point mutant constructs were generated using the QuikChange site-directed Mutagenesis kit (Stratagene). All of the constructs generated from PCR products were sequenced.
cIAP1 Auto-ubiquitylation by ME-BS

**FIGURE 1.** ME-BS sensitizes cancer cells to apoptosis. A, chemical structure of ME-BS. B, EBC-1, HT1080 and HeLaS3 cells were treated with CH11 (10, 100, and 100 ng/ml, respectively) with or without 30 μM ME-BS for 20 h. The dead cells were counted as described under "Experimental Procedures." The data are the means of triplicate determinations. Bars: S.D. C, HT1080 cells were treated with 100 ng/ml CH11 in the presence or absence of 30 μM ME-BS for 8 h, and the cell lysates were analyzed by Western blot (WB). D, HT1080 cells were treated for 24 h with 100 ng/ml TRAIL, 100 ng/ml CH11, 10 ng/ml tumor necrosis factor α (TNFα), 10 μg/ml cisplatin (cDDP), 30 μg/ml etoposide (ETP) in the presence or absence of 30 μM ME-BS, or 1 μg/ml cycloheximide (CHX). The data are the means of triplicate determinations. Bars, S.D.

Cell Culture, Drug Treatment, Transfection, and Western Blot Analysis—Human fibrosarcoma HT1080, cervical cancer cell lines (Ca-Ski and SKG-II), and monocytic leukemia THP1 cells were maintained in RPMI 1640 medium (Nissui Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin at 37 °C in a humidified atmosphere of 5% CO2. Human squamous cell lung carcinoma EBC-1 and human cervical carcinoma HeLaS3 cells were cultured in Eagle’s minimum essential medium (Nissui Co.) containing 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin at 37 °C in a humidified atmosphere of 5% CO2. Proteasome inhibitors (MG132 and lactacystin) were added to the culture 30 min before the ME-BS treatment. Transfection experiments were carried out with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. In brief, cells (1 × 105) were seeded in 12-well plates and transfected with a total of 1 μg of plasmid DNA for 18 h. The cells were harvested and lysed in 25 μl of lysis buffer (10 mM HEPES-KOH, pH7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride, and 1:100 (v/v) dilution of aprotinin. Cell lysates containing equal amounts of protein were separated by 4–20% gradient polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), and Western blotted using appropriate antibodies. Protein bands were detected using Enhanced Chemiluminescence detection (ECL) kits (GE Healthcare). We used the following antibodies for immunoprecipitation and Western blot analysis: anti-cIAP1 and anti-cIAP2 (R & D Systems), anti-XIAP and anti-ubiquitin (MBL), anti-tubulin (Serotec), anti-SMAC (Chemicon), horseradish peroxidase-conjugated anti-FLAG antibody (Sigma), and horseradish peroxidase-conjugated anti-actin antibody (Santa Cruz). Anti-SGVD that recognizes p12 fragment of active caspase-3 was generated as described previously (26).

Cell Death Assay—The cells were cultured overnight in 24-well plates and treated with various drugs for 24 h. Floating cells were counted as dead cells, which was confirmed by Trypan blue dye exclusion (HT1080 and EBC-1 cells). HeLaS3 cells were stained with propidium iodide to count the dead cells using a flow cytometer. In some experiments, cell viability was measured by staining the living cells with 0.05% methylene blue after treatment (24). Caspase activity was measured using Apo-ONE homogenous caspase-3/7 assay (Promega) according to the manufacturer’s instructions.

RNA Interference—The small interfering RNA oligonucleotides corresponding to the sequence of cIAP1 (UCCUGA-CAACUGGAGAGAA) and control (UUCUCGAACGUGUCACGU) were transfected into HT1080 for 48 h using OligofectAMINE, and then the cells were treated with CH11 or TRAIL for another 24 h. Floating dead cells were counted as above. The cell lysates were Western blotted to confirm down-regulation of the proteins at 72 h after transfection.

Aminopeptidase Assay—Aminopeptidase activity was measured as described previously (24). Briefly, cytosolic extract from HT1080 cells was incubated at room temperature for 30 min with a graded concentration of bestatin analogs in phosphate-buffered saline containing 0.5 mM Mg2+ and 0.9 mM Ca2+, and then 10 μM alanine- or arginine-4-methylcoumarine-7-amide were added as substrates for neutral and basic aminopeptidase, respectively. The amount of 7-amino-4-methylcoumarine

![Image](https://example.com/image1.png)

**A** Chemical structure of ME-BS. **B** EBC-1, HT1080 and HeLaS3 cells were treated with CH11 (10, 100, and 100 ng/ml, respectively) with or without 30 μM ME-BS for 20 h. The dead cells were counted as described under “Experimental Procedures.” The data are the means of triplicate determinations. Bars: S.D. **C** HT1080 cells were treated with 100 ng/ml CH11 in the presence or absence of 30 μM ME-BS for 8 h, and the cell lysates were analyzed by Western blot (WB). **D** HT1080 cells were treated for 24 h with 100 ng/ml TRAIL, 100 ng/ml CH11, 10 ng/ml tumor necrosis factor α (TNFα), 10 μg/ml cisplatin (cDDP), 30 μg/ml etoposide (ETP) in the presence or absence of 30 μM ME-BS, or 1 μg/ml cycloheximide (CHX). The data are the means of triplicate determinations. Bars, S.D.
lysates were analyzed by Western blot. Tagged wild type or H588A mutant cIAP1 for 24 h. The cells were treated with MG132 and ME-BS, and the cell lines were analyzed by Western blot with anti-cIAP1 antibody. Aliquots (1/40) of the cell extracts were Western blotted with horseradish peroxidase-conjugated anti-actin (WB : Actin) antibody. The reactions were analyzed by fluorometry using a fluorescence concentration analyzer (excitation at 365 nm and emission at 450 nm). The concentration of analogs required to inhibit the enzyme activity by 50% (IC50) was determined.

FIGURE 3. ME-BS induces RING-mediated poly-ubiquitylation of cIAP1. A, HT1080 cells were transfected with FLAG-tagged cIAP1 for 24 h, pretreated with MG132 for 30 min and then treated with ME-BS for the indicated times. The cell lysates were analyzed by Western blot (WB) with the indicated antibodies. B, HT1080 cells were treated with or without 10 µM MG132 for 3 h. Protease inhibitors (10 µM MG132, 10 µg/ml lactacystin, or 10 µM Z-LLH) were added to the culture 30 min prior to the ME-BS treatment. The cell lysates were analyzed by Western blot with the indicated antibodies.

Recombinant Proteins—cDNAs encoding full-length cIAP1, cIAP1(ΔBIR1–2) that has deleted N-terminal 268 amino acids, cIAP1(ΔBIR1–3) that has deleted N-terminal 336 amino acids, cIAP1-BIR3(253–341), and XIAP were inserted into pGEX4T-1 plasmid vector. Recombinant proteins were produced in Escherichia coli BL21 (DE3) cells and purified using GST-bind Kits (Novagen). The purity of the recombinant proteins was confirmed by SDS-PAGE and Coomassie staining, and aliquots of the proteins were stored at −80 °C.

In Vitro Ubiquitylation Assay—In vitro ubiquitylation assay was carried out as described (27). Briefly, recombinant IAPs (200 nM) were incubated with 100 nM rabbit E1, 400 nM human UbcH5a, 2 mM Mg-ATP, and 20 µM ubiquitin (Boston Biochem) in 20 µl of ubiquitylation assay buffer (50 mM Tris-HCl at pH 7.5, 50 mM NaCl). The reaction was terminated by boiling with an equal volume of 2× SDS sample buffer, followed by Western blot analysis with anti-cIAP1 antibody (R & D Systems).

SPR Analysis—BIAcore S511 (Biacore) was used to evaluate the binding of ME-BS to cIAP1 and GST-BIR3. Flow cells of a Series S Sensor Chip CM5 were coated with cIAP1 (R & D Systems), GST-BIR3 or GST using amine coupling at 30 µg/ml in 10 mM acetate buffer, pH 4.5 (cIAP1) or pH 5.0 (GST-BIR3 and GST), according to the manufacturer’s protocol. The chip was then flushed with 1 M ethanolamine hydrochloride, pH 8.5, and 50 mM HCl to eliminate unbound proteins. Approximately 10,000 (cIAP1 and GST-BIR3) and 7,000 (GST) resonance units were obtained after immobilization. Biosensor assays were performed at 25 °C with HBS-EP+ buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20) containing 5% Me2SO as running buffer. ME-BS was injected at different concentrations at a flow rate of 30 µl/min for 60 s and allowed to dissociate for an additional 70 s. Control sensorgrams were subtracted from the cIAP1 sensorgrams and analyzed by Biacore S51 evaluation software with steady state fitting methods.
ME-BS Sensitizes Cancer Cells to Apoptosis Induced by Various Stimuli—When various cancer cells were treated with the anti-Fas antibody CH11 in the presence or absence of ME-BS, increased number of cells underwent apoptosis in the presence of ME-BS (Fig. 1B). Consistent with this apoptosis sensitization, caspase-3 was more extensively activated in the cells co-treated with ME-BS and CH11 than with CH11 alone (Fig. 1C). ME-BS-sensitized cells were vulnerable to apoptosis induced by TRAIL, tumor necrosis factor α, and chemotherapeutic drugs such as cisplatin and etoposide. On the other hand, cycloheximide enhanced death receptor-mediated apoptosis but rather inhibited chemotherapy-induced apoptosis (Fig. 1D). This suggests that ME-BS and cycloheximide functions by different regulatory mechanisms. Because apoptosis signals initiated by death receptor ligation and DNA damage converge at mitochondria, ME-BS is likely to regulate apoptosis at a step downstream of mitochondria.

Induction of Proteasome-mediated Degradation of cIAP1 by ME-BS—Caspase activation via the apoptosome is a key step to promote apoptosis downstream of mitochondria (28, 29), and IAPs are regulators of caspase activation (1–3). Therefore, we examined levels of IAPs in the cells treated with ME-BS. As shown in Fig. 2A, endogenous cIAP1 protein was selectively reduced by ME-BS in various cancer cells, whereas this was not true of XIAP. The reduction of cIAP1 induced by ME-BS was blocked by MG132, an inhibitor of proteasome (supplemental Fig. S2). Exogenously expressed cIAP1, but not cIAP2 or XIAP, was also reduced by ME-BS treatment, which was protected by the proteasome inhibitors MG132 and lactacystin, but not by the calpain inhibitor Z-LLH (Fig. 2B). These results indicate that cIAP1 was selectively degraded by proteasome in the ME-BS-treated cells.

Because the proteasome degrades poly-ubiquitylated proteins, we examined ubiquitylation of cIAP1 in ME-BS treated cells. HT1080 cells expressing FLAG-tagged cIAP1 were analyzed by Western blot with anti-FLAG antibody. Fig. 3A shows that within 10 min a smear of protein bands that migrated slowly in the gel had accumulated in the cells co-treated with ME-BS and MG132 (lane 3). Consistently, cIAP1 began to decrease within 10 min of ME-BS treatment (lane 2). ME-BS did not show such effects on XIAP (supplemental Fig. S2A). These results suggest that poly-ubiquitylation of cIAP1 is induced immediately after ME-BS treatment. To confirm the poly-ubiquitylation of cIAP1, the cell lysates from ME-BS-treated cells were immunoprecipitated with anti-cIAP1, and the precipitates were subjected to Western blot analysis with anti-ubiquitin to detect the ubiquitylated cIAP1 (Fig. 3B). The smear bands with the higher molecular weight, which represents the poly-ubiquitylated cIAP1 (Fig. 3C), were observed in the cells treated with MG132 and more intensely in the cells co-treated with ME-BS and MG132. A similar experiment was performed with XIAP, but ubiquitylation was not stimulated by ME-BS (supplemental Fig. S2B). In addition, recombinant cIAP1ΔBIR1–2, which has deleted BIR1 and BIR2 domains (27), was poly-ubiquitylated in vitro by ME-BS, which was abolished by omitting UbcH5a, an E2 enzyme required for ubiquitylation (Fig. 3C and supplemental Fig. S3). Ubiquitylation of cIAP1ΔBIR1–3 and XIAP were not stimulated by ME-BS (supplemental Fig. S3). Collectively, these results make evident that ME-BS induces poly-ubiquitylation of cIAP1.

cIAP1 contains a RING domain at the C terminus that is involved in the auto-ubiquitylation of cIAP1 (13). To study the

Results

ME-BS Sensitizes Cancer Cells to Apoptosis Induced by Various Stimuli—When various cancer cells were treated with the anti-Fas antibody CH11 in the presence or absence of ME-BS, increased number of cells underwent apoptosis in the presence of ME-BS (Fig. 1B). Consistent with this apoptosis sensitization, caspase-3 was more extensively activated in the cells co-treated with ME-BS and CH11 than with CH11 alone (Fig. 1C). ME-BS-sensitized cells were vulnerable to apoptosis induced by TRAIL, tumor necrosis factor α, and chemotherapeutic drugs such as cisplatin and etoposide. On the other hand, cycloheximide enhanced death receptor-mediated apoptosis but rather inhibited chemotherapy-induced apoptosis (Fig. 1D). This suggests that ME-BS and cycloheximide functions by different regulatory mechanisms. Because apoptosis signals initiated by death receptor ligation and DNA damage converge at mitochondria, ME-BS is likely to regulate apoptosis at a step downstream of mitochondria.

Induction of Proteasome-mediated Degradation of cIAP1 by ME-BS—Caspase activation via the apoptosome is a key step to promote apoptosis downstream of mitochondria (28, 29), and cIAP1 contains a RING domain at the C terminus that is involved in the auto-ubiquitylation of cIAP1 (13). To study the

Results

ME-BS Sensitizes Cancer Cells to Apoptosis Induced by Various Stimuli—When various cancer cells were treated with the anti-Fas antibody CH11 in the presence or absence of ME-BS, increased number of cells underwent apoptosis in the presence of ME-BS (Fig. 1B). Consistent with this apoptosis sensitization, caspase-3 was more extensively activated in the cells co-treated with ME-BS and CH11 than with CH11 alone (Fig. 1C). ME-BS-sensitized cells were vulnerable to apoptosis induced by TRAIL, tumor necrosis factor α, and chemotherapeutic drugs such as cisplatin and etoposide. On the other hand, cycloheximide enhanced death receptor-mediated apoptosis but rather inhibited chemotherapy-induced apoptosis (Fig. 1D). This suggests that ME-BS and cycloheximide functions by different regulatory mechanisms. Because apoptosis signals initiated by death receptor ligation and DNA damage converge at mitochondria, ME-BS is likely to regulate apoptosis at a step downstream of mitochondria.

Induction of Proteasome-mediated Degradation of cIAP1 by ME-BS—Caspase activation via the apoptosome is a key step to promote apoptosis downstream of mitochondria (28, 29), and cIAP1 contains a RING domain at the C terminus that is involved in the auto-ubiquitylation of cIAP1 (13). To study the
FIGURE 5. Down-regulation of cIAP1 by ME-BS is relevant to apoptosis sensitization. A, HT1080 cells were transfected with plasmid DNA encoding 0.02 μg of Bax and 1 μg of FLAG-tagged cIAP1 (wild type and H588A mutant). Three hours after transfection, the cells were treated with 6 μM ME-BS for 17 h, and the dead cells were counted. The data are the means of triplicate determinations. Bars, S.D. B, HT1080 cells were transfected with small interfering RNA (siRNA) against c-IAP1 or control. After 48 h, the cells were treated with 100 ng/ml CH11 or 100 ng/ml TRAIL for another 24 h, and the dead cells were counted. The data are the means of triplicate determinations. Bars, S.D. The lower panels show Western blot (WB) analysis of lysates prepared from cells 72 h after transfection to confirm down-regulation of the proteins.

FIGURE 6. BIR3 domain plays a crucial role in ME-BS-induced cIAP1 down-regulation. A, domain diagram and the mutant constructs of cIAP1 and cIAP2 used in this study. The open and shaded columns indicate the domains derived from cIAP1 and cIAP2, respectively. B and C, HT1080 cells were transfected with chimeric mutants of cIAP1 and cIAP2 (B) and cIAP1 point mutants (C) for 24 h. The cells were treated with 30 μM ME-BS for 3 h, and the cell lysates were analyzed by Western blot (WB). WT, wild type.
ME-BS showed more potent activity to sensitize HeLa and EBC-1 cells to CH11 (supplemental Fig. S7). We next examined whether cIAP1 down-regulation is relevant for the apoptosis sensitization induced by ME-BS. Expression of Bax-induced apoptosis in HT1080 cells, which was inhibited by co-expression of wild type and H588A cIAP1. ME-BS treatment completely abrogated the apoptosis inhibition by wild type cIAP1 but not the inhibition by the H588A mutant (Fig. 5A). In addition, small interfering RNA-mediated down-regulation of cIAP1 sensitized the cells to apoptosis (Fig. 5B). These results indicate that the ME-BS-induced down-regulation of cIAP1 is, at least in part, relevant for the enhanced apoptosis.

ME-BS Interacts with BIR3 Domain of cIAP1—ME-BS destabilizes cIAP1 but not cIAP2, a close homolog of cIAP1 sharing 73% amino acid identity. To study the mechanism of ME-BS-induced destabilization of cIAP1 in more detail, we constructed a series of chimeric molecules containing portions of cIAP1 and cIAP2 (Fig. 6A). Chimera-2 protein, which contains the BIR1–3 domains from cIAP1 and the CARD-RING domains from cIAP2, responded well to ME-BS, whereas chimera-3 protein, which contains the BIR1–2 domains from cIAP1 and the BIR3-RING domains from cIAP2, did not. Chimera-4 protein, in which only the BIR3 domain of cIAP1 was replaced with the corresponding BIR3 domain from cIAP2, was not destabilized by ME-BS (Fig. 6B). Conversely, chimera-6 protein, in which the BIR3 domain of cIAP2 was replaced by that of cIAP1, was destabilized by ME-BS (supplemental Fig. S8). These results indicate that the BIR3 domain of cIAP1 plays a crucial role in ME-BS-induced destabilization. We further constructed cIAP1 mutants with a point mutation in a conserved cysteine residue in the BIR domains. As expected, the BIR3 mutant (C333A) was not reduced by ME-BS, whereas BIR1 (C110A) and BIR2 (C248A) mutants were reduced (Fig. 6C), which again suggests a crucial role of the BIR3 domain in ME-BS-induced destabilization of cIAP1.
cIAP1 Auto-ubiquitylation by ME-BS

response units at 100 μM over GST from the response units over GST-BIR3 showed that active analogs (ME-BS, BE32, and BE33) interacted with BIR3 more than bestatin and the inactive analogs (BE04, BE30 and BE54) (Fig. 7D). The interaction of glutathione with GST-BIR3 and with GST served as a control. These results indicate that ME-BS directly interacts with the BIR3 domain of cIAP1.

DISCUSSION

IAPs are a family of anti-apoptotic proteins that are frequently overexpressed in cancer cells (1–3). The anti-apoptotic activity of IAPs are negated by SMAC/DIABLO that is released from mitochondria into cytosol in response to apoptotic stimuli (30, 31). The IAP-binding motif at the N-terminal of mature SMAC/DIABLO plays a crucial role in the negation of IAP function, and a number of IAP antagonists that mimic the interaction of SMAC/DIABLO and IAPs have been developed (32–35). These IAP antagonists effectively block the interaction between IAPs and active caspase-9, a small subunit of which contains the IAP-binding motif, thereby promoting caspase activation and apoptosis. IAP antagonists that block XIAP-mediated caspase-3 inhibition were also developed to induce apoptosis in various cancer cells (36). We described in this paper a novel mechanism of cIAP1 antagonism, destabilization of cIAP1 by activating its auto-ubiquitylation.

Mutation analysis revealed the BIR3 domain of cIAP1 plays a crucial role in ME-BS-induced destabilization (Fig. 6), and ME-BS directly interacts with the BIR3 domain in vitro (Fig. 7). We assume that ME-BS associated with the BIR3 domain induces a conformational change in cIAP1 that triggers auto-ubiquitylation. Because SMAC/DIABLO interacts with the BIR3 domain of cIAP1 (30, 31), we examined a possible role of SMAC in ME-BS-induced destabilization of cIAP1. However, the binding of SMAC to cIAP1 was not inhibited by ME-BS. Expression of mature SMAC in cytosol through ubiquitin fusion (8, 37) did not affect the ME-BS-induced destabilization of cIAP1. Moreover, the E325A cIAP1 mutant was destabilized by ME-BS, although it does not bind to SMAC (supplemental Fig. S9). These results suggest that SMAC is not required for the ME-BS-induced destabilization of cIAP1.

The mechanism of how cIAP1 inhibits apoptosis is not clear, considering the inability of the cIAP1 to directly inhibit caspases (38). It is possible that binding of IAP antagonists such as SMAC/DIABLO and HtrA2/Omi would limit the ability of the proapoptotic proteins to block XIAP-mediated inhibition of caspases. Another explanation involves the regulatory role of cIAP1 in tumor necrosis factor α signaling pathway because cIAP1 interacts with TRAF proteins and participates in NFκB activation. Although the mechanism of the anti-apoptotic function of cIAP1 was not fully elucidated, cIAP1 is overexpressed in certain cancers because of the genetic amplification of cIAP1 locus (11q22), and the cIAP1 overexpression is involved in the resistance to therapy. Because a gene targeting study indicated that disruption of the cIAP1 gene in mice results in no obvious abnormality (39), cIAP1 could be a promising target for cancer therapy.

ME-BS is a derivative of bestatin that has immunomodulatory activity (19–21) and is approved in Japan to treat patients with adult acute nonlymphatic leukemia. A clinical trial with patients whose stage I squamous cell lung carcinoma has been completely resected showed bestatin as a postoperative adjuvant treatment significantly prolonged survival of these patients (22). Because bestatin has a weak effect on reducing cIAP1, it is tempting to speculate that the weak effect of bestatin on the down-regulation of cIAP1 is involved in the prolonged survival reported in the lung cancer patients. However, as we have been able to determine in a mouse model transplanted with human tumor xenograft overexpressing cIAP1, no therapeutic effects have been observed by the administration of bestatin, either alone or in combination with chemotherapeutics. Further study is required to evaluate the role of cIAP1 down-regulation in the survival benefit by bestatin treatment.

In summary, we demonstrated in this paper that selective destabilization of cIAP1 is achievable with a class of small molecules, ME-BS, by activating its ubiquitin-ligase activity. Given the structural similarity of the IAP proteins, it is also of interest to develop a small molecule to reduce other IAPs containing RING finger and UBC domains, such as XIAP, ML-IAP, and Apollon, the overexpression of which has been reported in various cancers (6, 7, 40–42).

Acknowledgments—We thank Drs. J. Inazawa and Y. Shimada for cell lines with cIAP1 gene amplification. We also thank T. Kumagai for technical assistance in SPR analysis. We appreciate Drs. H. Osada, Y. Hashimoto, A. Tanatani, A. Kitao, Y. Johchi, and N. Fujita for helpful discussions.

REFERENCES

1. Deveraux, Q. L., and Reed, J. C. (1999) Genes Dev. 13, 239–252
2. Salvesen, G. S., and Duckett, C. S. (2002) Nat. Rev. Mol. Cell. Biol. 3, 401–410
3. Vaux, D. L., and Silke, J. (2005) Nat. Rev. Mol. Cell. Biol. 6, 287–297
4. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) Nature 388, 300–304
5. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) EMBO J. 16, 6914–6925
6. Kasof, G. M., and Gomes, B. C. (2001) J. Biol. Chem. 276, 3238–3246
7. Vucic, D., Steen, Stenstie, H. R., Pisabarro, M. T., Salvesen, G. S., and Dixit, V. M. (2000) Curr. Biol. 10, 1359–1366
8. Hao, Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., Ohata, H., Katayama, R., Hashimoto, C., Zhang, X., Noda, T., Tsuuru, T., and Naito, M. (2004) Nat. Cell Biol. 6, 849–860
9. Bartke, T., Pohl, C., Pyrowolakis, G., and Jentsch, S. (2004) Mol. Cell 14, 801–811
10. Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) J. Biol. Chem. 273, 7787–7790
11. Srinivasula, S. M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R. A., Robbins, P. D., Fernandes-Alnemri, T., Shi, Y., and Alnemri, E. S. (2001) Nature 410, 112–116
12. Suzuki, Y., Nakabayashi, Y., and Takahashi, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8662–8667
13. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) Science 288, 874–877
14. Imoto, I., Yang, Z. Q., Pinkhaotham, A., Tsuda, H., Shimada, Y., Imamura, M., Ohki, M., and Inazawa, J. (2001) Cancer Res. 61, 6629–6634
15. Imoto, I., Tsuda, H., Hirasa, A., Miura, M., Sakamoto, M., Hirohashi, S., and Inazawa, J. (2002) Cancer Res. 62, 4860–4866
16. Dai, Z., Zhu, W. G., Morrison, C. D., Brenn, R. M., Smiraglia, D. J., Raval, A., Wu, Y. Z., Rush, L. J., Ross, P., Molina, J. R., Otterson, G. A., and Plass, C. (2003) Hum. Mol. Genet. 12, 791–801
cIAP1 Auto-ubiquitylation by ME-BS

17. Zender, L., Spector, M. S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S. T., Luk, J. M., Wigler, M., Hannon, G. J., Mu, D., Lucito, R., Powers, S., and Lowe, S. W. (2006) Cell 125, 1253–1267
18. Tamplin, J., Kornblau, S. M., Segal, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000) Clin. Cancer Res. 6, 1796–1803
19. Abe, F., Matsuda, A., Schneider, M., and Talmadge, J. E. (1990) Cancer Immunol. Immunother. 32, 75–80
20. Sasaki, S., Fukushima, J., Hamajima, K., Ishii, N., Tsuji, T., Xin, K. Q., Mohri, H., and Okuda, K. (1998) Clin. Exp. Immunol. 111, 75–80
21. Talmadge, J. E., Lenz, B. F., Pennington, R., Long, C., Phillips, H., Schneider, M., and Tribble, H. (1986) Cancer Res. 46, 4505–4510
22. Ichinose, Y., Genka, K., Koike, T., Kato, H., Watanabe, Y., Mori, T., Iioka, S., Sakuma, A., and Ohta, M. (2003) J. Natl. Cancer Inst. 95, 605–610
23. Sekine, K., Fujii, H., and Abe, F. (1999) Leukemia 13, 729–734
24. Sekine, K., Fujii, H., Abe, F., and Nishikawa, K. (2001) Int. J. Cancer 94, 485–491
25. Nishizawa, R., Saino, T., Takita, T., Suda, H., and Aoyagi, T. (1977) J. Med. Chem. 20, 510–515
26. Chen, Z., Seimiya, H., Naito, M., Mashima, T., Kizaki, A., Dan, S., Imaizumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999) Oncogene 18, 173–180
27. Yang, Q. H., and Du, C. (2004) J. Biol. Chem. 279, 16963–16970
28. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
29. Wang, X. (2001) Genes Dev. 15, 2922–2933
30. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Cell 102, 33–42
31. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., and Vaux, D. L. (2000) Cell 102, 43–53
32. Li, L., Thomas, R. M., Suzuki, H., De Brabander, J. K., Wang, X., and Harran, P. G. (2004) Science 305, 1471–1474
33. Oost, T. K., Sun, C., Armstrong, R. C., Al-Assaad, A. S., Betz, S. F., Decker, T. L., Ding, H., Elmore, S. W., Meadows, R. P., Olejniczak, E. T., Oleksijew, A., Olstersdorf, T., Rosenberg, S. H., Shoemaker, A. R., Tomasselli, K. J., Zou, H., and Fesik, S. W. (2004) J. Med. Chem. 47, 4417–4426
34. Sun, H., Nikolovska-Coleska, Z., Yang, C. Y., Xu, L., Tomita, Y., Krajewski, K., Roller, P. P., and Wang, S. (2004) J. Med. Chem. 47, 4147–4150
35. Zobel, K., Wang, L., Varfolomeev, E., Franklin, M. C., Elliott, L. O., Wallweber, H. J., Okawa, D. C., Flygare, J. A., Vucic, D., Fairbrother, W. J., and Deshayes, K. (2006) ACS Chem. Biol. 1, 525–533
36. Schimmer, A. D., Welsh, K., Pinilla, C., Wang, Z., Krajewski, M., Bonneau, M. J., Pedersen, I. M., Kitada, S., Scott, F. L., Bailly-Maitre, B., Glinsky, G., Scudiero, D., Sausville, E., Salvesen, G., Nefzi, A., Ostresh, J. M., Houghten, R. A., and Reed, J. C. (2004) Cancer Cell 5, 25–35
37. Hunter, A. M., Kottachchi, D., Lewis, J., Duckett, C. S., Korneluk, R. G., and Liston, P. (2003) J. Biol. Chem. 278, 7494–7499
38. Eckelman, B. P., and Salvesen, G. S. (2006) J. Biol. Chem. 281, 3254–3260
39. Conze, D. B., Albert, L., Ferrick, D. A., Goeddel, D. V., Yeh, W. C., Mak, T., and Ashwell, J. D. (2005) Mol. Cell. Biol. 25, 3348–3356
40. Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T., and Tsuruo, T. (1999) Biochem. Biophys. Res. Commun. 264, 847–854
41. Ferreira, C. G., van der Valk, P., Span, S. W., Jonker, J. M., Postmus, P. E., Kruyt, F. A., and Giaccone, G. (2001) Ann. Oncol. 12, 799–805
42. Krajewska, M., Krajewski, S., Baners, S., Huang, X., Turner, B., Buben- dorf, L., Kallioniemi, O. P., Shabaik, A., Vitelli, A., Pehl, D., Gao, G. J., and Reed, J. C. (2003) Clin. Cancer Res. 9, 4914–4925