Antitumor activity and pharmacodynamic biomarkers of a novel and orally available small molecule antagonist of Inhibitor of Apoptosis Proteins

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Inhibitor of apoptosis proteins (IAPs), which are key regulators of apoptosis, are inhibited by second mitochondria-derived activator of caspase (SMAC). Small molecule IAP antagonists have recently been reported as novel therapeutic treatments for cancer. In this study, we showed that the octahydro-pyrrolo[1,2-a]pyrazine derivative, T-3256336, is a novel and orally available small molecule IAP antagonist. T-3256336 selectively binds to and antagonizes protein interactions involving cellular IAP-1 (cIAP-1), cIAP-2, and X-linked IAP (XIAP). T-3256336 induced the rapid proteasomal degradation of cIAP-1 and activated tumor necrosis factor (TNF)-α-dependent extrinsic apoptosis signaling in cultured cells. In a MDA-MB-231-Luc breast cancer xenograft model, T-3256336 induced cIAP-1 degradation, TNF-α production, and caspase activation in tumors, which resulted in strong antitumor activities. T-3256336 induced increases in the plasma levels of TNF-α and fragmented cytokeratin 18, which correlated with the antitumor potency in MDA-MB-231-Luc xenograft models. This study provided further insights into biomarkers of IAP antagonists. Furthermore, our data provided evidence that T-3256336 is a promising new anticancer drug worthy of further evaluation and development.
The targeting of critical apoptosis inhibitors is an attractive cancer therapeutic strategy (1-3). Inhibitor of apoptosis proteins (IAPs) are a class of proteins that contain tandem duplications of a unique motif known as the baculoviral IAP repeat (BIR) motif, which was originally identified in baculoviruses (insect viruses; v-IAPs) (4). In mammals, 8 IAPs (X-linked IAP [XIAP], cellular IAP-1 [cIAP-1], cIAP-2, IAP-like protein-2 [ILP2], NAIAP, melanoma IAP [MLIAP], survivin, and BRUCE) have been identified (5). Among these IAP proteins, cIAP-1 and cIAP-2 play a critical role in the regulation of tumor necrosis factor (TNF) receptor-mediated apoptosis, and XIAP is a central regulator of both the death receptor-mediated and mitochondria-mediated apoptosis pathways (6-8). The third BIR domain (BIR3) of XIAP selectively binds to and inhibits the initiator caspase-9 (9), while the second BIR (BIR2) domain binds to and inhibits effector caspase-3/caspase-7 (10-12). Consistent with their role in the inhibition of apoptosis, XIAP and cIAP-1 are highly expressed in cancers of diverse tumor types (13-16) and are considered new cancer therapeutic targets (17, 18).

The second mitochondria-derived activator of caspase (SMAC)/direct IAP-binding protein with low pI (DIABLO) is an endogenous antagonist of IAP proteins (19, 20). The pro-apoptotic function of SMAC is dependent on a conserved 4-residue
IAP-interaction motif (Ala-Val-Pro-Ile) that is found at the amino terminus of the mature protein (21, 22). Recent independent studies have shown that IAP antagonists induce the rapid degradation of cIAP-1, which leads to nuclear factor-kappa B (NF-κB) activation, and the production and secretion of TNF-α, and the TNF-α-dependent apoptosis (23-26). Several IAP antagonists, including AT-406, LCL-161, GDC-0152, TL-32711, and HGS-1029, which mimic the interactions of the SMAC amino-terminal peptide with IAP proteins, have been developed and are currently being evaluated in clinical settings (27-30). The identification and establishment of biomarkers that report apoptotic cell death in tumors or in surrogate tissues, such as blood, is one of the key issues in the development of IAP antagonists.

In this study, we investigated the therapeutic potential of the novel and orally available IAP antagonist, T-3256336. We showed that T-3256336 effectively inhibited tumor growth and caused tumor regression without significant body weight loss. In the pharmacodynamic studies, we showed that the circulating levels of TNF-α, the marker of cell death, correlated with tumor growth inhibition (TGI). Our data provided evidence that T-3256336 is a promising new anticancer drug that is worthy of further evaluation and development. Our data also showed the potential of circulating TNF-α and cytokeratin-18 as biomarkers to predict the clinical efficacy of IAP antagonist.
Materials and Methods

Chemical synthesis

The \((7R)\text{-ethoxy-octahydro-pyrrolo[1,2-a]pyrazine}\) derivative, T-3256336, was synthesized and purified according to methods described in Supplementary information and the patents filed previously (31).

Cell lines, Proteins, Peptides, and Reagents

MDA-MB-231, MDA-MB-468, BT-474 SK-OV-3, and T-47D, HL-60 and NCI-H1703 cancer cell line and MRC5 normal lung fibroblasts were obtained from the ATCC (Manassas, VA, USA). The culture medium that was recommended by the suppliers was used for the cultivation of each cell line. MDA-MB-231 cells stably expressing luciferase (MDA-MB-231-Luc) were established at Takeda Pharmaceutical Company, Ltd. (TPC, Fujisawa, Kanagawa, Japan) by transfecting a firefly luciferase expression vector (Promega Corporation, Madison, WI, USA) into MDA-MB-231 cells. Commercially obtained cells were not authenticated by the authors. An antibody against cIAP-1 (AF8181), cIAP-2 (AF8171), and human TNF\(\alpha\) (MAB210) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-XIAP (610762) was purchased from BD Biosciences (Albertslund, Denmark). Anti-Livin antibody (88C570) was from
IMGENEX Corporation (San Diego, CA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; MAB374) antibody was from Millipore (Bedford, MA, USA). Anti-IκBα (#4814), anti-phospho-IκBα (Ser32) (#2859), anti-NF-κB p65 (#3034), anti-phospho-NF-κB p65 (Ser536) (#3033), anti-c-Jun N-terminal kinase (JNK) (#9258), anti-phospho-JNK (Thr183/Tyr185) (#9251), anti-phospho-p38 (Thr180/Tyr182) (#9211), anti-p38 (9217), anti-caspase-8 (#9746), and anti-caspase-3 (#9665) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). MG-132 (#474791), pan-caspase inhibitor z-BAD-fmk (#219007), caspase-8 inhibitor z-IETD-fmk (#218759) were purchased from Calbiochem (San Diego, CA, USA). The recombinant human XIAP (residues 124–357, XIAP_BIR2-BIR3) and caspase-9 were prepared at Takeda California, and the 6-His-tagged recombinant BIR3 domains of human cIAP-1 (residues 250–350, cIAP-1_BIR3) and cIAP-2 (residues 238–349, cIAP-2_BIR3) were prepared at TPC. The recombinant BIR3 domain of human XIAP (residues 252–356) fused to a N-terminal His-tag (XIAP_BIR3) was purchased from R&D Systems, Inc., and the SMAC-N7 peptide (AVPIAQK) was purchased from Merck KgaA (Darmstadt, Germany). C-terminal-biotinylated SMAC-N7 peptide [AVPIAQ-K(biotin)-NH2] (biotinyl-SMAC) was synthesized at Peptide Institute Inc (Osaka, Japan). The cryptate-conjugated mouse monoclonal antibody anti-6-Histidine (Anti-6HIS Cryptate), high-grade
XL665-conjugated streptavidin (SA-XL), and homogeneous time-resolved fluorescence resonance energy transfer (HTRF) detection buffer were purchased from Sceti Medical Labo K.K. (Tokyo, Japan). Recombinant human caspase-3 and caspase-7 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Binding activities using HTRF technology**

Five μL of IAP proteins (40 nM of XIAP_BIR3 and 8 nM of cIAP-1/-2_BIR3) and 5 μL of increasing concentrations of compounds were added to the wells containing assay buffer (25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 100 mM NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, pH 7.5). After shaking, 5 μL of biotinyl-SMAC (20 nM of XIAP_BIR3, 80 nM of cIAP-1_BIR3, and 120 nM of cIAP-2_BIR3 dissolved in assay buffer) was added to the well, which was followed by adding 5 μL of the mixture of Anti-6HIS Cryptate and SA-XL, which was diluted 100 times with HTRF detection buffer. After an overnight incubation at room temperature in the dark, HTRF measurements were conducted with a EnVision multi-label reader (PerkinElmer Inc., Waltham, MA). Fluorescence at 615 nm (F615 nm) is the total europium signal, and fluorescence at 665 nm (F665 nm) is the fluorescence resonance energy transfer signal. The ratio [(F665 nm/F615 nm) × 10,000] was calculated, and
IC50 values were determined using the ratio with nonlinear regression curve fitting with Prism (Version 5.01, GraphPad Software, Inc., La Jolla, CA, USA).

**Cell-free functional assay**

Various concentrations of T-3256336 or SMAC·N7, XIAP_BIR2+3 (40 nM for caspase-3, 4 μM for caspase-7 or 300 nM for caspase-9), and 1 unit of caspase-3/caspase-7/caspase-9 were added to wells in 384-well plates (Corning Incorporated, Lowell, MA) at a final volume of 30 μL in assay buffer (20 mM HEPES, 0.1% CHAPS, 1mM ethylenediaminetetraacetic acid [EDTA], 10% Sucrose, and 10 mM DTT, pH 7.5). After incubating at room temperature for 5 min, 10 μL of 40 μM or 160 μM Ac-DEVD-AMC solution (Enzo Life Sciences, Inc., Farmingdale, NY, USA) for caspase-3 and caspase-7, 50 μM of Ac-LEHD-AMC for caspase-9 were added to the wells, respectively. Following incubation for 30 min at room temperature with shaking, fluorescence at 380 nm excitation and 460 nm emission wavelengths was measured using a multi-mode microplate reader Spectra Max M5e (Molecular Devices, Inc., Sunnyvale, CA, USA). Activity was expressed as EC<sub>50</sub>, which was the concentration at which half-maximum recovery was achieved, with Prism.
Cell viability assay and measurement of caspase activity

Cells were seeded at $3 \times 10^3$ cells/well in 96-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and cultured overnight. On the following day, test compounds were diluted in growth medium to the desired final concentration and then added to the cells. After 24 h of incubation, caspase activities were measured with Caspase-Glo®-3/Caspase-Glo®-7, Caspase-Glo®-8, or Caspase-Glo®-9 Assays (Promega Corporation). After 3 days of incubation, cell viability was measured with a CellTiter-Glo® Luminescent Cell Viability Assay® (Promega Corporation). GI50 values were determined with the ratio by nonlinear regression curve fitting with Prism.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cells were lysed with 100 μL of SDS sample buffer (BioRad Laboratories, Inc., Hercules, CA, USA) and heated at 95°C for 5 min. Each cell lysate was subjected to SDS-PAGE and transferred onto Sequi-Blot™ PVDF Membranes (BioRad Laboratories, Inc.). The membranes were blocked with StartingBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and probed overnight with an antibody diluted 500- to 2000-fold with Can Get Signal® Immunoreaction Enhancer.
Solution I (Toyobo Co., Ltd., Osaka, Japan). The membrane was washed with phosphate-buffered saline containing 0.1% Tween-20 (Wako Pure Chemical Industries, Ltd.) and incubated with horseradish peroxidase-labeled secondary antibody (GE Healthcare, South Burlington, VT, USA) that was diluted 20,000-fold with Can Get Signal® Immunoreaction Enhancer Solution II (Toyobo Co., Ltd.) for 2 h at room temperature. The membrane was washed in the same manner as above, and proteins labeled with the antibody became chemically luminescent with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.) and were detected with the luminoimage analyzer LAS-1000 (Fujifilm Corporation, Tokyo, Japan).

**Quantitative real-time-polymerase chain reaction (RT-PCR)**

Cells were seeded at $5 \times 10^5$ cells/well in 6-well plates and cultured overnight. On the following day, test compounds were diluted in growth medium to the desired final concentration and then added to the cells. The isolation of total RNA was performed with a RNeasy mini kit (QIAGEN Inc., Valencia, CA, USA). About 1 ng of total RNA from each sample was reverse transcribed with the SuperScript® VILO™ cDNA synthesis kit (Life Technologies, Grand Island, NY, USA). For the quantitative
analysis of the levels of mRNA expression, a real-time PCR assay was performed (TaqMan with the 7900HT Fast Real-Time PCR System; Life Technologies) using TaqMan probes.

**In vivo Efficacy study**

Mice were housed and maintained within the facility at TPC in accordance with the Takeda Experimental Animal Care and Use Committee approved protocol. Athymic nude mice (BALB/cAcl·nu/nu) of approximately 5 weeks of age were obtained from CLEA Japan, Inc. (Tokyo, Japan). For the subcutaneous implanted tumor xenograft models, nude mice were injected with $5 \times 10^6$ cells/mouse. When tumor volumes reached approximately 200 mm$^3$, mice were randomly assigned to treatment groups. The compounds were orally administered to the mice in 0.5% methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan). Tumor growth and body weights were measured twice weekly, and average tumor volumes were calculated, as estimated from the formula $[(L \times W^2)/2]$ throughout the study.

**In vivo pharmacokinetics/pharmacodynamics study**

Nude mice were injected with $5 \times 10^6$ MDA-MB-231-Luc cells. When the tumor
volumes reached approximately 200 mm³, mice were randomly assigned to control or treatment groups. Tumors and plasma were collected at multiple time points after the oral administration of T-3256336. The concentrations of T-3256336 in the plasma and tumors were determined by liquid chromatography/tandem mass spectrometry.

Homogenization was performed with a Physcotron (NS-310E, Chiba, Japan) with RIPA buffer containing a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO, USA). The protein concentration in the tumor lysate was determined by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). cIAP-1 protein levels were determined as described above.

Plasma human TNF-α levels were determined with a human TNF-α Quantikine ELISA Kit (R&D Systems, Inc.). The levels of caspase-cleaved (M30) and total (M65) cytokeratin-18 in plasma were determined with an ELISA kit (Peviva AB, Bromma, Sweden).

**Measurement of caspase activity in tumors**

Caspase-3/caspase-7 activities in tumors were measured with a Caspase-Glo assay kit (Promega Corporation). Cytosolic extracts from MDA-MB-231-Luc xenografts were prepared by homogenization in extraction buffer (25 mM HEPES, pH 7.5; 5 mm
MgCl₂; 1 mm EDTA) and subsequently centrifuged (5 min, 10,000 rpm, 4°C). The protein concentration of the supernatant was adjusted to 1 mg/mL with extraction buffer, and an equal volume of reagents and 10 μg/mL cytosolic protein was mixed and incubated at room temperature for 30 min. The luminescence of each sample was measured in a luminometer.

**Histopathological examination of xenograft athymic nude mice**

To determine the effect of T-3256336 on xenograft model, necropsy was conducted on the day after 2-week po administration of T-3256336. The animals were euthanized and were macroscopically examined the external surface of the carcass, the thoracic and abdominal cavities, organs, and tissues. Tumor tissues, liver, spleen, skin and intestines from the control, 30 and 100 mg/kg groups were fixed in 10 vol% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin (H&E) and examined histopathologically.
T-3256336 bound to the BIR3 domains of cIAP-1, cIAP-2, and XIAP

We discovered the octahydro-pyrrolo[1,2-a]pyrazine derivative, T-3256336, was a novel small molecule IAP antagonist (Figure 1A). The binding affinities of T-3256336 to the BIR3 domains of human XIAP, cIAP-1, and cIAP-2 were tested in a HTRF binding assay (Figure 1B). T-3256336 showed high affinities for cIAP-1 and cIAP-2 with IC$_{50}$ values of 1.3 and 2.2 nM, respectively. The IC$_{50}$ value of T-3256336 for XIAP was 200 nM. The intermediate of T-3256336 showed very weak activity against XIAP, cIAP1 and cIAP2 (IC$_{50}$ values> 30 μM) (Supplementary information). We assessed T-3256336 for its ability to inhibit the function of XIAP in a cell-free functional assay. Recombinant human XIAP protein (XIAP_BIR2-BIR3) inhibited the activity of caspase-3 dose-dependently and achieved 80% inhibition at 40 nM (Figure 1C). In these conditions, T-3256336 dose-dependently antagonized XIAP and promoted the activities of caspase-3 with an EC$_{50}$ value of 1.3 μM (Figure 1D). In addition, T-3256336 promoted the activities of caspase-7 and caspase-9 dose-dependently (Supplementary Figure S1B). These data demonstrated that T-3256336 can bind to cIAP-1, cIAP-2, and XIAP and can functionally inhibit XIAP.
T-3256336 induced the proteasomal degradation of cIAP-1, the activation of NF-κB, and the extrinsic apoptosis in MDA-MB-231 breast cancer cells

It has been reported that IAP antagonists induce the degradation of cIAP-1, which leads activation of NF-κB through the stabilization of NIK and the recruitment of RIP1, resulting in TNF-α production and kills sensitive tumor cells through an extrinsic apoptosis in a subset of sensitive tumor cells apoptotic signaling pathway (23-26). We therefore tested the activity of our novel small molecule compound, T-3256336, with respect to cIAP-1 degradation, NF-κB activation, and caspase activation in MDA-MB-231 breast cancer cells. T-3256336 efficiently induced cIAP-1/-2 degradation (IC₅₀ < 5 nM), while it did not affect other IAPs (Figure 2A). The proteasome inhibitor, MG-132, prevented the T-3256336-induced degradation of cIAP-1 protein, which was consistent with the observations for other IAP antagonists (Supplementary Figure S2A). The rapid degradation of cIAP-1 was associated with the increased phosphorylation of IκBα and NF-κB p65, which was indicative of NF-κB activation (Supplementary Figure S2B). TNF-α mRNA levels were time-dependently induced by treatment with T-3256336 (Figure 2B). TNF-α secretion into culture medium was induced by treatment with T-3256336 (Supplementary Figure S3A). The cleavage of caspase-8 and caspase-3, which is indicative of their activations, was also observed (Supplementary Figure S2B).
T-3256336 activated the executioner caspase-3/caspase-7 and the initiator caspase-8 but not caspase-9 with 4 h of treatment (Figure 2C). T-3256336 inhibited the proliferation of MDA-MB-231 breast cancer cell with a mean GI50 value of 1.8 nM, while proliferation of the normal human lung fibroblast MRC5 cells was not inhibited (Figure 2D). T-3256336 also inhibited the growth of MDA-MB-468, NCI-H1703 and SK-OV-3 cells but not that of T-47D and BT-474 cells (Supplementary Figure S4). The precise mechanisms underlying the different sensitivity remain to be elucidated. Human TNF neutralizing antibody, Z-Bad-FMK, which is a pan-caspase inhibitor, and Z-IETD-FMK, which is a selective caspase-8 inhibitor, markedly inhibited the activity of T-3256336 (Supplementary Figure S3B). These data indicated that T-3256336 functions as a potent and selective antagonist of IAPs in cells.

**Pharmacokinetic profile of orally administered T-3256336 in nude mice**

To investigate the pharmacokinetic properties of T-3256336, T-3256336 was orally administered to mice with xenograft tumors. The concentration of T-3256336 was measured during the time range of 0 to 72 h in plasma and xenograft tumors. The area under the curve (AUC) values of the concentration of T-3256336 were 0.29, 1.85, 3.53, and 8.03 μg/mL in plasma and 4.02, 11.20, 23.05, and 63.09 μg·h/mg in tumors when
administered at doses of 10, 30, 50, and 100 mg/kg, respectively (Figure 3, Supplementary Table S1). The AUC values in the tumors were 6- to 14-fold higher than those in plasma. These data clearly showed that T-3256336 was orally absorbed and efficiently distributed to tumor tissues.

**T-3256336 induced rapid degradation of cIAP-1 TNF-α-dependent apoptosis in tumor tissues**

To investigate the in vivo activities, T-3256336 was orally administered to mice bearing xenografts of MDA-MB-231-Luc cells at doses of 10, 30, 50, and 100 mg/kg. A single administration of T-3256336 at 10 mg/kg markedly decreased the levels of cIAP-1 protein in tumors within 30 min and the effect lasted for about 24 h. The cIAP-1 protein degradation was induced more rapidly with 100 mg/kg (Figure 4A, Supplementary Figure S5). Human TNF-α levels in plasma were dose-dependently increased, and the maximum level was observed 6 h after administration (Figure 4B). Robust activations of caspase-3/caspase-7 in tumors were observed with 30 mg/kg or more, and the effects were dose-dependently prolonged (Figure 4C). In addition, we analyzed the levels of caspase-cleaved cytokeratin (M30) and total cytokeratin 18 (M65). Both M30 and M65 levels in plasma were dose-dependently increased with 30 mg/kg or more (Figure 4D).
and 4E). These data showed that orally administered T-3256336 exerted its effects in xenograft tumors and that the effect could be monitored with serum biomarkers.

**In vivo efficacy of T-3256336 in xenograft mice models**

To evaluate the therapeutic potential, T-3256336 was administered to mice bearing MDA-MB-231-Luc xenograft tumors once a day for 14 days, and the effects on tumor growth, as well as on body weight, were examined. Treatment with T-3256336 at 10 mg/kg completely inhibited tumor growth during the treatment (T/C = <5%). Treatment with T-3256336 at 30, 50, and 100 mg/kg reduced the tumor volume from around 200 mm³ to 84 mm³, 56 mm³, and 39 mm³, respectively, at the end of the treatment, which was a reduction of 79%, 86%, and 90%, respectively (Figure 5A, Supplementary Table S2). Importantly, no significant body weight loss was observed in mice when 10, 30 or 50 mg/kg of T-3256336 was administered (Figure 5B). In HL-60 xenograft model, T-3256336 dose-dependently inhibited tumor growth, and after the 2-week treatment, dose-dependent increases of necrotic area and apoptotic cells in tumor were observed (Supplementary Figure S6A and S6B). In a histopathological analysis, atrophy of hair follicles and hyperkeratosis in the skin and slight increase of granulopoiesis in the spleen were noted at 100 mg/kg. No histopathological
abnormalities in the liver and intestines were detected up to 100 mg/kg (Supplementary Figure S7). Taken together, our data showed that T-3256336 can exert antitumor effects without severe adverse effects.

**Correlation between pharmacodynamic biomarkers and antitumor effects in the MDA-MB-231-Luc xenograft model**

To analyze the correlations between pharmacodynamics and anti-tumor efficacy, we calculated the area under the effect (AUE) of pharmacodynamic parameters. The total pharmacodynamic responses of cIAP-1 degradation [AUE cIAP-1 degradation (0–24 h)] in the tumor were saturated around 10 mg/kg (Figure 6A). The AUE of TNF-α secretion [AUE TNF-α (0–24 h)], M30 [AUE M30 (0–48 h)], and M65 [AUE M65 (0–48 h)] in plasma increased dose-dependently with 10 to 50 mg/kg of T-3256336. However, they were similar at around 50 mg/kg (Figure 6B, 6D, and 6E). In contrast, the AUE of caspase activation [AUE caspase activation (0–48 h)] in tumors increased dose-dependently up to 100 mg/kg (Figure 6C). Tumor growth inhibition was positively correlated with the AUE of TNF-α, M30, and M65 levels in plasma in MDA-MB-231-Luc xenograft models. (Supplementary Figure S8). These data suggested that pharmacodynamic parameter levels, such as TNF-α, M30, and M65 in plasma, could predict the effects of the compound in the clinic.
Targeting of the IAP family is a widely accepted cancer therapeutic strategy for the induction of tumor-selective cell death (23-26). In this study, we developed a novel and orally available small molecule IAP antagonist, T-3256336, that binds specifically to cIAP-1, cIAP-2, and XIAP. We showed that T-3256336 induced the rapid degradation of cIAP-1, activation of NF-\(\kappa\)B, the production and secretion of TNF-\(\alpha\), and TNF-\(\alpha\)-dependent apoptosis. In addition, we showed that the oral administration of T-3256336 significantly induced rapid cIAP-1 degradation and apoptosis in tumor tissues. Consistent with its potent activity in apoptosis induction in xenograft tumor tissues, T-3256336 was highly effective in the inhibition of tumor growth xenograft mice model.

The binding-inhibitory activities of T-3256336 against cIAP-1-BIR3 and cIAP-2-BIR3 were stronger than the binding-inhibitory activity against XIAP-BIR3. These cIAP-dominant profiles of T-3256336 were confirmed by a co-crystal structural analysis of T-3256336 with both XIAP and cIAP-1, which indicated a higher binding affinity of the T-3256336 against cIAP-1 (manuscript in preparation). A cell-free functional assay with recombinant XIAP containing BIR2-BIR3 showed that T-3256336 had XIAP inhibition potency, which has been reported to be important for the sufficient
induction of apoptosis by IAP antagonists. In the pharmacokinetic analysis, dose-dependent pharmacokinetics and a relatively high tumor concentration were observed. The concentration of T-3256336 in the tumors at 30, 50, and 100 mg/kg dosing was sufficient for the functional inhibition of XIAP. Therefore, these data suggested that T-3256336 has the potential to show strong efficacy as an orally available IAP antagonist in mouse models.

Our in vivo data clearly showed that a single dose of T-3256336 was effective in inducing rapid cIAP-1 protein degradation, TNF-α secretion, caspase activation, and apoptosis in the MDA-MB-231-Luc tumor tissues. cIAP-1 protein levels were decreased within 30 min after a single administration of T-3256336 at 10, 30, 50, and 100 mg/kg, and the effect lasted for at least 24 h. Furthermore, T-3256336 induced TNF-α secretion (maximum concentration at 6 h post-dose) and caspase activation (maximum activity at 12 h post-dose). The time difference in the maximum concentration between cIAP-1 protein degradation and TNF-α secretion/caspase activation was a reasonable response based on the mechanisms of action of IAP antagonists. We found that AUE cIAP-1 degradation (0–24 h) and AUE TNF-α (0–24 h) increased dose-dependently in the dose range of 10 to 50 mg/kg in the MDA-MB-231-Luc xenograft model. However, there was no difference between cIAP-1 protein degradation and TNF-α secretion levels above 50 mg/kg,
thus, the E_{max} for the pharmacodynamic response could be saturated at a dose around 30 to 50 mg/kg. Both cIAP-1 degradation and TNF-\(\alpha\) secretion were expected to reflect the cIAP-1 inhibition potency. Therefore, the concentration of T-3256336 in tumor tissues could be adequate for triggering the induction of TNF-\(\alpha\)-dependent apoptosis in MDA-MB-231-Luc xenograft tumors based on the strong cIAP-1 inhibition potency of T-3256336. However, we found that AUE_{caspase activation (0–48 h)} increased dose-dependently. Caspase activation at the peak time was almost the same as those for doses at 50 and 100 mg/kg of T-3256336. However, caspase activation lasted longer for doses at 100 mg/kg of T-3256336 compared to doses of 50 mg/kg of T-3256336, reflecting the prolonged concentration of T-3256336 in tumors at 100 mg/kg. Therefore, XIAP inhibition potency could contribute to the prolonged caspase activation in MDA-MB-231-Luc xenograft models. As a reflection of this prolonged caspase activation, T-3256336 showed strong tumor growth inhibition potency with a once-a-week administration regimen of 100 mg/kg in MDA-MB-231-Luc xenograft models (data not shown). These data suggested that both cIAP-1 inhibition and XIAP inhibition are necessary for efficient apoptosis induction in TNF-\(\alpha\)-dependent tumors. Consistent with this profile of T-3256336, our data indicated that T-3256336 exhibited a very strong in vivo antitumor activity at nontoxic dose schedules. However, an optimal human dose
and schedule need to be determined in clinical studies.

Cytokeratins are expressed in most epithelial cells, and, in many carcinomas, fragmented or complexed cytokeratins have been detected in the circulation of patients with epithelial malignancies. Thus, they have been evaluated as tumor biomarkers (32-35). The M65 assay detects full-length and caspase-cleaved cytokeratin 18 and thus has been proposed as a biomarker of caspase-dependent and caspase-independent cell death (36). The M30 assay detects only a cytokeratin 18 neoepitope that is generated following caspase cleavage and that is considered a specific assay for epithelial apoptosis (37-39). Several reports have recently suggested that the circulating form of cytokeratin 18 is predictive of tumor response to drug treatment and may have prognostic significance (40-42). In this study, both the circulating levels of M30 and M65 in plasma increased dose-dependently, peaking 9 h after a single dose of T-3256336. Recently, several reports have revealed that the loss of cIAP proteins can modulate programmed necrosis, necroptosis, as well as apoptosis (43, 44). Therefore, not only M30 but also M65 levels could be increased dose-dependently as a response to T-3256336 administration. In addition, both AUE M30 (0–48 h) and AUE M65 (0–48 h) were positively correlated with the TGI in MDA-MB-231·Luc xenograft models. These data suggested that the measurement of circulating M30 and M65 levels in patient samples could be a
promising method for determining the efficiency of IAP antagonists in the clinic.

In this study, we showed that cIAP-1 expression levels and caspase activation in tumor tissues and circulating TNF-α, M30, and M65 levels were useful for detecting the in vivo efficiency of IAP antagonists at an early stage of treatment as these pharmacodynamic parameters correlated with TGI. This study provided further insights into the biomarkers of IAP antagonists. Circulating TNF-α, M30, and M65 levels may be potential biomarkers for detecting cell death and clinical efficiency as invasive biomarkers in the clinic. Furthermore, our data provided evidence that T-3256336 is a promising new anticancer drug worthy of further evaluation and development.
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Figure 1. Chemical structure and binding affinity to the baculoviral IAP repeat-3 (BIR3) domain of X-linked inhibitor of apoptosis protein (XIAP), cellular IAP (cIAP)-1, and cIAP-2.

A: Chemical structure of T-3256336.

B: Inhibition Curves of T-3256336 on binding to the BIR3 domain of XIAP, cIAP-1, or cIAP-2 as determined with a HTRF method.

C: XIAP_BIR2+3 protein inhibited caspase-3 activity dose-dependently.

D: Recovery effect of T-3256336 on caspase-3 activity that was inhibited by XIAP_BIR2+3. Caspase-3 was incubated with various concentrations of T-3256336 and XIAP_BIR2+3 for 5 min at room temperature. Ac-DEVD-AMC was then added and incubated for 30 min. Fluorescence detection of substrate cleavage was assessed.

Figure 2. Biochemical activity of T-3256336 in MDA-MB-231 cells.

A: T-3256336 causes a rapid loss of c-IAP1. MDA-MB-231 cells were treated with T-3256336 at the indicated concentration for 8 h, and cell lysates were examined by western blotting with antibodies against c-IAP1, c-IAP2, XIAP, and Livin.

B: T-3256336 stimulates tumor necrosis factor (TNF)-α mRNA expression. A
quantitative real-time polymerase chain reaction analysis of TNF-α mRNA expression levels was done on the RNA samples derived from MDA-MB231 treated with T-3256336 (0.5 μmol/L) for the indicated time periods. All values were normalized to a GAPDH internal control.

C: T-3256336 treatment activated caspase-8 and caspase-3/caspase-7. MDA-MB-231 cells were treated with T-3256336 (0.5 μmol/L) for the indicated time periods and caspase activity was determined.

D: T-3256336 inhibits the cellular proliferation of MDA-MB231 cells, but not in MRC-5 cells. MDA-MB-231 and MRC-5 cells were treated with the indicated concentrations of T-3256336 for 3 days and cell viability was determined.

Figure 3. Pharmacokinetic analysis of T-3256336 in mice bearing MDA-MB-231-Luc cancer cells. Concentrations of T-3256336 in plasma (A) and tumors (B) after the oral administration at doses of 10, 30, 50, and 100 mg/kg were plotted. Data represent the means and standard deviations of 3 samples.

Figure 4. T-3256336 shows pharmacodynamic activity in MDA-MB-231-Luc xenograft models. MDA-MB-231-Luc tumor-bearing mice were orally administered T-3256336 at
10, 30, 50, or 100 mg/kg. Mice were sacrificed at the indicated time after the single treatment of T-3256336, and tumor and plasma pharmacodynamics parameters were measured. A: Tumor cIAP1 protein levels were determined by western blotting. Plasma human TNF-α levels (B) were determined by ELISA. C: Tumor caspase-3/caspase-7 activity was determined after the indicated time periods. Plasma M30 (D) and M65 (E) levels were determined by ELISA. The data are presented as means and standard deviations from 3 mice in each group (N = 3).

**Figure 5.** Single agent efficacy of T-3256336 in MDA-MB-231-Luc xenograft models. MDA-MB-231-Luc tumor-bearing mice were treated with T-3256336 daily at 10, 30, 50, or 100 mg/kg/day for 14 days. A tumor growth curve is shown in (A), and body weight changes are shown in (B). The data represent the means and standard deviations from 5 mice in each group. Statistical significance was determined by Shirley-Williams test, *, P < 0.05, **, P < 0.01. vs vehicle treatment.

**Figure 6.** Total pharmacodynamic responses of the pharmacodynamic parameter area under the effects (AUEs) in MDA-MB-231-Luc xenograft models. The AUEs of cIAP-1 degradation (A) and human TNF-α (B) up to 24 h after a single dose of T-3256336.
Caspase activation (C) and M30 (D), and M65 (E) levels up to 48 h after a single dose of T-3256336 were calculated by multiplying the cumulative mean values by time (1 h equals 1 unit).
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Antitumor activity and pharmacodynamic biomarkers of a novel and orally available small molecule antagonist of Inhibitor of Apoptosis Proteins

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