Antitumor Activity and Pharmacodynamic Biomarkers of a Novel and Orally Available Small-Molecule Antagonist of Inhibitor of Apoptosis Proteins

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Abstract

Inhibitor of apoptosis proteins (IAP), 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 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. Mol Cancer Ther; 12(2); 230–40. ©2012 AACR.

Introduction

The targeting of critical apoptosis inhibitors is an attractive cancer therapeutic strategy (1–3). Inhibitor of apoptosis proteins (IAP) 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; ref. 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 TNF receptor-mediated apoptosis, and XIAP is a central regulator of both the death receptor- and mitochondria-mediated apoptosis pathways (6–8). The third BIR domain (BIR3) of XIAP selectively binds to and inhibits the initiator caspase-9 (9), whereas 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 pl (DIABLO) is an endogenous antagonist of IAPs (19, 20). The proapoptotic 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 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)-ethoxy-octahydro-ppyrollo[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 American Type Culture Collection. 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) by transfecting a firefly luciferase expression vector (Promega Corporation) 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-α (MAB210) was purchased from R&D Systems, Inc. Anti-XIAP (610762) was purchased from BD Biosciences. Anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH; MAB374) antibody was obtained from Novus Biologicals, Inc. Anti-Livin antibody (88C570) was from Biochemical Sciences, Inc. Anti-XIAP (610762) was purchased from BD Biosciences. Anti-IAP antibody against cIAP-1 (AF8181), cIAP-2 (AF8171), and IAP-BIR3 and 8 nmol/L of cIAP-1/-2_BIR3) and 5 µL of increasing concentrations of compounds were added to the wells containing assay buffer [25 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mmol/L NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, pH 7.5]. After shaking, 5 µL of biotinyl-SMAC (20 mmol/L of XIAP_BIR3, 80 mmol/L of cIAP-1_BIR3, and 120 mmol/L 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 an EnVision multi-label reader (PerkinElmer Inc.). Fluorescence at 615 nm (F615 nm) is the total europium signal, and fluorescence at 665 nm (F665 nm) is the FRET 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.).

Cell-free functional assay
Various concentrations of T-3256336 or SMAC-N7, XIAP_BIR2+3 (40 nmol/L for caspase-3, 4 µmol/L for caspase-7, or 300 nmol/L for caspase-9), and 1 unit of caspase-3/caspase-7/caspase-9 was added to wells in 384-well plates (Corning Incorporated) at a final volume of 30 µL in assay buffer [20 mmol/L HEPES, 0.1% CHAPS, 1 mmol/L EDTA, 10% sucrose, and 10 mmol/L diithiothreitol (DTT), pH 7.5]. After incubating at room temperature for 5 minutes, 10 µL of 40 or 160 µmol/L Ac-DEVD-AMC solution (Enzo Life Sciences, Inc.) for caspase-3 and caspase-7, 50 µmol/L of Ac-LEHD-AMC solution (Enzo Life Sciences, Inc.) for caspase-9 were added to the wells, respectively. Following incubation for 30 minutes at room temperature with shaking, fluorescence at 380 nm excitation and 460 nm emission wavelengths was measured using a multimode microplate reader Spectra Max M5e (Molecular Devices, Inc.). Activity was expressed as EC50, 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 × 103 cells per well in 96-well plates (Sumitomo Bakelite Co., Ltd.) 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 hours 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). GI$_{50}$ values were determined with the ratio by nonlinear regression curve fitting with Prism.

**SDS-PAGE and Western blotting**

Cells were lysed with 100 µL of SDS sample buffer (BioRad Laboratories, Inc.) and heated at 95°C for 5 minutes. 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.) and probed overnight with an antibody diluted 500- to 2,000-fold with Can Get Signal Immunoreaction Enhancer Solution I (Toyobo Co., Ltd.). The membrane was washed with PBS containing 0.1% Tween-20 (Wako Pure Chemical Industries, Ltd.) and incubated with horseradish peroxidase-labeled secondary antibody (GE Healthcare) that was diluted 20,000-fold with Can Get Signal Immunoreaction Enhancer Solution II (Toyobo Co., Ltd.) for 2 hours 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 luminomage analyzer LAS-1000 (Fujifilm Corporation).

**Quantitative real-time PCR**

Cells were seeded at $5 \times 10^5$ cells per 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 conducted with an RNeasy Mini Kit (Qiagen Inc.). About 1 ng of total RNA from each sample was reverse transcribed with the SuperScript Mini Kit (Qiagen Inc.). The isolated RNA was then amplified using TaqMan probes.

**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 oral 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 and eosin (H&E), and examined histopathologically.

**Results**

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 (Fig. 1A). The binding affinities of T-3256336 to...
the BIR3 domains of human XIAP, cIAP-1, and cIAP-2 were tested in an HTRF-binding assay (Fig. 1B). T-3256336 showed high affinities for cIAP-1 and cIAP-2 with IC50 values of 1.3 and 2.2 nmol/L, respectively. The IC50 value of T-3256336 for XIAP was 200 nmol/L. The intermediate of T-3256336 showed very weak activity against XIAP, cIAP-1, and cIAP-2 (IC50 > 30 μmol/L; 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 nmol/L (Fig. 1C). In these conditions, T-3256336 dose dependently antagonized XIAP and promoted the activities of caspase-3 (Fig. 1D). In addition, T-3256336 promoted the activities of caspase-7 and caspase-9 dose dependently (Supplementary Fig. S1B). These data showed 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 nmol/L), whereas it did not affect other IAPs (Fig. 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 Fig. S2A). The rapid degradation of cIAP-1 was associated with the increased phosphorylation of IkBζ and NF-κB p65, which was indicative of NF-κB activation (Supplementary Fig. S2B). TNF-α mRNA levels were time dependently induced by treatment with T-3256336 (Fig. 2B). TNF-α secretion into culture medium was induced by treatment with T-3256336 (Supplementary Fig. S3A). The cleavage of caspase-8 and caspase-3, which is indicative of their activations, was also observed (Supplementary Fig. S2B). T-3256336 activated the executioner caspase-3/caspase-7 and the initiator caspase-8 but not caspase-9 with 4 hours of treatment (Fig. 2C). T-3256336 inhibited the proliferation of MDA-MB-231 breast cancer cell with a mean GI50 value of 1.8 nmol/L, whereas proliferation of the normal human lung fibroblast MRC5 cells was not inhibited (Fig. 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 Fig. 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 Fig. 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 hours 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/mL in tumors when administered at doses of 10, 30, 50, and 100 mg/kg, respectively (Fig. 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-325636 was orally absorbed and efficiently distributed to tumor tissues. T-325636 induced rapid degradation of cIAP-1 TNF-α–dependent apoptosis in tumor tissues

To investigate the in vivo activities, T-325636 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-325636 at 10 mg/kg markedly decreased the levels of cIAP-1 protein in tumors within 30 minutes and the effect lasted for about 24 hours. The cIAP-1 protein degradation was induced more rapidly with 100 mg/kg (Fig. 4A, Supplementary Fig. S5). Human TNF-α levels in plasma were dose dependently increased, and the maximum level was observed 6 hours after administration (Fig. 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 (Fig. 4C). In addition, we analyzed the levels of caspase-cleaved cytokeratin-18 (M30) and total cytokeratin 18 (M65). Both M30 and M65 levels in plasma were dose dependently increased with 30 mg/kg or more (Fig. 4D and E). These data showed that orally administered T-325636 exerted its effects in xenograft tumors and that the effect could be monitored with serum biomarkers.

In vivo efficacy of T-325636 in xenograft mice models

To evaluate the therapeutic potential, T-325636 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-325636 at 10 mg/kg completely inhibited tumor growth during the treatment (T/C < 5%). Treatment with T-325636 at 30, 50, and 100 mg/kg reduced the tumor volume from around 200 to 84, 56, and 39 mm³, respectively, at the end of the treatment, which was a reduction of 79%, 86%, and 90%, respectively (Fig. 5A, Supplementary Table S2). Importantly, no significant body weight loss was observed in mice when 10, 30, or 50 mg/kg of T-325636 was administered (Fig. 5B). In HL-60 xenograft model, T-325636 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 Fig. S6A and S6B). In a histopathologic 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 histopathologic abnormalities in the liver and intestines were detected up to 100 mg/kg (Supplementary Fig. S7). Taken together, our data showed that T-325636 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 antitumor efficacy, we calculated the area under the effect (AUE) of pharmacodynamic parameters. The total pharmacodynamic responses of cIAP-1 degradation [AUEcIAP-1 degradation(0–24 h)] in the tumor were saturated around 10 mg/kg (Fig. 6A). The AUE of TNF-α secretion [AUETNF-α(0–24 h)], M30 [AUEM30(0–48 h)], and M65 [AUEM65(0–48 h)] in plasma increased dose dependently with 10 to 50 mg/kg of T-325636. However, they were similar at around 50 mg/kg (Fig. 6B, D, and E). In contrast, the AUE of caspase activation [AUEcaspase activation(0–48 h)] in tumors increased dose dependently up to 100 mg/kg (Fig. 6C). TGI was positively correlated with the AUE of TNF-α, M30, and M65 levels in plasma in MDA-MB-231-Luc xenograft models (Supplementary Fig. 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.

Discussion

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-κB, the production and secretion of TNF-α, and TNF-α–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-
degradation, TNF-α secretion/caspase activation was a reasonable response based on the mechanisms of action of IAP antagonists. We found that $AUE_{CIA}-1$ degradation $\alpha -24 h$ and $AUE_{TNF-\alpha}$ 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, and, 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-α 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-α-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 than 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 TGI 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-α-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 -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 hours 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.

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 (A) is shown, and body weight changes (B) are shown. The data represent the means and SDs from 5 mice in each group. Statistical significance was determined by Shirley–Williams test. $P < 0.05$; $P < 0.01$ versus vehicle treatment. q.d., once daily dosing.

Tumor vol. (mm$^3$)

Body weight (g)

Days after inoculation

Days after inoculation
Therefore, not only M30 but also M65 levels could be increased dose dependently as a response to T-3256336 administration. In addition, both AUEM30(0–48 h) and AUEM65(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.

Disclosure of Potential Conflicts of Interest
All authors are employees of Takeda Pharmaceutical Company, Ltd.
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Acknowledgments

The authors thank Akihiro Ohashi, Takeo Arita, Akito Nakamura, Yuichi Hikishi, and Osamu Nakashima for helping with insightful discussions, suggestions, and reagents and the entire IAP team members for their support and many contributions to this project.

Grant Support

All works were funded by Takeda Pharmaceutical Company, Ltd. All authors have not received any grant.

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Received July 6, 2012; revised October 30, 2012; accepted November 29, 2012; published OnlineFirst December 12, 2012.

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