Preclinical Development

Antitumor Action of the MET Tyrosine Kinase Inhibitor Crizotinib (PF-02341066) in Gastric Cancer Positive for MET Amplification

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Abstract
Therapeutic strategies that target the tyrosine kinase MET hold promise for gastric cancer, but the mechanism underlying the antitumor activity of such strategies remains unclear. We examined the antitumor action of the MET tyrosine kinase inhibitor crizotinib (PF-02341066) in gastric cancer cells positive or negative for MET amplification. Inhibition of MET signaling by crizotinib or RNA interference–mediated MET depletion resulted in induction of apoptosis accompanied by inhibition of AKT and extracellular signal–regulated kinase phosphorylation in gastric cancer cells with MET amplification but not in those without it, suggesting that MET signaling is essential for the survival of MET amplification–positive cells. Crizotinib upregulated the expression of BIM, a proapoptotic member of the Bcl-2 family, as well as downregulated that of survivin, X-linked inhibitor of apoptosis protein (XIAP), and c-IAP1, members of the inhibitor of apoptosis protein family, in cells with MET amplification. Forced depletion of BIM inhibited crizotinib-induced apoptosis, suggesting that upregulation of BIM contributes to the proapoptotic effect of crizotinib. Crizotinib also exhibited a marked antitumor effect in gastric cancer xenografts positive for MET amplification, whereas it had little effect on those negative for this genetic change. Crizotinib thus shows a marked antitumor action both in vitro and in vivo specifically in gastric cancer cells positive for MET amplification. Mol Cancer Ther; 11(7); 1–8. ©2012 AACR.

Introduction
Gastric cancer is the second most frequent cause of cancer deaths worldwide (1). Chemotherapy has a beneficial effect on survival in individuals with advanced-stage gastric cancer, but even so overall survival is usually still only about 1 year (1, 2). Substantial advances in the development of molecularly targeted therapies for gastric cancer have been achieved in recent years (3). Amplification of the proto-oncogene MET is a frequent molecular abnormality in gastric cancer (4–6), and a MET-tyrosine kinase inhibitor (TKI) has been shown to induce apoptosis in gastric cancer cells with MET amplification (7, 8).

Crizotinib (PF-02341066; Fig. 1A) was recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with lung cancer positive for fusion of the echinoderm microtubule-associated protein–like 4 (EMLA) and anaplastic lymphoma kinase (ALK) genes. This agent also inhibits MET in addition to oncogenic fusion variants of the tyrosine kinase ALK (9, 10), and it may thus be an attractive therapeutic option for individuals with gastric cancer positive for MET amplification. We have therefore now investigated the effects of crizotinib on cell survival and signal transduction in gastric cancer cells with MET amplification. We further examined the molecular mechanism underlying the antitumor action of MET inhibition.

Materials and Methods

Cell culture and reagents

Human gastric cancer cell lines positive (SNU5, Hs746T, MKN45, HSC58, 58As1, 58As9) or negative (SNU1, N87, AGS, MKN1, MKN7, NUGC3, AZ521, MKN28, HSC39, SNU216) for MET amplification were obtained as previously described (8, 11). All cells were cultured under a humidified atmosphere of 5% CO2 at 37°C in RPMI-1640 medium (Sigma) supplemented with 10% FBS and were passaged for 3 months before renewal from frozen early-passage stocks obtained from the respective sources. Cells were regularly screened for mycoplasma with the use of a MycoAlert Mycoplasma detection kit (Lonza). Crizotinib was kindly provided by...
Pfizer Global Research & Development, and BEZ235 and AZD6244 were from ShangHai Biochempartner.

Immunoblot analysis
Cells were washed twice with ice-cold PBS and then lysed with 1× Cell Lysis Buffer (Cell Signaling Technology) containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA (disodium salt), 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, leupeptin (1 μg/mL), and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration of cell lysates was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% or 12% gel (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque) for 20 minutes at room temperature before incubation overnight at 4°C with primary antibodies. Rabbit polyclonal antibodies to phosphorylated human MET (pY1234/pY1235), total AKT, phosphorylated AKT, phosphorylated extracellular signal–regulated kinase (ERK), total STAT3, phosphorylated STAT3 (phospho-Tyr705), PARP, caspase-3, BIM, Bcl-2, and X-linked inhibitor of apoptosis protein (XIAP) were obtained from Cell Signaling Technology; those to total ERK were from Santa Cruz Biotechnology; those to total MET were from Zymed/Invitrogen; those to survivin were from Novus; those to c-IAP1 were from R&D Systems; and those to β-actin were from Sigma. All antibodies were used at a 1:1,000 dilution, with the exception of those to β-actin (1:200). The membrane was then washed with PBS containing 0.05% Tween-20 before incubation for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Immune complexes were finally detected with ECL Western Blotting Detection Reagents (GE Healthcare).

Gene silencing
Cells were plated at 50% to 60% confluence in 6-well plates or 25-cm² flasks and then incubated for 24 hours before transient transfection for the indicated times with siRNAs mixed with the Lipofectamine reagent (Invitrogen). The siRNAs specific for MET (MET-1, 5’-ACAA-GAUCGUCAACAAAAA-3’; MET-2, 5’-CUACAGAAAU-GGUUUCAAA-3’), BIM (BIM-1, 5’-GGAGGGUAUU-UUGAAUAA-3’; BIM-2, 5’-AAGGAGGUAAUUUUGA-AUA-3’), or AKT (AKT-1, 5’-CAACGGCAAUCCAGACUGU-3’) mRNAs, as well as corresponding scrambled (control) siRNAs were obtained from Nippon EGT. The data presented for the effects of MET, BIM, or AKT depletion were obtained with the corresponding siRNA-1, but similar results were obtained with each siRNA-2.

Annexin V–binding assay
The binding of Annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche).
Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × g for 5 minutes. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 minutes at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

**Cell growth inhibition assay**

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of crizotinib for 72 hours. Tetra Color One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku Kogyo) was then added to each well, and the cells were incubated for 3 hours at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of crizotinib resulting in 50% growth inhibition (IC₅₀) was calculated.

**Growth inhibition assay in vivo**

All animal studies were conducted in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University (Osaka, Japan). The ethical procedures followed conformed to the guidelines of the United Kingdom Co-ordinating Committee on Cancer Research (12). Tumors cells (5 × 10⁶) were injected subcutaneously into the axilla of 5- to 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan). Treatment was initiated when tumors in each group of 6 mice achieved an average volume of 300 to 900 mm³. Treatment groups consisted of vehicle control and crizotinib (25 or 50 mg/kg). Crizotinib was administered by oral gavage daily for 4 weeks, with control and crizotinib (25 or 50 mg/kg). Crizotinib was administered by oral gavage daily for 4 weeks, with control animals receiving a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula LW²/2. Both tumor size and body weight were measured twice per week.

**Statistical analysis**

Quantitative data are presented as means ± SD or SEM from 3 independent experiments or for 6 animals per group, unless indicated otherwise, and were analyzed with the Student 2-tailed t test. A P value of <0.05 was considered statistically significant.

**Results**

**Crizotinib inhibits the proliferation of gastric cancer cells with MET amplification**

We first examined the effect of the MET-TKI crizotinib on the proliferation of gastric cancer cells positive or negative for MET amplification. Six cell lines with MET amplification (MKN45, HSC58, 58As1, 58As9, SNU5, Hs746T) were sensitive to crizotinib, with IC₅₀ values of less than 200 nmol/L (Table 1). In contrast, crizotinib did not substantially inhibit the proliferation of gastric cancer cells without such gene amplification (Table 1). Transcripts of the EML4–ALK fusion gene were not detected in the crizotinib-sensitive gastric cancer cell lines by reverse transcription PCR analysis (data not shown). These data suggested that crizotinib has a marked anti-proliferative effect specifically in gastric cancer cells with MET amplification.

**Effects of crizotinib on downstream signaling of MET and on apoptosis in gastric cancer cells with or without MET amplification**

We next examined the effects of crizotinib on phosphorylation of ERK, AKT, and STAT3 in gastric cancer cell lines. Crizotinib markedly inhibited the phosphorylation of ERK, AKT, and STAT3, as well as that of MET in cells with MET amplification (Fig. 1B; Supplementary Fig. S1). In contrast, crizotinib had little effect on the phosphorylation of ERK, AKT, or STAT3 in gastric cancer cells without amplification of MET (Fig. 1B). Determination of cell-cycle distribution in SNU5, HSC58, 58As1, and 58As9 cells, all of which manifest MET amplification, revealed that treatment with crizotinib for 72 hours increased the size of the cell population in sub-G₁ phase, indicative of the induction of apoptosis, as well as reduced that of the cell population in S-phase (Supplementary Fig. S2). We further investigated the effect of crizotinib on apoptosis.

<table>
<thead>
<tr>
<th>Crizotinib response</th>
<th>Cell line</th>
<th>Crizotinib IC₅₀, μmol/L</th>
<th>MET amplification</th>
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<tbody>
<tr>
<td>Sensitive</td>
<td>MKN45</td>
<td>0.04</td>
<td>+</td>
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<tr>
<td></td>
<td>HSC58</td>
<td>0.05</td>
<td>+</td>
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<tr>
<td></td>
<td>58As1</td>
<td>0.06</td>
<td>+</td>
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<td></td>
<td>58As9</td>
<td>0.17</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SNU5</td>
<td>0.03</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hs746T</td>
<td>0.05</td>
<td>+</td>
</tr>
<tr>
<td>Resistant</td>
<td>MKN1</td>
<td>8.57</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AZ521</td>
<td>1.96</td>
<td>–</td>
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<tr>
<td></td>
<td>SNU216</td>
<td>4.19</td>
<td>–</td>
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<tr>
<td></td>
<td>N87</td>
<td>6.10</td>
<td>–</td>
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<tr>
<td></td>
<td>MKN7</td>
<td>&gt;10</td>
<td>–</td>
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<tr>
<td></td>
<td>SNU1</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>HSC39</td>
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<td>–</td>
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<tr>
<td></td>
<td>AGS</td>
<td>0.90</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MKN28</td>
<td>3.73</td>
<td>–</td>
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<tr>
<td></td>
<td>NUGC3</td>
<td>2.65</td>
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NOTE: Data are means of triplicates from experiments that were repeated a total of 3 times with similar results.

Table 1. IC₅₀ values of crizotinib for inhibition of the growth of gastric cancer cells in vitro
Immunoblot analysis showed that crizotinib triggered the generation of the cleaved forms of caspase-3 and PARP in cells with MET amplification but not in those without it (Fig. 1B). Consistent with these results, an Annexin V–binding assay revealed that crizotinib induced a substantial level of apoptosis in MET amplification–positive cells but was largely without effect in cell lines without MET amplification (Fig. 1C). These data thus suggested that crizotinib inhibits the phosphorylation of ERK, AKT, and STAT3, resulting in induction of apoptosis, in gastric cancer cells with MET amplification, whereas such effects were not observed in cells without MET amplification.

Effects of crizotinib on the expression of apoptosis-related proteins in MET amplification–positive gastric cancer cells

Given that crizotinib induced apoptosis in MET amplification–positive gastric cancer cells, we examined the effects of this drug on the expression of apoptosis-related proteins in such cells. Crizotinib upregulated the expression of BIM, a proapoptotic member of the Bcl-2 family of proteins, whereas it had little effect on the expression of other Bcl-2 family members including Bcl-2 (Fig. 2A). Furthermore, crizotinib downregulated the expression of members of the IAP family including survivin, XIAP, and c-IAP1 in cells with MET amplification (Fig. 2A).

To verify that the effects of crizotinib on the expression of apoptosis-related proteins in MET amplification–positive gastric cancer cells are indeed mediated by MET inhibition rather than by nonspecific inhibition of other kinases, we transfected gastric cancer cells with siRNAs specific for MET mRNA. Transfection with MET siRNAs resulted in a marked decrease in the abundance of MET, which was accompanied by generation of the cleaved forms of both caspase-3 and PARP, in SNU5 and 58As9 cells, both of which manifest MET amplification (Fig. 2B). Similar to the effects of crizotinib, depletion of MET by RNA interference (RNAi) also resulted in upregulation of BIM and downregulation of members of the IAP family, whereas it had little effect on the expression of Bcl-2 in such cells (Fig. 2B). These data thus suggested that the upregulation of BIM and the downregulation of members of the IAP family, including survivin, XIAP, and c-IAP1, are related to the induction of apoptosis by the MET inhibitor in gastric cancer cells with MET amplification.

Inhibition of the MEK-ERK or PI3K-AKT pathways results in BIM upregulation and survivin downregulation, respectively, in MET amplification–positive cells

To identify the signaling pathways responsible for upregulation or downregulation of apoptosis-related proteins by crizotinib, we examined the effects of specific inhibitors of the mitogen-activated protein (MAP)/ERK kinase (MEK) and of phosphoinositide 3-kinase (PI3K) in MET amplification–positive cell lines. The MEK inhibitor AZD6244 induced BIM expression without affecting the abundance of the other proteins examined (Fig. 3A), suggesting that expression of BIM is regulated by the MEK-ERK pathway. On the other hand, the PI3K inhibitor BEZ235 reduced the abundance of survivin without affecting that of the other IAP family proteins including c-IAP1 and XIAP (Fig. 3A). We also found that depletion of AKT by RNAi resulted in downregulation of survivin but not of XIAP or c-IAP1 in the MET amplification–positive SNU5 and 58As9 cell lines (Fig. 3B). Together, these data suggested that crizotinib regulates BIM and survivin expression through inhibition of the MEK-ERK and PI3K-AKT signaling pathways, respectively, in MET amplification–positive gastric cancer cells.
Role of BIM induction in crizotinib-induced apoptosis in gastric cancer cells with MET amplification

Induction of the proapoptotic BH3-only protein BIM has been found to be important for TKI-induced apoptosis in EGF receptor (EGFR) gene mutation–positive lung cancer and HER2 amplification–positive breast cancer (13–17). To investigate whether the upregulation of BIM is related to the induction of apoptosis by crizotinib, we transfected MET amplification–positive gastric cancer cells with siRNAs specific for BIM mRNAs. Such transfection resulted in marked inhibition of the upregulation of BIM by crizotinib (Fig. 4A). Immunoblot analysis showed that the attenuation of BIM induction was associated with inhibition of crizotinib-induced apoptosis, as revealed by a reduced extent of caspase-3 and PARP cleavage (Fig. 4A). The Annexin V-binding assay also revealed that such transfection resulted in inhibition of crizotinib-induced apoptosis (Fig. 4B). These data thus suggested that the induction of apoptosis by crizotinib in gastric cancer cells with MET amplification is mediated, at least in part, by upregulation of BIM.

Effect of crizotinib on the growth of gastric cancer cells in vivo

To determine whether the antitumor action of crizotinib observed in vitro might also be apparent in vivo, we injected 58As9 cells (positive for MET amplification),
SNU5 cells (positive for MET amplification), AZ521 cells (negative for MET amplification), or MKN28 cells (negative for MET amplification) into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with vehicle (control) or with crizotinib at a daily dose of 25 or 50 mg/kg by oral gavage for 4 weeks. Crizotinib at either dose eradicated tumors in mice injected with 58As9 or SNU5 cells (Fig. 5; Supplementary Fig. S3). In contrast, tumors in mice injected with AZ521 or MKN28 cells were not affected by crizotinib treatment even at the dose of 50 mg/kg/d (Fig. 5; Supplementary Fig. S3). Treatment with crizotinib at either dose was well-tolerated by the mice, with no signs of toxicity or weight loss during therapy (data not shown). Crizotinib thus exerted a marked antitumor action in gastric cancer cells with MET amplification. In such gastric cancer cells, attenuation of MET function either by treatment with crizotinib or by MET-targeted RNAi resulted in inhibition of AKT and ERK signaling as well as in the induction of apoptosis, indicating that tumor cells with MET amplification are dependent on MET signaling for their growth and survival. Targeting of MET signaling by MET-TKIs is thus a potentially valuable therapeutic strategy for patients with gastric cancer with MET amplification.

We also investigated the mediators of crizotinib-induced apoptosis in MET amplification–positive gastric cancer cells. We found that crizotinib induced upregulation of BIM, a key proapoptotic member of the Bcl-2 family of proteins that initiates apoptosis signaling by binding to and antagonizing the function of prosurvival Bcl-2 family members (18). Furthermore, depletion of BIM by RNAi resulted in inhibition of crizotinib-induced apoptosis in gastric cancer cells with MET amplification, indicating that upregulation of BIM contributes to the induction of apoptosis by the MET-TKI in such cells. These findings are consistent with those of previous studies of lung cancer.

**Discussion**

Aberrant activation of receptor tyrosine kinase signaling pathways contributes to the development of various types of cancer. Small-molecule inhibitors that target these activated kinases have been developed and have shown substantial efficacy in clinical trials. The identification of patient subgroups that might actually benefit from treatment with such drugs would be expected to optimize their efficacy. The receptor tyrosine kinase MET is considered one such potential target in cancer, and several MET-TKIs are currently undergoing clinical trials in humans. Amplification of MET is often responsible for activation of MET signaling, with such amplification occurring frequently in gastric cancer (4–6). We have now shown that crizotinib exerted a marked antitumor action in gastric cancer cells with MET amplification. In such gastric cancer cells, attenuation of MET function either by treatment with crizotinib or by MET-targeted RNAi resulted in inhibition of AKT and ERK signaling as well as in the induction of apoptosis, indicating that tumor cells with MET amplification are dependent on MET signaling for their growth and survival. Targeting of MET signaling by MET-TKIs is thus a potentially valuable therapeutic strategy for patients with gastric cancer with MET amplification.
cells with EGFR mutations and breast cancer cells with HER2 amplification (13–17). However, our observations revealed that depletion of BIM did not completely abolish crizotinib-induced apoptosis, suggesting that another apoptotic regulator might contribute to MET-TKI-induced apoptotic cell death. We also found that crizotinib induced downregulation of survivin, a member of the IAP family that protects cells against apoptosis by either directly or indirectly inhibiting the activation of effector caspases (19). We found that a PI3K inhibitor or RNAi-mediated depletion of AKT reduced the abundance of survivin, indicating that the expression of survivin is regulated by PI3K-AKT signaling in MET amplification–positive gastric cancer cells. Previous studies have shown that the expression of survivin is dependent on PI3K-AKT signaling that operates downstream of receptor tyrosine kinases and is essential for cell survival in EGF–mutation–positive non–small cell lung cancer cells as well as in breast cancer cells positive for HER2 amplification (16, 17). These results suggest that downregulation of survivin via inhibition of the MET–PI3K–AKT pathway likely also contributes to the induction of apoptosis by crizotinib in MET amplification–positive gastric cancer cells.

We have shown that crizotinib induced downregulation of XIAP and c-IAP1 in gastric cancer cells with MET amplification. We further showed that depletion of MET by RNAi induced downregulation of XIAP and c-IAP1, indicating that these proteins are also regulated by MET signaling in MET amplification–positive gastric cancer cells. We investigated which signaling pathway is responsible for downregulation of XIAP and c-IAP1 resulting from inhibition of MET. Given that crizotinib inhibited both ERK and AKT phosphorylation in MET amplification–positive cell lines, we examined the effects both of specific inhibitors of MEK and PI3K as well as of siRNAs specific for AKT mRNA in such cells. However, none of these agents induced downregulation of XIAP or c-IAP1 in gastric cancer cells with MET amplification. We previously showed that activation of STAT3 is linked to MET signaling and that forced expression of a constitutively active form of STAT3 attenuated MET-TKI–induced apoptosis in MET-activated gastric cancer cells, suggesting that inhibition of STAT3 activity contributes to MET-TKI–induced apoptosis (20). To investigate whether the regulation of XIAP or c-IAP1 expression is mediated by STAT3 signaling, we transfected SNU5 and 58As9 cells, both of which manifest MET amplification, with an siRNA that targets STAT3 mRNA. However, depletion of STAT3 by RNAi had no substantial effect on expression of XIAP and c-IAP1 in such cells (data not shown). Previous studies have shown that XIAP and c-IAP1 were not substantially affected by EGFR-TKIs in EGF–mutation–positive non–small cell lung cancer cells or by HER2-targeting agents in breast cancer cells positive for HER2 amplification (16, 17). Given that inhibition of MET results in downregulation of XIAP and c-IAP1 in MET amplification–positive gastric cancer cells, the mechanism by which the expression of such proteins is regulated likely differs between MET and other receptor tyrosine kinases including EGFR and HER2. However, the signaling pathway responsible for downregulation of XIAP and c-IAP1 by MET inhibition remains unknown. Further studies are thus required to clarify the regulation of XIAP and c-IAP1 and the contribution of members of the IAP family to MET-TKI–induced apoptosis in gastric cancer cells with MET amplification.

In conclusion, our results have shown that crizotinib has pronounced effects on signal transduction and survival in gastric cancer cells with MET amplification. Crizotinib has recently been approved for treatment of ALK-driven lung cancer by the FDA on the basis of safety and effectiveness data. The IC50 values of crizotinib for inhibition of the growth of MET amplification–positive gastric cancer cell lines were lower than the mean trough concentration of the drug achieved in the plasma of patients at steady state (292 ng/mL or 644 nmol/L; ref. 21). Indeed, crizotinib was recently found to exhibit antitumor activity in 2 of 4 patients with MET amplification–positive gastroesophageal cancer (22), supporting further study of the molecular mechanism underlying its antitumor action. In the present study, we showed that BIM and IAP family members including survivin, XIAP, and c-IAP1 play a role in crizotinib-induced apoptosis in association with inhibition of MET signaling in gastric cancer cells with MET amplification. Our observations provide a basis for the further development of MET-targeted therapy for patients with gastric cancer with MET amplification.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Okamoto, I. Okamoto, K. Kuywata, E. Hata-Shita, H. Yamaguchi, K. Sakai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Okamoto, I. Okamoto, K. Kuywata, E. Hata-Shita, H. Yamaguchi
Writing, review, and/or revision of the manuscript: W. Okamoto, I. Okamoto, K. Nishio, K. Nakagawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Arao, K. Kuywata, E. Hata-Shita, H. Yamaguchi, K. Sakai, K. Yanagihara
Study supervision: I. Okamoto, K. Nakagawa

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