Crizotinib Induces PUMA-Dependent Apoptosis in Colon Cancer Cells

Xingnan Zheng¹, Kan He¹, Lin Zhang², and Jian Yu¹

Abstract

Oncogenic alterations in MET or anaplastic lymphoma kinase (ALK) have been identified in a variety of human cancers. Crizotinib (PF02341066) is a dual MET and ALK inhibitor and approved for the treatment of a subset of non–small cell lung carcinoma and in clinical development for other malignancies. Crizotinib can induce apoptosis in cancer cells, whereas the underlying mechanisms are not well understood. In this study, we found that crizotinib induces apoptosis in colon cancer cells through the BH3-only protein PUMA. In cells with wild-type p53, crizotinib induces rapid induction of PUMA and Bim accompanied by p53 stabilization and DNA damage response. The induction of PUMA and Bim is mediated largely by p53, and deficiency in PUMA or p53, but not Bim, blocks crizotinib-induced apoptosis. Interestingly, MET knockdown led to selective induction of PUMA, but not Bim or p53. Crizotinib also induced PUMA-dependent apoptosis in p53-deficient colon cancer cells and synergized with gefitinib or sorafenib to induce marked apoptosis via PUMA in colon cancer cells. Furthermore, PUMA deficiency suppressed apoptosis and therapeutic responses to crizotinib in xenograft models. These results establish a critical role of PUMA in mediating apoptotic responses of colon cancer cells to crizotinib and suggest that mechanisms of oncogenic addiction to MET/ALK-mediated survival may be cell type-specific. These findings have important implications for future clinical development of crizotinib.

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Introduction

Receptor tyrosine kinases (RTK) are cell surface receptors that act upon a variety of ligands including growth factors, cytokines, and hormones. RTKs are vital regulators of normal cell physiology and play critical roles in the development and progression of human cancer (1, 2). MET is an extensively studied RTK and the receptor for hepatocyte growth factor (HGF; ref. 3). The activation of MET by HGF ligation initiates various signaling cascades, such as the PI3K/AKT and RAS/RAF/MAPK pathways, to induce cell survival, proliferation, migration, and tissue regeneration (3). Aberrant activation of MET can result from gene amplification, transcriptional upregulation, missense mutations, or ligand autocrine loops, and is implicated in the pathogenesis of many human cancers (2, 3). In colon cancer, MET overexpression and gene amplification are associated with advanced diseases and poor prognosis (4, 5).

Recent efforts in cancer genomics continue to identify aberrantly activated oncogenic kinases and facilitate the development of targeted agents. This approach is expected to ultimately deliver safer and more effective cancer therapeutics (6). MET-targeting agents currently in clinical use include the monoclonal antibody MetAb (Roche; ref. 7) and small-molecule tyrosine kinase inhibitors (TKI), such as ARQ197 (ArQule/Daiichi Sankyo; ref. 8), INCB28060 (Incyte; ref. 9), and crizotinib (PF02341066, Pfizer; ref. 10). Crizotinib was initially designed as a selective ATP-competitive MET inhibitor and later found to inhibit several related kinases, including anaplastic lymphoma kinase (ALK; ref. 11), C-ros oncogene1, and receptor tyrosine kinase (ROS1; ref. 11). Crizotinib has received the approval of the U.S. Food and Drug Administration for the treatment of ALK-rearranged non–small cell lung carcinoma (NSCLC), and is being evaluated in patients with other malignancies. Crizotinib has garnered much attention as it inhibits MET- and ALK-dependent tumor cell growth, migration, and invasion via both HGF-dependent and -independent mechanisms (12). Crizotinib also induces apoptosis in cancer cells; however, the underlying mechanisms are not well understood. Currently, there is no reliable biomarker for crizotinib response other than ALK or ROS1 rearrangement (11, 13, 14).

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Apoptosis plays an important role in the antitumor activities of conventional chemotherapeutic agents and targeted therapies (1, 15, 16). The Bcl-2 family proteins are the central regulators of mitochondria-mediated apoptosis. The BH3-only family members are first engaged in response to distinct as well as overlapping signals. Several of them, such as Bim and PUMA, are potent inducers of apoptosis by activating Bax/Bak following the neutralization of antiapoptotic Bcl-2 family members (15, 17). We and others have shown that PUMA functions as a critical initiator of apoptosis in both p53-dependent and -independent manners in a wide variety of cell types (18). PUMA transcription is directly activated by p53 in response to DNA damage (18), and lack of its induction renders p53-deficient cancer cells refractory to chemotherapeutic drugs and radiation. PUMA induction by nongenotoxic stimuli is generally p53-independent, and mediated by transcription factors such as p73 (19, 20), forkhead box O3a (FoxO3a; ref. 21, 22), and NF-κB (23, 24). Upon induction, PUMA potently induces apoptosis by antagonizing antiapoptotic Bcl-2 family members and/or directly activating the proapoptotic members Bax and Bak, leading to mitochondrial dysfunction and caspase activation cascade (18).

In this study, we investigated the underlying mechanisms of crizotinib-induced apoptosis in colon cancer cells, and found that both p53-dependent and -independent induction of PUMA contributes to crizotinib-induced apoptosis. These results provide novel mechanistic insight into the therapeutic responses of crizotinib, a rationale for manipulating PUMA and BH3-only proteins to improve the efficacy of targeted therapies, as well as therapy-induced changes in their expression as potential biomarkers.

Materials and Methods

Cell culture and drug treatment

Human colorectal cancer cell lines, including HCT116, RKO, LoVo, DLD1, and HT29 were obtained from American Type Culture Collection. The isogenic cell lines, including PUMA-KO (25), p53-KO (26), and p53-binding site knockout (BS-KO; ref. 27) HCT116 cells, PUMA-KO DLD1 cells (27), and p53-KO RKO cells (28) have been described. More details are found in the Supplementary Data for drug treatments. We examine loss of expression of targeted protein by Western blotting and conduct mycoplasma testing by PCR during culture routinely; no addition authentication was done by the authors.

Western blotting

Western blotting was carried out as previously described (29). More details on antibodies are found in the Supplementary Data.

Real-time reverse transcription PCR

Total RNA was isolated from untreated or drug-treated cells using the Mini-RNA Isolation II Kit (cat. #R1055, Zymo Research) according to the manufacturer’s protocol. Total RNA (2 μg) was used to generate complementary DNA using SuperScript III reverse transcriptase (Invitrogen). The following primers were used for PUMA: Forward: 5'-CGACCTCAAGGACAGTAC-GA-3', Reverse: 5'-AGGCACCTAATGCTCCAT-3', β-Actin: Forward: 5'-GACCTCAAGACTACCTCAT-3', Reverse: 5'-AGACACACTGTGTTGGCTA-3'.

Transfection and siRNA

The gene-specific siRNA, including MET siRNA (30) and PUMA siRNA (31) were synthesized by Dharmacon (Lafayette) and transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 hours of transfection, cells were treated with PF02341066 for further analysis. More details are found in the Supplementary Data.

Analysis of apoptosis, growth, and mitochondria-associated events

Apoptosis was analyzed by counting cells with condensed chromatin and micronucleation following nuclear staining with Hoechst 33258 (Invitrogen; ref. 27). The methods of colony formation, changes in the mitochondrial membrane potential, and cytochrome c release have been previously described (25, 32). More details are found in the Supplementary Data.

Reporter assays

PUMA reporters with or without p53-bindings sites have been described previously (20). Reporter assays were carried out in 12-well plates as described (33). Normalized relative luciferase units were plotted. All reporter experiments were carried out in triplicate and repeated 3 times.

Xenograft studies

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Female 5- to 6-week-old Nu/Nu mice (Charles River) were housed in a sterile environment with microisolator cages and allowed access to water and chow ad libitum. Mice were injected subcutaneously in both flanks with 4 million wild-type (WT) or PUMA-KO HCT116 cells. Following tumor growth for 7 days, mice were treated daily by oral gavage for 9 consecutive days with 35 mg/kg PF02341066 in 10% ethanol or 10% ethanol without PF02341066 (control buffer), the total volume being approximately 100 μL/mouse. Detailed methods on tumor measurements, harvests, and histologic analysis are found in the Supplementary Data as previously described (34–36).

Statistical analysis

Statistical analyses were carried out in Microsoft Excel. P values were calculated by the Student t test, and were considered significant if P < 0.05. The means ± 1 SD are displayed in the figures.
Results

**PUMA is induced by crizotinib in colon cancer cells**

We first investigated the effects of crizotinib (PF02341066, PF) on MET signaling and PUMA expression in HCT116 colon cancer cells. Crizotinib treatment led to rapid dephosphorylation of MET and AKT without affecting their total levels. However, the levels of phosphorylated extracellular signal-regulated kinase (ERK) only decreased transiently, and recovered within 6 hours (Fig. 1A). PUMA protein and mRNA were induced within 6 hours of treatment, suggesting transcriptional regulation (Fig. 1A and B). Interestingly, the expression of Bcl-2 family members, such as Bim and Mcl-1, also increased, whereas that of Bad, Bid, Bcl-2, and Bcl-xL remained unchanged by 48 hours (Supplementary Fig. S1A). PUMA induction was dose-dependent and stimulated by as little as 1 \( \mu \)mol/L crizotinib (Fig. 1C). To determine whether MET regulates PUMA expression directly, we depleted MET by siRNA. MET knockdown led to increased PUMA mRNA and protein (Fig. 1D), but not that of Bim (Supplementary Fig. S1B). Taken together, these data suggest that MET inhibition selectively induces PUMA, whereas crizotinib has a broader effect on the levels of PUMA and other BH3-only proteins.

**PUMA mediates crizotinib-induced apoptosis**

Next, we determined the role of PUMA in crizotinib-induced apoptosis using isogenic PUMA-KO HCT116 cells and siRNA. Crizotinib treatment induced approximately 15% to 65% apoptosis from 24 to 48 hours in WT HCT116 cells, which was associated with the activation of caspase-3 and caspase-9, mitochondrial membrane depolarization, and cytochrome c release (Fig. 2A and B). In contrast, apoptosis in PUMA-KO cells was suppressed by more than 70% with little or no activation of caspases, mitochondrial membrane depolarization, or cytochrome c release at 48 hours (Fig. 2A–C). Annexin V/propidium iodide staining confirmed the apoptotic resistance of PUMA-KO cells (Supplementary Fig. S2A). Consistent with blocked apoptosis, PUMA-KO cells showed much improved clonogenic survival (Fig. 2D). We have shown previously that PUMA induces Bax-dependent apoptosis (25, 29). As expected, BAX-KO HCT116 cells were also resistant to crizotinib-induced apoptosis (Fig. 2E and Supplementary Fig. S2B).
Supplementary Fig. S2B). Transient PUMA knockdown with siRNA also led to the reduced apoptosis in HCT116 and LoVo cells following crizotinib treatment (Supplementary Fig. S2C and S2D). Despite Bim induction, Bim knockdown by siRNA did not render HCT116 cells resistant to crizotinib (Supplementary Fig. S1B and S2E). Collectively, these results show an essential role of PUMA and the mitochondrial pathway in crizotinib-induced apoptosis in colon cancer cells.

p53-Dependent induction of PUMA by crizotinib

Both HCT116 and LoVo cells contain WT p53 gene, and p53 mediates PUMA induction following DNA damage (18). Earlier work suggested that other small-molecule MET inhibitors can cause DNA double-strand breaks (37, 38). We found that crizotinib treatment resulted in increased phosphorylation of p53 and H2AX, as well as p53 stabilization in HCT116, LoVo, and RKO cells, all with WT p53 (Fig. 3A and Supplementary Fig. S3A). Induction of PUMA, and BIM to a lesser extent but not MCL-1, was observed in HCT 116 cells and reduced in p53-KO HCT116 cells (Fig. 3B). Furthermore, p53 deficiency attenuated crizotinib-induced apoptosis and caspase activation in HCT116 cells (Fig. 3C and Supplementary Fig. S3B), as well as in RKO cells (Supplementary Fig. S3C and S3D). In time...
course experiments, PUMA and Bim were induced in HCT116 p53-KO or RKO p53-KO cells after 24 hours, though at much lower levels compared with WT cells (Fig. 3D and Supplementary Fig. S3D). To further investigate p53-dependent activation of PUMA, we used a series of PUMA promoter luciferase reporters (within ~2 kb; ref. 20), and found that the reporters containing the 2 p53-binding sites, such as fragments "A", "abc," and "E" had high activities after crizotinib treatment (Fig. 3E). Notably, the most proximal fragment "de" (~200 bp), lacking 2 p53-binding sites, still had a moderate activity (Fig. 3E). The higher activity of the shorter fragment "abc" may be due to the removal of GC-rich sequences and binding sites for transcriptional repressors (18). Together, these data suggest that PUMA induction by crizotinib is mediated by both p53-dependent and -independent mechanisms, and p53 is the major transcriptional activator of PUMA in WT p53 colon cancer cells.

**P53-Independent induction of PUMA by crizotinib**

To further probe p53-independent induction of PUMA, we treated HCT116 cells harboring a deletion of 2 p53-binding sites in the PUMA promoter (RS-KO; ref. 27) with crizotinib, and found much reduced PUMA induction compared with WT HCT116 cells (Fig. 4A). Moreover, PUMA, but not Bim, was induced by crizotinib in 2 p53-mutant colon cancer cell lines DLD1 and HT29 in the absence of p53 phosphorylation or stabilization (Fig. 4B). Other transcription factors, such as NF-κB subunit p65 and FoxO3a can bind to respective sites located proximal to p53-binding sites in the PUMA promoter. Despite phosphorylation changes associated with activation of p65 and FoxO3a, p65 or FoxO3a knockdown did not affect PUMA induction following crizotinib treatment (Supplementary Fig. S4). Crizotinib also induced apoptosis in both DLD1 and HT29 cells, which was suppressed by PUMA gene ablation or siRNA (Fig. 4C and 4D). DLD1 cells showed lower
PUMA induction and were more resistant to crizotinib-induced apoptosis, compared with HT29 cells (Fig. 4B–D). These results indicate that PUMA plays a critical role in the apoptotic responses to crizotinib in both p53 WT and mutant colon cancer cells.

Crizotinib synergizes with gefitinib or sorafenib to induce apoptosis via PUMA

Cooperative signaling of MET and the EGF receptor (EGFR) can contribute to EGFR-TKI resistance (39). HCT116 cells are highly resistant to gefitinib-induced apoptosis or PUMA expression (Fig. 5A and B). Therefore, we hypothesized that the combination of gefitinib and crizotinib may enhance apoptosis. Indeed, this combination induced a much stronger induction of apoptosis, caspase activation, and PUMA and Bim in HCT116 cells, compared with either agent alone (Fig. 5A–C). Similarly, crizotinib and erlotinib combination resulted in a strong PUMA-dependent synergy in cell killing (data not shown).

Crizotinib treatment decreased phosphorylation of both AKT and ERK in HCT116 cells. However, ERK dephosphorylation was only transient and restored after 6 hours, long before apoptosis was detected (Fig. 1A). We reasoned that a more durable inhibition of ERK phosphorylation may potentiate crizotinib in cell killing. The combination of crizotinib and sorafenib, a Raf inhibitor, markedly induced apoptosis, expression of PUMA and Bim, and long-term growth suppression, compared with the single agent (Supplementary Fig. S5). Apoptosis and long-term growth suppression were attenuated in PUMA-KO cells (Supplementary Fig. S5A, S5B, and S5D). Of note, AKT phosphorylation was completely inhibited by all 3 agents, but not ERK phosphorylation, and reduction of either p-AKT or p-ERK is not sufficient for the enhanced induction of PUMA and Bim, or apoptosis in the combination treatments (Fig. 5C and Supplementary SSC). In addition, Mcl-1 levels did not change or decreased.

Figure 4. Crizotinib induces PUMA- and p53-independent apoptosis in colon cancer cells. A, PUMA protein levels in WT and BS-KO HCT116 cells treated with 12 μmol/L PF02341066 at the indicated times were analyzed by Western blotting. BS-KO HCT116 cells harbor the deletion of 2 p53-binding sites in the PUMA promoter. B, p53-mutant colon cancer cell lines DLD1 and HT29 were treated with 12 μmol/L PF02341066 for 24 hours. The expression levels of PUMA, Bim, p-p53, and total p53 were analyzed by Western blotting. C, top, PUMA expression was analyzed by Western blotting in WT and PUMA-KO DLD1 cells. Bottom, apoptosis was analyzed by nuclear fragmentation in WT and PUMA-KO DLD1 cells treated with the indicated doses of PF02341066 for 48 hours. ** P < 0.001; WT versus p53-KO. D, HT29 cells were transfected with either a scrambled siRNA or PUMA siRNA for 24 hours and then treated with 12 μmol/L PF02341066 for 48 hours. Left, Western blotting confirmed PUMA depletion by siRNA in HT29 cells. Right, apoptosis was determined by a nuclear fragmentation assay. ** P < 0.001; si-PUMA versus Scrambled. β-Actin was used as a loading control for Western blot analyses.
following crizotinib combination with gefitinib or sorafenib (Fig. 5C and Supplementary Fig. S5C). These results suggest that the combinations of crizotinib with additional TKIs are required to effectively target nonoverlapping survival pathways in cancer cells to induce apoptosis.

**PUMA contributes to the antitumor activity of crizotinib in a mouse xenograft model**

To determine whether PUMA-mediated apoptosis plays a critical role in the antitumor activity of crizotinib in vivo, we established WT and PUMA-KO HCT116 cells treated with 6 μmol/L PF02341066, 20 μmol/L gefitinib, or their combination for 24 hours. The expression levels of p-AKT, total AKT, p-ERK, total ERK, PUMA, and Bim were analyzed by Western blotting. D, WT and PUMA-KO HCT116 cells were treated with 6 μmol/L PF02341066, 20 μmol/L gefitinib, or their combination for 30 hours and were then subjected to a colony formation assay as described in Materials and Methods. Colony numbers were scored 14 days after plating. Representative pictures of colonies (top) and relative clonogenic survival (bottom) of WT and PUMA-KO HCT116 cells compared with untreated cells are shown. ***, P < 0.001; combination versus single agent in WT cells and KO versus WT in combination. β-Actin was used as a loading control for Western blot analyses.

Figure 5. Crizotinib synergizes with gefitinib to induce PUMA-dependent apoptosis in colon cancer cells. A, WT and PUMA-KO HCT116 cells were treated with 6 μmol/L PF, 20 μmol/L gefitinib, or their combination for 48 hours. Apoptosis was determined by a nuclear fragmentation assay. **, P < 0.001; combination versus single agent in WT cells and KO versus WT in combination. Right, chemical structure of gefitinib. B, caspase-3 activation was analyzed by Western blotting in WT and PUMA-KO HCT116 cells treated as in A. C, HCT116 cells were treated with 6 μmol/L PF02341066, 20 μmol/L gefitinib, or their combination for 24 hours. The expression levels of p-AKT, total AKT, p-ERK, total ERK, PUMA, and Bim were analyzed by Western blotting. D, WT and PUMA-KO HCT116 cells were treated with 6 μmol/L PF02341066, 20 μmol/L gefitinib, or their combination for 30 hours and were then subjected to a colony formation assay as described in Materials and Methods. Colony numbers were scored 14 days after plating. Representative pictures of colonies (top) and relative clonogenic survival (bottom) of WT and PUMA-KO HCT116 cells compared with untreated cells are shown. ***, P < 0.001; combination versus single agent in WT cells and KO versus WT in combination. β-Actin was used as a loading control for Western blot analyses.

Aberrantly activated oncogenic kinases are promising drug targets for small molecules; however, biomarkers and resistance mechanisms of most clinically useful kinase inhibitors remain largely unknown. Crizotinib has been approved in ALK-rearranged NSCLC (3, 13), and clinical interest is expanding to other solid tumors with genetic alterations in c-MET, ALK, and ROS-1 (14, 40). The antitumor activities of crizotinib include the induction of cell-cycle arrest, apoptosis, and inhibition of cell

**Discussion**

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proliferation and invasion (3, 13). Our results indicated that activation of mitochondrial pathway and PUMA plays a key role in crizotinib-induced cancer cell death in vitro and in vivo. In addition, the combinations of crizotinib with EGFR or Raf inhibitors resulted in the potent induction of apoptosis in colon cancer cells via PUMA.

Crizotinib-induced apoptosis can be attributed to Bim in lung cancer cells with MET amplification but not with MET mutations (41, 42). In colon cancer cells, Bim induction, though not required, likely potentiates PUMA-dependent apoptosis by antagonizing antiapoptotic Bcl-2 family proteins including Mcl-1, whose levels remain high after treatment. It is likely that multiple BH3-only proteins are used in the apoptotic response to crizotinib, whereas a distinct member may serve as cell or tissue-specific initiator. The coregulation of Bim and PUMA by crizotinib is interesting and somewhat unexpected, and requires further investigation. Bim and PUMA are located on different chromosomes and their basal expression levels are quite different. It is possible that higher order chromatin changes may be involved in addition to loading of stress-induced transcription factors, such as p53 onto their promoters. The mechanisms of MET inhibitor-induced DNA damage response (37, 38), and p53-independent induction of PUMA and Bim remain unclear.

Despite a plethora of oncogenic activities ascribed to MET and its related kinases (3, 13), our data suggest that crizotinib effectively inhibits MET signaling, and induces PUMA-dependent apoptosis in colon cancer cells. Surprisingly, MET siRNA did not induce obvious apoptosis or p53 stabilization in HCT116 cells but only a modest PUMA induction. One possible explanation is that crizotinib treatment is more effective in blocking MET signaling than MET siRNA. Another more likely explanation is that crizotinib inhibits other RTKs and activates p53-dependent DNA damage responses in some cells. In addition, PUMA induction may further engage cytoplasmic function of p53 to trigger apoptosis (43, 44). These issues might be relevant as the clinically efficacious concentration of crizotinib used in vitro for cell killing (1–10 μmol/L) are much higher than those required to selectively inhibit MET or ALK.

Crizotinib and other TKI combinations enhance apoptosis and the induction of PUMA and Bim. Interestingly, the inhibition of AKT phosphorylation is not sufficient for cell killing or strong PUMA induction. Our data are consistent with the emerging concept that cross-talk between RTKs is a major mechanism for cancer progression and therapeutic resistance, and successful therapy will likely require targeting multiple survival pathways (1, 6, 45). However, several challenges are faced in the clinical applications of kinase inhibitors: (i) genetic alterations in EGFR, MET, or ALK, and possibly ROS1 are infrequent, (ii) not all tumors with same alterations respond, (iii) tumor heterogeneity plus preexisting or de...


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