Aurora Kinase Inhibition Induces PUMA via NF-κB to Kill Colon Cancer Cells

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Abstract
Aurora kinases play a key role in mitosis and are frequently overexpressed in a variety of tumor cells. Inhibition of aurora kinases results in mitotic arrest and death of cancer cells, and has been explored as an anticancer strategy. However, how aurora inhibition kills cancer cells is poorly understood. In this study, we found that inhibition of aurora kinases by siRNA or small-molecule inhibitors led to induction of p53 upregulated modulator of apoptosis (PUMA), a BH3-only Bcl-2 family protein, in colorectal cancer cells irrespective of p53 status. Deficiency in PUMA increased polyploidy, improved cell survival, and abrogated mitochondria-mediated apoptosis induced by aurora kinase inhibitors. In response to aurora kinase inhibition, PUMA was directly activated by p65 through the canonical NF-κB pathway following AKT inhibition. Furthermore, PUMA was necessary for the chemosensitization and in vivo antitumor effects of aurora kinase inhibitors in colon cancer cells. These results suggest that PUMA induction mediates the apoptotic response to mitotic arrest imposed by aurora kinase inhibition, and may be a useful indicator for the anticancer activity of aurora kinase inhibitors. Mol Cancer Ther; 13(5); 1298–308. ©2014 AACR.

Introduction
Aurora kinases are a group of highly conserved serine/threonine kinases that play a key role in mitosis. They are frequently overexpressed in a variety of tumors and promote cell-cycle progression (1). There are three aurora kinases in mammalian cells, including aurora A, B, and C, among which aurora A and B are most abundantly expressed in cancer cells (2). Small-molecule inhibitors of aurora kinases have been developed and shown promises in recent preclinical studies (3). In response to aurora kinase inhibition, cancer cells undergo mitotic arrest and eventually, cell death (4). Accumulating evidence suggests that aurora kinase inhibitors kill cancer cells by inducing apoptosis via mitochondrial dysfunction (5). However, the mechanisms by which mitotic arrest imposed by aurora kinase inhibitors triggers apoptotic cell death are poorly understood (6).

p53 upregulated modulator of apoptosis (PUMA) is a BH3-only Bcl-2 family member, which functions as a critical initiator of apoptosis in cancer cells (7). It is transcriptionally activated by p53 in response to DNA damage, and is indispensable for p53-dependent apoptosis induced by radiation and cytotoxic chemotherapeutic drugs (8). PUMA can also be induced in a p53-independent manner by a variety of nongenotoxic stimuli, such as kinase inhibitors, growth factor deprivation, and inflammatory cytokines (9–14). p53-independent PUMA induction can be mediated by different transcription factors, including the p53 homologue p73, Forkhead Box O3a (FoxO3a), and NF-κB (9–14). Upon its induction, PUMA potently induces apoptosis in cancer cells by acting upon other Bcl-2 family members such as Bax, Bcl-2, and Bcl-XL, resulting in mitochondrial outer membrane permeabilization and activation of the caspase cascade (15–17). PUMA was previously shown to be induced by anti-mitotic drugs such as microtubule-targeting agents (18), and upregulated in response to polyoidy (19, 20), suggesting it may play a role in initiating the apoptotic response to mitotic arrest. In this study, we found that PUMA is transcriptionally activated through the canonical NF-κB pathway following aurora kinase inhibition, which contributes to the in vitro and in vivo anticancer activities of aurora kinase inhibitors. Our results suggest that PUMA induction may be a useful indicator for the therapeutic effects of aurora kinase inhibitors.

Materials and Methods
Cell culture and drug treatment
The human colorectal cancer cell lines, including HCT116, DLD1, RKO, HT29, SW480, and SW48 were obtained from the American Type Culture Collection. Cell...
lines were last tested and authenticated for genotypes, drug response, morphology, and absence of mycoplasma in October 2012. p53-knockout (KO) and PUMA-KO colon cancer cell lines and wild-type (WT) and PUMA-KO mouse embryonic fibroblasts (MEF) cells were previously described (13). All cell lines were maintained at 37°C in 5% CO2. Colon cancer cell lines were cultured in McCoy’s 5A (Invitrogen). MEF cells were cultured in DMEM media (Invitrogen). Cell culture media were supplemented with 10% defined FBS (HyClone), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). For drug treatment, cells were plated in 12-well plates at 20% to 30% density 24 hours before treatment. The dimethyl sulfoxide (DMSO; Sigma) stocks of agents used, including ZM-447439 (Selleck Chemicals), VX-680, Gefinitib (LC Laboratories), 5-fluorouracil (5-FU; Sigma), BAY 11-7082 (Merck Chemicals), GX15-070 (Cayman Chemical), were diluted to appropriate concentrations with the cell culture medium. Human TNF-α (R&D Systems) was diluted with PBS. For NF-κB inhibition, cells were pretreated with BAY 11-7082 for 1 hour before ZM-447439 treatment. Transfection of expression constructs of wild-type and constitutive AKT was performed as described (12).

Real-time reverse transcription-PCR
Total RNA was isolated from cells using the Mini RNA Isolation II Kit (Zymo Research) according to the manufacturer’s protocol. Total RNA (1 µg) was used to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was carried out for PUMA and glyceraldehyde-3-phosphate dehydrogenase as described (13).

Western blotting
Antibodies used for Western blotting included those against active caspase-3, p-ERK (T202/Y204), IκB, p-IκB (S32/S36), p-GSK3β, p65 (total), p-p65 (S536), FoxO3a (total), AKT (total), p-AKT (S473; Cell Signaling Technology), cytochrome c, α-tubulin, Bcl-XL (BD Biosciences), caspase-9 (Stressgen Bioreagents), cytochrome oxidase subunit IV (Cox IV; Invitrogen), Bcl-2 (Dako), Flag (Sigma), PUMA (17), p53, Bim, Bid, Noxa, and β-actin (EMD Millipore). Western blotting analysis was performed as previously described (13). The release of cytochrome c was detected in the cytosol following subcellular fractionations as described (13).

Transfection and siRNA knockdown
Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Knockdown experiments were performed 24 hours before ZM-447439 or VX-680 treatment using 400 pmol of siRNA. All siRNA have been previously described and were from Dharmacon (Lafayette), including those for aurora A (21), aurora B (22), GSK3β (sc-35527; Santa Cruz; ref. 13), p65 (11), p73 (9), FoxO3a (10), and the control scrambled siRNA. A nondegradable IκBα super repressor mutant (S52A/S36A; IκBαM) was previously described (11).

Analysis of NF-κB nuclear translocation
HCT116 cells pretreated with BAY 11-7082 were subjected to ZM-447439 or TNF-α for 3 hours. NF-κB nuclear translocation was analyzed by nuclear fractionation. Briefly, nuclear extracts were isolated from cells plated and treated in 75 cm2 flasks using the NE-PER Nuclear/Cyttoplasmic Extraction Kit (Thermo Fisher) according to the manufacturer’s instructions, and probed by Western blotting for p65.

Luciferase assays
PUMA luciferase reporter constructs have been previously described (9). Mutations were introduced into the p65 binding sites of Fragment A using QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) as previously described (13). Cells were transfected with PUMA reporters containing either wild-type or mutant p65 binding sites (13), along with the transfection control β-galactosidase reporter pCMVβ (Promega), and treated with 15 µmol/L ZM-447439 for 24 hours. Cell lysates were collected and luciferase activities were measured as previously described (13). All reporter experiments were done in triplicate and repeated 3 times.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was done using the ChIP Assay kit (Millipore) with p65 (Santa Cruz) antibody for chromatin precipitation as described (13). The precipitates were analyzed by PCR using primers 5’-GTGGCTCTGTGTACGCATCG-3’ and 5’-CCCGCGTGAGCTACGCCC-3’ as previously described (13).

Apoptosis assays
Adherent and floating cells were harvested, stained with Hoechst 33258 (Invitrogen), and analyzed for apoptosis by nuclear staining assay. A minimum of 300 cells were analyzed for each treatment. For colony formation assays, equal numbers of cells were subjected to various treatments and plated into 12-well plates at different dilutions. Colonies were visualized by crystal violet (Sigma) staining 14 days after plating as previously described (13). Each experiment was performed in triplicate and repeated at least twice.

Xenograft tumors
All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Wild-type and PUMA-KO HCT116 xenografts were established and measured as described (13). In brief, 5- to 6-week-old female athymic nude mice (Harlan) were inoculated with 5 x 106 cells per site on both flanks. Tumors were allowed to establish for 7 days. The mice were treated by intraperitoneal injection for 14 consecutive days with 80 mg/kg/d ZM-447439 diluted in 10% DMSO, or vehicle control. The tumor volumes were measured in 2 dimensions using a vernier caliper. Mice were randomized into groups such that the average tumor volume across the groups was the same.
before treatment. For all in vivo experiments, tumor volumes were measured every other day in 2 dimensions and volumes were determined in mm$^3$ using the formula $l \times b^2 \times 0.5$ (where $l$ is the larger diameter and $b$ is the smaller diameter of the tumor). Mice were euthanized 5 (for Western blot analysis) or 21 days after the treatment. Tumors were dissected and fixed in 10% formalin and embedded in paraffin. Active caspase-3 immunostaining was performed on 5 μm paraffin-embedded tumor sections as previously described (23), with an Alexa Fluor 594–conjugated secondary antibody (Invitrogen) for signal detection.

**Statistical analysis**

Statistical analyses were carried out using GraphPad Prism IV software. $P$ 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

**p53-independent PUMA induction in response to aurora kinase inhibition**

Aurora kinases, in particular aurora A and B, are frequently overexpressed in colon cancer cells (2). To determine how aurora kinases are involved in cell survival, we transfected p53-wild-type HCT116 colon cancer cells with siRNA for aurora A or aurora B. Knockdown of either aurora A or B led to the induction of PUMA (Fig. 1A). Treatment with ZM-447439, a selective inhibitor of aurora A and B, at 5 to 20 μmol/L induced PUMA expression in a dose-dependent manner (Fig. 1B, top), and suppressed the activating phosphorylation of T288 of aurora A and T232 of aurora B (Fig. 1C; ref. 1). Treatment with the pan-aurora inhibitor VX-680 (Tozasertib) at 1 to 40 μmol/L also strongly induced PUMA (Fig. 1B, bottom and Supplementary Fig. S1A). Following ZM-447439 or VX-680 treatment, PUMA mRNA was induced as early as 4 hours,
whereas PUMA protein started to accumulate between 8 and 12 hours (Fig. 1D and Supplementary Fig. S1B). Both ZM-447439 and VX-680 induced p53 in HCT116 cells (Fig. 1B and data not shown). However, the induction of PUMA by these agents was intact in p53-KO HCT116 cells (Fig. 1B and Supplementary Fig. S1A), and was observed in colon cancer cells with different p53 statuses, including p53-WT RKO and SW48 cells, and p53-mutant DLD1, HT29, and SW480 cells (Fig. 1E and Supplementary Fig. S1C). Furthermore, the Bcl-2 family members, including Bax, Bid, Bim, and Bcl-2, were upregulated, whereas Mcl-1 was depleted following ZM-447439 treatment (Fig. 1F). Therefore, the expression of multiple Bcl-2 family proteins including PUMA is modulated in response to aurora kinase inhibition, which may collectively initiate an apoptotic response in colon cancer cells.

**Increased polyploidy, improved survival, and reduced apoptosis in PUMA-deficient cells upon aurora kinase inhibition**

To determine whether PUMA induction plays a functional role in response to aurora kinase inhibition, we analyzed the DNA content and cell-cycle progression of wild-type and PUMA-KO HCT116 cells following aurora kinase inhibition. Knockdown of *aurora B*, but not *aurora A*, by siRNA resulted in a higher fraction of polyploid PUMA-KO cells compared to wild-type HCT116 cells (Fig. 2A and data not shown). ZM-447439 treatment also induced a higher level of polyploidy (Fig. 2B), and enhanced accumulation of the G2 population in PUMA-KO cells relative to wild-type cells (Fig. 2C). Consistent with these findings, PUMA-KO cells had improved survival than wild-type HCT116 cells after ZM-447439 or VX-680 treatment in a long-term colony formation assay (Fig. 2D and Supplementary Fig. S2A).

PUMA-KO cells were found to be defective in apoptosis induced by aurora kinase inhibitors. Nuclear staining revealed that apoptosis induced by ZM-447439 or VX-680 at different concentrations was markedly reduced in PUMA-KO cells than wild-type cells (Fig. 2E and Supplementary Fig. S2B). Annexin V/PI staining confirmed the reduction of ZM-447439- and VX-680-induced apoptosis in the absence of PUMA (Fig. 2F and Supplementary Fig. S2C). ZM-447439-induced and PUMA-dependent apoptosis is not cell line specific, and was observed in p53-mutant DLD1 colon cancer cells (Supplementary Fig. S2D), and in MEFs (Supplementary Fig. S2E). Furthermore, PUMA deficiency abrogated ZM-447439-induced mitochondrial events including activation of caspase-3, -8, and -9 (Fig. 2G) and cytochrome c release (Fig. 2H). Together, these results suggest that cells undergoing mitotic arrest following aurora kinase inhibition are eliminated in part through PUMA-dependent apoptosis.

**Direct activation of PUMA by NF-κB in response to aurora kinase inhibition**

We then investigated the mechanism by which aurora kinase inhibitors induce PUMA in the absence of p53 by examining several transcription factors. The expression of FoxO3a and p73, which can induce PUMA in p53-deficient cells (9, 24), was unchanged after ZM-447439 or VX-680 treatment (data not shown). Knockdown of FoxO3a or p73 by siRNA also did not affect PUMA induction by ZM-447439 (Supplementary Fig. S3A–S3C), indicating that FoxO3a and p73 are not involved in PUMA induction by aurora kinase inhibitors. The p65 subunit of NF-κB was recently identified as a transcriptional activator of PUMA in response to TNF-α or the c-Raf inhibitor sorafenib (11, 13). Suppression of p65 expression by siRNA abrogated PUMA induction following ZM-447439 treatment in HCT116 cells (Fig. 3A), and in p53-KO HCT116 and p53-deficient DLD1 cells (Fig. 3B). Knockdown of p65 also attenuated PUMA induction following VX-680 treatment (Supplementary Fig. S3D).

To determine whether NF-κB directly activates PUMA transcription in response to aurora kinase inhibition, p53-KO HCT116 cells were transfected with luciferase reporter constructs containing different regions of the PUMA promoter (fragments A-D; Fig. 3C, left; ref. 9). Upon ZM-447439 treatment, only the proximal 495-bp region of the PUMA promoter (fragments A and E) was found to be strongly activated (Fig. 3C, right). In contrast, the NF-κB responsive element distal within fragment D, which is required for PUMA induction by TNF-α (11), was not activated (Fig. 3C). The activated PUMA promoter region contains at least 5 putative κB sites (Fig. 3D, left; ref. 13), among which site 5 seems to be most critical for activation of the PUMA promoter by ZM-447439 treatment (Fig. 3D, right, and Supplementary Fig. S3E). But complete blockage of PUMA promoter activation was only observed when all 5 κB sites were mutated (Fig. 3D, right), suggesting that multiple, if not all, of the 5 κB sites contribute to the activation of the PUMA promoter. Furthermore, ChIP analysis revealed increased recruitment of p65 to the region containing the κB sites following ZM-447439 treatment (Fig. 3E). These results indicate that p65 directly binds to multiple κB sites in the PUMA promoter to drive its transcription in response to aurora kinase inhibition.

**PUMA induction by aurora kinase inhibitors through the canonical NF-κB pathway and AKT inhibition**

Activation of NF-κB signaling is characterized by p65 phosphorylation on several residues and its subsequent translocation to the nucleus, where it activates transcription of NF-κB target genes (25). We found that ZM-447439 treatment enhanced the phosphorylation of S536, the major regulatory site of p65 (25), in both wild-type and p53-KO HCT116 cells (Fig. 4A). Probing p65 in nuclear fractions detected its nuclear translocation in cells treated with ZM-447439 or the control TNF-α (Fig. 4B). Suppressing p65 nuclear translocation, by pretreating cells with the NF-κB inhibitor BAY 11-7082 (Fig. 4B, left), impeded PUMA induction by ZM-447439 or TNF-α (Fig. 4B, right), suggesting that PUMA induction following aurora kinase inhibition is mediated by p65 nuclear translocation. The canonical NF-κB pathway activated by agents such as TNF-α is mediated by IkB S32/S36 phosphorylation and
subsequent degradation (25). ZM-447439 treatment led to IkB phosphorylation and degradation (Fig. 4C). Transfecting cells with IkBaM, a nondegradable mutant of IkB (11), reduced ZM-447439–induced PUMA expression and p65 phosphorylation in wild-type and p53-KO HCT116 cells (Fig. 4D and Supplementary Fig. S4A), suggesting that ZM-447439–induced p65 activation is mediated by IkB depletion through the canonical NF-κB pathway.

Figure 2. PUMA mediates apoptosis induced by aurora kinase inhibitors through the mitochondrial pathway. A, WT and PUMA-KO HCT116 cells were transfected with control scrambled siRNA or aurora B siRNA. Cell nuclei were stained with Hoechst 33258 48 hours after siRNA transfection. Left, representative pictures (400×C2) are shown with arrows indicating polyploid nuclei; right, quantification of polyploid cells. B, WT and PUMA-KO HCT116 cells were treated with 15 μmol/L ZM-447439 (ZM) for 48 hours. Cell nuclei were stained with Hoechst 33258 after treatment. Left, representative pictures (400×C2) with arrows indicating polyploid nuclei; right, quantification of polyploid cells. C, cell-cycle profiles in WT and PUMA-KO HCT116 cells treated with ZM-447439 as in B were determined by flow cytometry. Sub-G1 cells were excluded from the analysis. D, colony formation assay was done by seeding an equal number for ZM-447439–treated (10 μmol/L) WT and PUMA-KO HCT116 cells in 12-well plates, and then staining attached cells with crystal violet 14 days later. Left, representative pictures of colonies; right, quantification of colony numbers. E, WT and PUMA-KO HCT116 cells were treated with ZM-447439 at the indicated concentrations for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei following nuclear staining with Hoechst 33258. F, WT and PUMA-KO HCT116 cells were treated with 15 μmol/L ZM-447439 for 48 hours. Cells were stained with annexin V/propidium iodide and analyzed by flow cytometry. The percentages of annexin V–positive cells are indicated in the 2 right quadrants. G, Western blot analysis of active caspase-3, -8, and -9 in WT, p53-KO, and PUMA-KO HCT116 cells with or without 15 μmol/L ZM-447439 treatment for 24 hours. H, cytosolic and mitochondrial fractions isolated from WT and PUMA-KO HCT116 cells treated with 15 μmol/L ZM-447439 for 36 hours were probed for cytochrome c by Western blotting. α-Tubulin and cytochrome oxidase subunit IV (Cox IV), which are expressed in cytoplasm and mitochondria, respectively, were analyzed as the control for loading and fractionation. Results in A, B, D, and E were expressed as means ± SD of 3 independent experiments. * * , P < 0.01; * , P < 0.05.
We further analyzed the signaling events leading to NF-κB activation, focusing on several kinases known to act upon the NF-κB pathway (25). ZM-447439 treatment did not significantly affect the activity of ERK kinase (Supplementary Fig. S4B), but suppressed the inhibitory S9 phosphorylation of GSK3β (ref. 26; Supplementary Fig. S4C). However, knockdown of GSK3β by siRNA did not affect the induction of PUMA by ZM-447439 in both wild-type and p53-KO HCT116 cells (Supplementary Fig. S4D). AKT phosphorylation was decreased shortly after ZM-447439 or VX-680 treatment (Fig. 4E). Exogenous expression of active AKT suppressed PUMA induction, IκB phosphorylation, and p65 phosphorylation following ZM-447439 treatment (Fig. 4F), and also downregulated endogenous PUMA expression in untreated cells (Supplementary Fig. S4E). Furthermore, knockdown of aurora B recapitulated these effects of ZM-447439 on AKT, IκB, and p65 phosphorylation (Fig. 4G). Collectively, these observations suggest that aurora kinase inhibitors induce PUMA through a pathway involving AKT inhibition, IκB degradation, and subsequent p65 activation.

**PUMA-mediated chemosensitization by aurora kinase inhibitors**

Aurora kinase inhibitors have been used in combination with conventional cytotoxic drugs in clinical studies.
We reasoned that PUMA induction may mediate the chemosensitization effects of aurora kinase inhibitors, because of concurrent PUMA induction by aurora kinase inhibitors and other agents through different mechanisms. Indeed, we found that the combination of ZM-447439 with other anticancer agents, at a concentration that did not robustly induce PUMA or apoptosis, such as the DNA-damaging drug 5-FU, the EGFR inhibitor gefitinib, or the BH3-mimetic GX15-070 (Obatoclax mesylate; ref. 27), induced PUMA at a much higher level compared with a single agent alone (Fig. 5A). Accordingly, the levels of apoptosis and caspase-3 activation were also significantly higher in HCT116 cells following the combination treatments (Fig. 5B and C). These data suggest that PUMA mediates the chemosensitization effects of aurora kinase inhibitors, and robust induction of PUMA is indicative of effective drug combinations.

**PUMA-dependent in vivo therapeutic activity of aurora kinase inhibitors**

We then used a xenograft tumor model to determine whether PUMA is necessary for the antitumor effects of aurora kinase inhibitors. Wild-type and PUMA-KO HCT116 cells were injected subcutaneously into nude mice to establish xenograft tumors. Mice were then treated with 80 mg/kg/d ZM-447439 or the control vehicle by intraperitoneal injection for 14 consecutive days. Wild-type and PUMA-KO tumors without drug treatment were not different in their growth (Fig. 6A and B). Although...
ZM-447439 treatment resulted in 72.8% growth inhibition relative to the vehicle control in wild-type tumors, it only led to 25.7% growth inhibition in PUMA-KO tumors (Fig. 6A and B). The differences between wild-type and PUMA-KO groups were statistically significant in the ZM-447439 arm ($P < 0.001$), but not in the control vehicle arm (Fig. 6A), indicating that PUMA accounts for a substantial portion of the antitumor activity of ZM-447439. Increased p65 phosphorylation and PUMA expression were detected in the ZM-447439–treated wild-type tumors (Fig. 6C). Substantial apoptosis induction was revealed by TUNEL staining in the ZM-447439–treated wild-type tumors, but not in the control tumors. In contrast, apoptosis was barely detectable in the PUMA-KO tumors after ZM-447439 treatment (Fig. 6D). Staining for active caspase-3 verified defective apoptosis in ZM-447439–treated PUMA-KO tumors (Fig. 6E). Therefore, the in vivo antitumor activity of ZM-447439 is PUMA dependent, and also involves NF-kB activation.

Discussion

Upregulation of mitotic kinases such as aurora and polo-like kinases promotes cell division and contributes to sustained cell-proliferative signaling, a hallmark of cancer cells (28). Several lines of evidence suggest that aurora B is the prominent aurora kinase in colorectal cancer cells (29–31). Aurora B is frequently overexpressed in colorectal tumors (1), and its overexpression correlates with advanced tumor stages (29). Inhibition of aurora B seems to have more profound effects in colon cancer cells than that of other aurora kinases. For example, inhibition of aurora B, but not aurora A, sensitized colon cancer cells to the Bcl-2 inhibitor ABT-263 (30). Aurora B mutations were found to be enriched in cells with acquired resistance to ZM-447439 (31). In our study, knockdown of aurora B recapitulated the effects of ZM-447439 on PUMA induction, AKT inhibition, and IκB and p65 phosphorylation (Figs. 1A and 4G). Therefore, targeting aurora kinases in colorectal cancer may benefit from development of more specific inhibitors for aurora B.

Our results demonstrate that PUMA is induced following mitotic arrest and AKT inhibition in response to aurora kinase inhibition, and contributes to apoptosis initiation in colon cancer cells via the mitochondrial pathway. Consistent with this notion, PUMA and PUMA-dependent apoptosis can be induced by polyploidy (19, 20), or by AKT inhibition (9, 10, 12, 32). PUMA seems to function as a nodule of multiple killing pathways, and can initiate the apoptotic response to a variety of kinase inhibitors, including the pan-CDK inhibitor UCN-01 (10), the EGFR inhibitors gefitinib and erlotinib (12), the c-MET/ALK inhibitor crizotinib (33), and the multikinase inhibitor Bcr-Abl (34).
Inhibitor drugs sorafenib and sunitinib (13, 14). In addition to PUMA, other Bcl-2 family members are also modulated following aurora kinase inhibition and contribute to apoptosis induction. Multiple proapoptotic BH3-only proteins are induced by ZM-447439 and VX-680 in colon cancer cells (Fig. 1F and Supplementary Fig. S1D; ref. 30), and ZM-447439 could induce Bax-mediated apoptosis in HCT116 cells (34). A recent study suggests that downregulation of Mcl-1 following aurora kinase inhibition has a functional role in colon cancer cells by increasing the reliance on Bcl-XL for cell survival (30).

PUMA mediates apoptosis induced by aurora kinase inhibitors in a p53-independent manner, although p53 can be stabilized and promotes apoptosis in response to aurora kinase inhibitors (34, 35). The residual apoptosis in ZM-447439–treated p53-deficient cells may be attributable to p53 induction and its effects on other Bcl-2 family members (Fig. 2E), as well as other cell death mechanisms. It is unexpected that aurora kinase inhibitors activate NF-kB, better known as a pro-survival factor, to induce PUMA; however, NF-kB clearly can promote apoptosis under certain conditions (36). Unlike the induction of PUMA by sorafenib, which does not involve IκB degradation and results from GSK3β and ERK inhibition (13), the induction of PUMA by aurora kinase inhibitors is mediated by the canonical NF-κB pathway via IκB degradation and subsequent p65 nuclear translocation following AKT inhibition. These results reinforce the multifaceted nature of NF-κB signaling, and suggest a broader functional role of NF-κB signaling in mediating therapeutic response to targeted drugs, which has not been sufficiently appreciated. In addition to p65, other aurora kinase inhibitors may function through different transcription factors, such as p73 (35), to induce PUMA and other Bcl-2 family members to induce apoptosis.

For robust induction of apoptosis, most of the experiments in this study were performed by using ZM-447439 or VX-680 in a dose range of 10 to 20 μmol/L, which are substantially higher than those known to be effective for aurora kinase inhibition (3). The results cannot be solely explained by inhibition of aurora kinase activity. A recent phase I dose escalation study showed that the plasma...
concentration of VX-680 (MK-0457) can reach 1 to 5 μmol/L in patients 24 hours after drug infusion (37). We found that VX-680 in this dose range was sufficient to induce PUMA expression (Fig. 1B, bottom). PUMA is also necessary for the chemosensitization effects of ZM-447439 at 5 μmol/L (Fig. 5). These observations suggest that PUMA might be involved in the effects of aurora kinase inhibitors at clinically relevant doses.

There is great enthusiasm for developing new antimitotic agents including aurora kinase inhibitors, largely because of the success of microtubule-targeting drugs such as taxanes and vinca alkaloids. To date, at least 30 aurora kinase inhibitors have been developed, some of which have advanced into clinical trials for treating a variety of tumors (2, 4). However, these new antimitotic agents have not met the initial expectation with regard to their clinical efficacy and toxicities, raising doubt on the rationale of targeting mitosis for anticancer therapy (38). Understanding the killing mechanisms of aurora kinase inhibitors may help to improve their clinical applications (39). For example, it might be possible to use aurora kinase inhibitors at reduced doses in combination with agents with different PUMA induction mechanisms and nonoverlapping toxicities. It also seems to be plausible to use apoptosis-targeting agents such as BH3 and SMAC mimetics to potentiate aurora kinase inhibitors. PUMA induction might be a useful biomarker for clinical trials testing aurora kinase inhibitors. Furthermore, delineating a critical mediator of their killing activity may help to develop more specific and more effective aurora kinase inhibitors with less toxicity.

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

Authors’ Contributions
Conception and design: J. Sun, J. Yu, L. Zhang
Development of methodology: J. Sun, C. Dudgeon, J. Yu, L. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Sun, D. Chen, J. Yu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Sun, K. Knickelbein, Y. Shu, J. Yu, L. Zhang
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Sun, K. He, Y. Shu, L. Zhang
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