Cabozantinib induces PUMA-dependent apoptosis in colon cancer cells via AKT/GSK-3β/NF-κB signaling pathway

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Abstract
Cabozantinib is a multi-kinase inhibitor targeting MET, AXL, and VEGFR2, and has been approved for use in multiple malignancies. The means by which Cabozantinib acts to target colorectal cancer (CRC) cells remains poorly understood, and we sought to investigate how this drug disrupts cell growth in CRC cells and how it interacts to enhance the efficacy of other chemotherapeutic agents. In this study, we found that Cabozantinib activated a p65-dependent signaling pathway in response to both inhibition of AKT and activation of glycogen synthase kinase 3β (GSK3β), leading to upregulation of PUMA in CRC cells regardless of p53 activity. PUMA upregulation facilitates CRC apoptosis in response to Cabozantinib, which also acts synergistically with the chemotherapeutic agents Cetuximab and 5-FU to induce robust apoptosis in a PUMA-dependent manner. Eliminating PUMA expression ablated this apoptosis induced by Cabozantinib in xenograft mouse model. Our findings revealed that Cabozantinib acts to drive CRC cells apoptosis via a PUMA-dependent mechanism, thus identifying PUMA expression as a potential predictor of Cabozantinib efficacy and a potential novel therapeutic target.

Introduction
Colorectal cancer (CRC) is a leading cause of cancer-related death [1, 2]. Initial treatment is often surgical in nature, but because the disease is often not diagnosed until a more advanced and aggressive stage the application of chemotherapeutic agents is often essential to disease treatment [3, 4]. There is a wide range of heterogeneity between colon cancer types, with nearly 30% of cases stemming from hereditary mutations and the remainder arising from de novo acquired mutations [5, 6]. This heterogeneity can lead to the development of colon cancer via a number of pathways whereby adenocarcinomas develop from increasingly mutated gastrointestinal epithelial cells through the mutation of different oncogenes or tumor suppressor genes, resulting in differing levels of sensitivity to specific chemotherapeutic agents depending on the particular mutations driving a given case of the disease [7–9].

Cabozantinib is a small molecule multiple tyrosine kinase inhibitor that has been demonstrated to disrupt signaling through kinases including VEGFR-2, MET, RET, KIT, AXL, TIE2, and FLT3 [10, 11]. As many of these tyrosine kinases are often mutated and aberrantly activated in tumors including colon cancers, targeting them with a small molecule inhibitor is an optimal therapeutic strategy to block tumor growth, survival, and eventual metastasis [12, 13].

In this study, our findings demonstrated the induction of PUMA by Cabozantinib via AKT/GSK-3β/NF-κB signaling pathway. Once induced, PUMA acts to potentiate cellular apoptosis in response to Cabozantinib in CRC, making its upregulation essential to the efficacy of this chemotherapeutic agent. Our results indicated that PUMA induction is indicative of the therapeutic efficacy of Cabozantinib, and likely other targeted agents as well.
Materials and methods

Cell culture and drug treatment

The human colon cancer cell lines including HCT116, DLD1, HT29, Lim2405, and LoVo were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All colon cancer cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated newborn calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The anticancer agents and chemicals including Cabozantinib (Selleckchem, Houston, TX, USA), Cetuximab (InvivoGen, San Diego, CA, USA), BAY 11-7082 (Merck, Kenilworth, NJ, USA), 5-fluorouracil (5-FU, Sigma-Aldrich, St. Louis, MO, USA) were diluted with DMSO. Constitutively active AKT was got from addgene.

Real-time (RT) quantitative PCR

Cellular RNA was obtained through TRIzol extraction (Invitrogen), and 1 μg of the RNA was then used with the SuperScript II reverse transcriptase (Invitrogen) to produce cDNA [14, 15]. Real-time RT-polymerase chain reaction (PCR) using cDNA was performed using appropriate primers and probes in a 20 μL volume with the SsoFast™ Probes Supermix (Bio-Rad, Shanghai, China) and the Bio-Rad CFX96™ Real-time PCR System. Transcript quantification was achieved via a comparative Ct method (ΔΔCt) based on previously described and established protocols [14], with the 2-ΔΔCt approach being used to compare relative gene expression between samples. For all real-time RT-PCR assays, β-actin served as a normalizing control. The primers used in this study are listed as follows: PUMA, sense: 5′-ATGGCCGAGCAGCCTCAAC-3′ and anti-sense: 5′-AGTCCCATGAAGAGATTGTACATGAC-3′; β-actin, sense: 5′-GTGGGCCGCTTAGGGTTCAGGGGGG-3′; and anti-sense: 5′-CGTTGGCCTTAGGGTTCAGGGGGG-3′.

Western blotting

Western blotting was performed as previously described [14, 16], with antibodies for PUMA (ab33906) (Abcam, Cambridge, MA, USA), AKT (#9272), phospho-AKT (#4060), Bid (#2002), cleaved-caspase 3 (#9661), cleaved-caspase 9 (#9502), p65 (#8242), phospho-p65 (#4887), phospho-FoxO3a (#2599), glycogen synthase kinase 3β (GSK3β) (212456), phospho-GSK3β (55558), Bak (#6947), FoxO3a (#2497), cytochrome oxidase subunit IV (Cox IV) (#4850), p-STAT1 (#9167), STAT1 (#9172) (Cell Signaling Technology, Beverly, MA, USA), cytochrome C (sc-13561), Lamin A/C (sc-7292), β-actin (sc-1616), Bim (sc-374358) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Mcl-1 (559027), and Bcl-XL (610746) (BD, San Jose, CA, USA).

Apoptosis assays

Apoptosis was analyzed by nuclear staining with Hoechst 33258 [17]. Annexin V/propidium iodide (PI) staining was performed using annexin-Alexa 488 (Invitrogen) and PI. For analysis of cytochrome C release, cytosolic fractions were isolated by differential centrifugation, and probed by western blotting for cytochrome C. For colony formation assays, the treated cells were plated in 12-well plates at appropriate dilutions and allowing for cell growth for 10 days, followed by crystal violet staining of cell colonies.

Transfection and siRNA/shRNA knockdown

HCT116 cells at exponential stage were used for transfection. Before transfection, 2 × 10^5 cells were cultured in 12-well plate with 1 mL complete medium for 24 h. Lipofectamine 2000 (Invitrogen) was used in accordance with manufacturer’s protocols to transfect cells with the indicated constructs. For AKT overexpression, 0.4 μg AKT overexpression plasmid were transfected into the cells 24 h prior to treatment. For knockdown experiments, 24 h before Cabozantinib treatment 300 picomoles of either a scrambled control siRNA or siRNA specific for p65 (sc-29410) or GSK3β (sc-35527) purchased from Santa Cruz Biotechnology was transfected into cells. For shRNA-mediated stable gene knockdown, plasmids were obtained that contained either the p53-specific (CACCCTC- ACTACAACAT), the PUMA-specific sequence (CCTGGAGGGTCATGTACAATCTCTT), or a scrambled control sequence. HCT116 cells then had these plasmids transfected into them, after which they were added to a 96-well plate with puromycin (5 μg/mL) as a selection agent. Protein levels of target proteins of interest were then assessed by western blotting.

NF-κB nuclear translocation assessment

HCT116 cells in which genes of interest had been knocked down were treated with Cabozantinib for 6 h. A NE-PER nuclear/cyttoplasmic extraction kit (Thermo Fisher) was then used to isolate nuclei from these cells in accordance with the manufacturer’s instructions, and western blotting was used to assess NF-κB nuclear translocation via measuring p65 levels.
Chromatin immunoprecipitation

p65 antibody (Cell Signaling Technology) was used to perform chromatin immunoprecipitation (ChIP) in conjunction with a Chromatin Immunoprecipitation Assay Kit (Millipore). PUMA levels in kit precipitates were analyzed with primers for a region of the gene containing potential κB binding sites with the sequences 5′-GTCGGTCTGTGTACGCATCG-3′ and 5′-CCCCGCGTACGCTACGGCCC-3′.

Mouse model

All work with animals was conducted in accordance with the ethical guidelines of Chinese Medicine University. Totally, 4 × 10⁶ HCT116 cells in a 0.2 mL volume were implanted subcutaneously into Female athymic nude mice. After 7 days, mice were treated daily for 12 days with 30 mg/kg Cabozantinib via oral gavage. Tumor volume was measured regularly, and when size reached ~1.0 cm³ mice were euthanized. Tumors were embedded in paraffin after
being fixed with 10% formalin. Embedded tumor sections were used to assess TUNEL staining and active caspase 3 via immunostaining with a secondary antibody conjugated to AlexaFluor 488 (Invitrogen).

**Statistical analysis**

Data were shown as mean ± SD. Statistical analysis of the data was performed using the one-way ANOVA by SPSS software. $P < 0.05$ was considered statistically significant.

**Results**

**Cabozantinib induces PUMA expression in p53-independent manner in CRC**

To investigate the effect of Cabozantinib on CRC, we treated CRC cells with Cabozantinib. After treatment with 5 μmol/L Cabozantinib, HCT116 cells exhibited significant upregulation of PUMA at both the protein and mRNA level in a time-dependent manner (Fig. 1a–c). Knockdown of p53 in HCT116 cells (p53-KD) did not adversely affect PUMA
induction in response to Cabozantinib (Fig. 1a, d), and occurred in additional tested colon cancer cell lines such as the p53-WT Lim2405 and LoVo cell lines and the p53-mutant DLD1 and HT29 cell lines (Fig. 1e). In addition, Cabozantinib treatment did not lead to the upregulation of the proapoptotic Bcl-2 family proteins Bim and Bid. However, Cabozantinib treatment decreased the level of the antiapoptotic proteins Mcl-1 and Bcl-XL (Fig. 1f). These findings demonstrate that Cabozantinib induces PUMA regardless of p53 status, potentially mediating additional anticancer effects.

PUMA is required for Cabozantinib-induced apoptosis

To investigate the role of PUMA for Cabozantinib-induced apoptosis, we generated HCT116 cells in which PUMA was stably knocked down (PUMA-KD). Cabozantinib-dependent apoptosis was markedly decreased in response to PUMA knockdown (Fig. 2a), which was confirmed by Annexin V/PI staining (Fig. 2b). Similarly, Cabozantinib-induced caspase 3 and 9 activation (Fig. 2c) as well as release of cytochrome C (Fig. 2d), events consistent with
mitochondrial apoptotic processes, were decreased in PUMA-KD HCT116 cells. A long-term colony formation assay confirmed the enhancement of survival in these PUMA-KD cells upon Cabozantinib treatment (Fig. 2e). PUMA expression is thus essential for Cabozantinib-mediated apoptosis in CRC.

**NF-κB mediates PUMA activation in response to Cabozantinib**

We next studied how p53-independent PUMA induction occurs in response to Cabozantinib in CRC. We surveyed a range of different transcription factors with the potential to activate PUMA expression and found that FoxO3a was not relevant to this process as its phosphorylation was unchanged upon treatment (Fig. 3a). p73 and STAT1 were similarly unchanged in response to Cabozantinib (Fig. 3a).

The p65 subunit of NF-κB has been previously shown to induce transcription of PUMA upon TNF-α exposure, or treatment with Aurora Kinase inhibitors or Cetuximab. Cabozantinib treatment led to phosphorylation of the regulatory residue S536 of p65 in HCT116 cells (Fig. 3b). Knockdown of p65 eliminated PUMA induction by Cabozantinib in HCT116 cells (Fig. 3c). Following Cabozantinib treatment, nuclear fractionation demonstrated detectable p65 nuclear translocation (Fig. 3d). Treatment with BAY 11-7082, an inhibitor of NF-κB, blocked this nuclear translocation (Fig. 3d). Importantly, BAY 11-7082 treatment eliminated Cabozantinib-induced PUMA expression in addition to blocking p65 activation (Fig. 3e), suggesting a role for p65 nuclear translocation in Cabozantinib-mediated PUMA induction. ChIP was used to investigate whether NF-κB directly induces PUMA transcription, and results of this study showed that...
p65 binds directly to sites within the PUMA promoter region upon Cabozantinib treatment (Fig. 3f). Our results show that p65 binds directly to the PUMA promoter at specific κB sites in order to induce transcription upon Cabozantinib treatment.

**GSK3β activation-mediated p53-independent induction of PUMA by Cabozantinib**

Further analysis found that GSK3β facilitates Cabozantinib-induced p65 activation. When GSK3β was knocked down via siRNA this suppressed Cabozantinib-induced p65 nuclear translocation (Fig. 4a). Similarly, GSK3β depletion impaired Cabozantinib-induced PUMA upregulation in HCT116 cells (Fig. 4b). In control and p53-KD cells, Cabozantinib blocked the phosphorylation of Serine 9 (Ser 9) on GSK3β, blocking the kinase activity (Fig. 4c). The kinase AKT is known to promote GSK3β phosphorylation at Ser 9, and we observed that Cabozantinib treatment blocked the phosphorylation of AKT at Ser473 (Fig. 4d). Overexpressing exogenous AKT in an active form blocked PUMA induction upon Cabozantinib treatment (Fig. 4e). These results indicate that Cabozantinib treatment leads to inhibition of AKT, which in turn releases GSK3β from inhibition, thereby activating p65.

**PUMA mediates the chemosensitization effects of Cabozantinib**

Previous studies have combined Cabozantinib with other chemotherapeutic agents in clinical studies [18–20], and so we sought to assess whether PUMA induction facilitated chemosensitization effects in response to Cabozantinib. We observed that combination treatment with 5-FU and Cabozantinib led to elevated PUMA expression levels relative to single agent treatments (Fig. 5a). This supports the presence of multiple distinct modes of PUMA induction in response to these agents, potentially via a combination of p53-dependent and -independent manner. Apoptotic cell death and caspase-3 activation were significantly enhanced by this dual agent treatment in parental but not PUMA-KD HCT116 cells (Fig. 5a, b). Comparable results were also observed in response to combination treatment with Cabozantinib and Cetuximab (Fig. 5c, d). The above results indicate that PUMA can facilitate chemosensitization effects of Cabozantinib, potentially indicating that altering...
PUMA-mediated apoptosis is an effective strategy for enhancing Cabozantinib efficacy.

**PUMA enhances the antitumor activity of Cabozantinib in vivo**

We next used a xenograft model to assess how PUMA mediates Cabozantinib efficacy in vivo. Subcutaneous injection was used to implant nude athymic mice with both parental and PUMA-KD HCT116 cells. Mice were then treated daily with 30 mg/kg Cabozantinib or control via oral gavage for 12 days. Cabozantinib treatment suppressed the growth of parental HCT116 tumors by 80–90% (Fig. 6a). In contrast, tumors in mice given PUMA-KD cells were far less sensitive to the Cabozantinib treatment relative to control tumors (Fig. 6a), indicating that PUMA expression was required for Cabozantinib antitumor activity. Both PUMA upregulation and p65 phosphorylation were elevated in Cabozantinib-treated tumors (Fig. 6b). TUNEL staining also exhibited significant increases in apoptosis in tumors from Cabozantinib treated mice relative to control. In contrast, there was little detectable apoptosis in PUMA-KD tumors (Fig. 6c). Cleaved caspase 3 staining validated the presence of PUMA-dependent apoptosis in tumors treated with Cabozantinib (Fig. 6d). These results show that the in vivo efficacy of Cabozantinib depends on PUMA expression and engages the NF-κB signaling pathway.
Discussion

Our study reveals a novel mechanism by which Cabozantinib exerts therapeutic efficacy in CRC via inhibiting AKT, leading to activation of GSK3β, p65 activation and nuclear localization, and subsequent PUMA upregulation which leads to apoptosis. p65 is also known to be a key mediator of apoptosis induced in response to other chemotherapeutic agents such as Aurora Kinase inhibitors and Cetuximab, indicating that NF-κB signaling acts broadly in similar therapeutics to mediate cancer cell death [21, 22]. Other mechanisms that contribute to Cabozantinib-mediated apoptosis may include the depletion of Mcl-1 that arises in response to the treatment.

The data presented herein indicate that PUMA is induced following AKT inhibition upon Cabozantinib treatment, and these results further suggest that PUMA mediates activation of the mitochondrial apoptosis pathway. The importance of PUMA induction is not unique to Cabozantinib, and it serves as an important mediator of the efficacy of many chemotherapeutic agents making its upregulation a relevant marker of tumor chemosensitivity. Consistent with this model, previous studies have indicated that the degree of PUMA upregulation in response to EGFR TKIs in head and neck cancer cells is closely linked to the chemosensitivity of these cells, with ablation of PUMA signaling negating this sensitivity [23]. Other studies found that when exposed to the Bcl-2 homology 3 domains of PUMA, mitochondria isolated from tumor cells induced apoptotic pathways consistent with patient chemotherapy responses, suggesting a potential mechanism whereby PUMA mediates tumor cell death [24, 25]. PUMA is thus a key biomarker for predicting tumor response and sensitivity to Cabozantinib both in CRC and a range of other cancers. Cabozantinib additionally modulates the activation of other Bcl-2 family proteins upon treatment, suggesting that although it is a key mediator of this process. PUMA is not the only means by which Cabozantinib can induce tumor cell apoptosis. The remaining apoptosis in PUMA-KD cells treated with Cabozantinib cells may be linked to the induction of p53 and subsequent Bcl-2 family activation, and to other mechanisms of cell death. Although rarely is it practical to collect tumor biopsies from colorectal cancer patients after chemotherapy and surgical interventions, identifying PUMA levels in tumor cells through assessment of its expression in circulating tumor cells may allow for a non-invasive assessment of this key chemosensitivity biomarker in patients.

In summary, Cabozantinib exerts p53-independent effects on CRC, disrupting their growth via induction of apoptosis. Cabozantinib activates both caspase 3 and 9 cleavage in HCT116 cells, and our results demonstrate that inhibition of AKT and activation of GSK-3β/p65 signaling may drive Cabozantinib-mediated PUMA expression. This induction of PUMA may serve as an ideal biomarker for Cabozantinib clinical trials and therapeutic management, guiding future applications and development of this chemotherapeutic agent.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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