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PUMA induction by FoxO3a mediates the anticancer activities of the broad-range kinase inhibitor UCN-01

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Abstract

Most targeted anticancer drugs are inhibitors of kinases that are aberrantly activated in cancer cells. However, the mechanisms by which kinase inhibitors suppress tumor growth remain unclear. In this study, we found that UCN-01, a staurosporine analogue and broad-range kinase inhibitor used in clinical trials, inhibits colon cancer cell growth by inducing apoptosis via PUMA, a BH3-only Bcl-2 family member and a p53 target. PUMA expression was markedly elevated in a p53-independent fashion following UCN-01 treatment. The induction of PUMA by UCN-01 was mediated by direct binding of FoxO3a to the *PUMA* promoter following inhibition of AKT signaling. Deficiency in PUMA abrogated UCN-01-induced apoptosis, caspase activation, and mitochondrial dysfunction, and rendered UCN-01 resistance in a clonogenic assay, whereas elevated PUMA expression or a BH3 mimetic sensitized UCN-01-induced apoptosis. Chemosensitization by UCN-01 appeared to involve simultaneous PUMA induction through both p53-dependent and -independent mechanisms. Furthermore, deficiency in PUMA suppressed the anti-tumor effects of UCN-01 in a xenograft model, concurrent with reduced apoptosis and caspase activation *in vivo*. These results suggest that PUMA-mediated apoptosis is pivotal for the anticancer activities of UCN-01, and possibly other clinically used kinase inhibitor drugs, and that PUMA manipulation may be useful for improving their anticancer activities.

Keywords

PUMA; FoxO3a; UCN-01; apoptosis; mitochondria; AKT; colon cancer

Introduction

Inhibition of aberrant kinase signaling in cancer cells represents one of the most effective approaches for anticancer therapy. Most recently approved anticancer drugs are inhibitors of kinase signaling pathways required for tumor cell growth. However, there is little understanding of how kinase inhibition leads to therapeutic response. Virtually all kinase inhibitor drugs inhibit multiple kinases, and their anticancer activities are often attributed to off-target effects. One critical anticancer mechanism of kinase inhibitors is their ability to

induce apoptosis (1). For example, clinical response to epidermal growth factor receptor (EGFR) targeted anticancer therapies is correlated with induction of apoptosis in tumor cells (2).

It is well known that several broad-range kinase inhibitors, such as staurosporine (STS) and its more selective derivative UCN-01 (7-hydroxystaurosporine), have anticancer activities. UCN-01 is being evaluated in a number of clinical trials as a single agent or chemosensitizer (<http://clinicaltrials.gov>). It can potentiate cell cycle arrest and apoptosis induced by a variety of chemotherapeutic agents, such as cisplatin, topotecan, and 5-Fluorouracil (5-FU) (3). UCN-01 inhibits a variety of kinases involved in regulating cell cycle progression and apoptosis, such as cyclin-dependent kinases (CDKs), checkpoint kinase 1 (CHK1), protein kinase C (PKC), phosphoinositide-dependent protein kinase 1 (PDK1), and AKT (4). Although the effects of UCN-01 on cell cycle checkpoints have been well characterized, the mechanism by which UCN-01 promotes apoptosis remains unclear. Recent studies suggest that UCN-01 can modulate Bcl-2 family members to potentiate apoptosis in cancer cells (5,6).

PUMA, p53-upregulated modulator of apoptosis, is a BH3-only Bcl-2 family member and a potent inducer of apoptosis. Transcription of PUMA is activated by p53 in response to DNA damaging agents such as γ -irradiation and common chemotherapeutic drugs (7). PUMA binds to all five anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, which relieves their inhibition of Bax and Bak, leading to mitochondrial membrane permeabilization, and subsequently caspase cascade activation (7). *PUMA* knockout renders resistance to p53-dependent apoptosis induced by genotoxic agents in human cancer cells and mice (8–10). Nevertheless, p53-dependent regulation of PUMA is dysfunctional in most cancer cells due to p53 abnormalities, causing survival of tumor cells and therapeutic resistance. PUMA also mediates p53-independent apoptosis induced by a variety of non-genotoxic stimuli, such as TNF- α (11), serum starvation (12), cytokine withdrawal (13), STS (10,14), glucocorticoids (9,10), and ischemia/reperfusion (15). Several transcription factors, including p65, p73, and Forkhead Box O3a (FoxO3a), have been implicated in p53-independent *PUMA* induction. For example, PUMA is induced in response to cytokine deprivation by FoxO3a (13,16), whose activity is negatively regulated by phosphorylation via AKT (17).

In this study, we investigated how PUMA is induced by the kinase inhibitors UCN-01 and STS, and its role in UCN-01-induced apoptosis and chemosensitization. We found FoxO3a-mediated PUMA induction is pivotal for the anticancer effects of UCN-01. The results provide novel mechanistic insight into therapeutic response to kinase inhibitors, and may have broad implications for their future applications.

Materials and Methods

Cell culture and treatment

The human colorectal cancer cell lines, including HCT116, RKO, Lim2405, LOVO, (all WT p53), and HT29 and DLD1, (both mutant p53), were obtained from American Type Culture Collection (Manassas, VA) before 2002. The isogenic cell lines included the previously described *p53*-knockout (KO), *p21*-KO, *PUMA*-KO, *p21*-KO/*PUMA*-KO (from Dr. Bert Vogelstein at Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins in 2002) (8), *p21*-KO/p53 binding site-KO (*BS*-KO) HCT116 cells, and *PUMA*-KO DLD1 cells created in our laboratory in 2007 (14). Cell lines were last tested and authenticated for absence of mycoplasma, genotypes, drug response, and morphology in our laboratory in April, 2010.

All cell lines were cultured in McCoy's 5A modified media (Invitrogen, Carlsbad, CA) supplemented with 10% defined FBS (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Cells were maintained in a 37°C incubator at 5% CO₂. For

drug treatment, cells were plated in 12-well plates at 20–30% density 24 hr prior to treatment. The DMSO (Sigma, St. Louis, MO) stocks of the agents used, including UCN-01, STS, Wortmannin, LY294002 (Sigma), and triciribine (Enzo Life Sciences AG, Lausen, Switzerland), and MK-2206 (MediMol, Centereach, NY, USA), were diluted into appropriate concentrations with the cell culture medium. Cisplatin (Sigma) was diluted with 0.9% NaCl.

Western blotting and treatments

Western blotting was performed as previously described (11). The following antibodies were used: PUMA (8), FoxO3a (07702MI, Millipore, Billerica, MA), phospho-FoxO3a (9464, Cell Signaling, Beverly, MA), AKT (9272, Cell Signaling), phospho-AKT (S473) (4058, Cell Signaling), active caspase 3 (9661, Cell Signaling), CoxIV (A21348, Invitrogen), cytochrome *c* (7159, Santa Cruz Biotechnology, Santa Cruz, CA), β -actin (A5441, Sigma), and α -tubulin (CP06, Oncogene Science, Cambridge, MA).

Real-time Reverse Transcriptase (RT) PCR

Total RNA was isolated from UCN-01- or STS-treated cells using the Mini RNA Isolation II Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. One μ g of total RNA was used to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was carried out as before for *PUMA* and *GAPDH* (11).

Transfection and siRNA knockdown

Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. pCMV, FoxO3a triple mutant (FoxO3a TM; Addgene, Cambridge, MA), WT and constitutively active AKT vectors were used in the transfection. siRNA knockdown was performed 24 hr before UCN-01 or STS treatment using 400 pmoles of *FoxO3a* (ON-TARGETplus J-003007-10) or the control scrambled siRNA (Dharmacon, Chicago, IL).

Luciferase assays

PUMA luciferase reporter construct was generated by cloning a genomic fragment (WT fragment: –500 to +739 bp) containing two FoxO3a sites located within the first intron of *PUMA* into the pBV-Luc plasmid as previously described (12). Mutations were introduced into the FoxO3a binding sites using QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). For reporter assays, cells were transfected with the WT or mutant *PUMA* reporter along with the transfection control β -galactosidase reporter pCMV β (Promega, Madison, WI, USA). Cell lysates were collected and luciferase activities were measured as previously described (12). All reporter experiments were performed in triplicate and repeated three times.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology, Lake Placid, NY, USA) as previously described (14), with FoxO3a antibody for chromatin precipitation. The precipitates were analyzed by PCR using primers 5' GCGCACAGGTGCCTCGGC 3' and 5' TGGGTGTGGCCGCCCT 3'.

Stable knockdown of FoxO3a

The sequence of *FoxO3a* siRNA used for transient knockdown was cloned into the pSUPER vector (OligoEngine, Seattle, WA). The shRNA construct was transfected into HCT116 cells, and cells were plated in 96-well plates in the presence of puromycin (2 μ g/ml, Invitrogen). After puromycin-resistant clones were isolated, Western blotting was used to identify clones with reduced FoxO3a levels.

Analysis of apoptosis

Analysis of apoptosis by nuclear staining with Hoechst 33258 (Invitrogen) was performed as previously described (18). Annexin V/propidium iodide (PI) staining was performed using annexin Alexa 488 (Invitrogen) and PI as described (14). For analysis of cytochrome *c* release, mitochondrial and cytosolic fractions were isolated by the differential centrifugation method previously described (19), 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 allowed to grow for 10–14 days before staining with crystal violet (Sigma). For detection of mitochondrial membrane potential change, the treated cells were stained by MitoTracker Red CMXRos (Invitrogen) for 15 min at room temperature, and then analyzed by flow cytometry.

Xenograft studies

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Female 5–6 week-old Nu/Nu mice (Charles River, Wilmington, MA) were housed in a sterile environment with micro isolator cages and allowed access to water and chow *ad libitum*. Mice were injected subcutaneously in both flanks with 4×10^6 *p21*-KO or *p21*-KO/*PUMA*-KO HCT116 cells. Following tumor growth for 7 days, mice were injected intraperitoneally (i.p.) for 5 consecutive days with 9 mg/kg UCN-01 (20) diluted in 20 mM sodium citrate buffer, pH 6. UCN-01 used for the animal study was obtained from Kwoya Hakko Kirin, Co. Ltd. (Tokyo, Japan) through the Developmental Therapeutics Program at the National Cancer Institute (NCI). Mice were euthanized when tumors reached $\sim 1 \text{ cm}^3$ in size. Tumors were dissected and fixed in 10% formalin before paraffin embedding.

Immunostaining

Immunostaining was carried out on 5 μM paraffin-embedded tumor sections. TUNEL and active caspase 3 immunofluorescence was performed as previously described (21), with an AlexaFluor 594-conjugated secondary antibody (Invitrogen) for detection. p-AKT and p-FoxO3a immunohistochemistry was performed using the same antibodies used for Western blotting, and the ABC/DAB method for detection.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism IV software. *p* values were calculated by the student's *t*-test and were considered significant if $p < 0.05$. The means \pm one standard deviation (s.d.) were displayed in the figures.

Results

PUMA is induced by UCN-01 in a p53-independent manner

The requirement for PUMA in STS-induced apoptosis (10,14) prompted us to investigate whether it contributes to apoptosis induced by UCN-01, a STS derivative and more selective kinase inhibitor that has been tested in clinical trials (Fig. 1A). PUMA was found to be induced by 0.1–1.0 μM of UCN-01 in a dose-dependent manner in both WT and *p53*-KO HCT116 colon cancer cells (Fig. 1B). Both PUMA mRNA and protein were induced by UCN-01 within several hours, with the peak level of *PUMA* mRNA induction at 8 hr (Fig. 1C, left panel), and that of protein at 24 hr (Fig. 1C, right panel). This induction time course was similar to that of STS (Fig. S1A and B), which also induced PUMA independently of p53 (Fig. S1C). The induction of PUMA by UCN-01 or STS could be blocked by the transcription inhibitor actinomycin D (data not shown). Furthermore, UCN-01 treatment resulted in PUMA induction in five additional colon cancer cell lines regardless of their p53 status (Fig. 1D). Taken together, these data indicate that PUMA can be induced by the broad-spectrum kinase inhibitor UCN-01 through p53-independent transcription.

FOXO3a directly activates *PUMA* transcription following UCN-01 treatment

PUMA can be induced by the transcription factor FoxO3a in T and mast cells following cytokine deprivation (13,16). Since UCN-01 was shown to inhibit AKT (22), which phosphorylates FoxO3a and prevents its nuclear localization (17), we determined whether FoxO3a and AKT are involved in *PUMA* induction by UCN-01. Expression of a constitutively active FoxO3a mutant (FoxO3aTM) induced *PUMA* in *p53*-KO cells (Fig. S2A). siRNA depletion of FoxO3a in WT and *p53*-KO HCT116 cells abrogated *PUMA* induction by UCN-01 or STS (Fig. 2A and data not shown), indicating the requirement of FoxO3a for the induction of *PUMA* following kinase inhibition.

A conserved FoxO binding element (AAACA) was previously identified in the human and mouse *PUMA* promoters (13). We noted that in addition to this site, there is another FoxO3a binding element 2 bases apart within the first intron of *PUMA*, but in the reverse orientation (Fig. 2B). To determine the role of these putative FoxO3a binding sites in *PUMA* induction, luciferase reporters containing a 1.1-kb fragment including these two sites were constructed and analyzed. Both FoxO3a binding sites were also mutated to dissect out their specific role in *PUMA* induction (Fig. 2B). Transfection of FoxO3aTM or treating cells with UCN-01 or STS markedly activated the WT *PUMA* reporter. In contrast, the binding site mutations abolished the responsiveness of the *PUMA* reporter to FoxO3aTM, and also abrogated the effects of STS and UCN-01 (Fig. 2C). To determine whether FoxO3a directly activates the *PUMA* promoter, chromatin immunoprecipitation (ChIP) was performed on lysates from STS- or UCN-01-treated cells using the FoxO3a antibody. The binding of FoxO3a to the *PUMA* promoter was significantly enhanced after treatment with either compound (Fig. 2C). Taken together, these data suggest that FoxO3a can directly bind to the *PUMA* promoter to activate its transcription following STS or UCN-01 treatment.

AKT inhibition drives *PUMA* induction by UCN-01

We then determined whether AKT, an antiapoptotic kinase often activated aberrantly in cancer cells, is involved in the effect of UCN-01 on *PUMA* expression. UCN-01 treatment markedly inhibited AKT phosphorylation at S473, concurrent with reduced FoxO3a phosphorylation (Fig. 3A), which is known to prevent its nuclear translocation and ensuing transactivation (17). On the other hand, transfection with WT or constitutively active AKT induced FoxO3a phosphorylation, suppressed basal *PUMA* expression (Fig. 3B), and overcame the induction of *PUMA* by UCN-01 (Fig. 3C). Furthermore, treating cells with the AKT inhibitor triciribine or MK-2206, an AKT inhibitor in clinical trials, was sufficient to induce *PUMA*, subsequent to diminished AKT and FoxO3a phosphorylation (Fig. 3D). In addition, *PUMA* could also be induced similarly by the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002 in *p53*-KO cells (Fig. S2B). Therefore, the induction of *PUMA* by FoxO3a following UCN-01 treatment seemed to be mediated through AKT inhibition.

PUMA plays an essential role in UCN-01-induced apoptosis

In order to investigate the role of *PUMA* in UCN-01-induced apoptosis, we analyzed *p21*-KO cells with different *PUMA* status because they harbor a more pronounced apoptotic phenotype due to defects in cell cycle checkpoints (23). UCN-01 treatment induced dose-dependent apoptosis in *p21*-KO cells, with over 50% apoptotic cells detected following treatment with 3 μ M UCN-01 (Fig. 4A). Remarkably, UCN-01-induced apoptosis was almost completely abolished in *PUMA*-deficient *p21*-KO cells (Fig. 4A). The requirement of *PUMA* in UCN-01-induced apoptosis is not cell line specific, as the apoptosis was also abolished in the previously described *PUMA*-knockout DLD1 colon cancer cells (14) (Fig. S3A). Analysis of apoptosis by Annexin V/PI staining confirmed reduced apoptosis in *PUMA*-deficient cells (Fig. S3B). *PUMA* deficiency abrogated UCN-01-induced caspase 9 and caspase 3 activation (Fig. 4B), cytosolic release of cytochrome *c* (Fig. 4C), as well as mitochondrial membrane potential

change (Fig. S3C). PUMA-deficient cells were also found to be significantly more resistant to UCN-01 than WT cells in a long-term clonogenic assay (Fig. 4D). As a control, we also analyzed *p21*-KO cells with a deletion of the p53 binding sites in the *PUMA* promoter (*p21*-KO/*BS*-KO), which abolishes apoptosis induced by p53 and DNA damaging agents (14). The apoptotic effect of UCN-01 remained intact in these cells, confirming the dispensability of p53. Furthermore, transient knockdown of FoxO3a by a siRNA or stable depletion of FoxO3a with a shRNA suppressed PUMA transcription and UCN-01-induced apoptosis (Fig. S3D and data not shown). Collectively, these results demonstrate that PUMA is essential for UCN-01-induced apoptosis in colon cancer cells.

PUMA mediates chemosensitization by UCN-01 and enhances its anticancer activity

UCN-01 has mostly been used as a chemosensitizer and can potentiate the effects of common chemotherapeutic drugs such as cisplatin (3). Since both UCN-01 and cisplatin induce PUMA-dependent apoptosis (Fig. 4, S4A and S4B), we asked whether chemosensitization by UCN-01 involves PUMA induction. A combination of UCN-01 and cisplatin induced PUMA at a much higher level in HCT116 cells compared with UCN-01 or cisplatin alone (Fig. 5A), consistent with two distinct p53-dependent and -independent mechanisms of PUMA induction by these agents. Apoptosis and caspase 3 activation was also markedly enhanced by the combination treatment in HCT116 cells (Fig. 5A and 5B). However, the enhanced apoptosis and caspase activation by the combination treatment was greatly reduced in the *PUMA*-KO cells (Fig. 5B and S4C), suggesting that p53-independent induction of PUMA by UCN-01 mediates its chemosensitization effect. We also found that an adenovirus expressing PUMA (Ad-PUMA), but not a BH3 domain-deleted mutant (Ad- Δ BH3), enhanced UCN-01-induced apoptosis in HCT116 cells (Fig. 5C). Furthermore, GX15-070, a PUMA-like BH3 mimetic compound capable of binding to all five antiapoptotic Bcl-2 family members, significantly enhanced UCN-01 induced apoptosis (Fig. 5D). These observations suggest that manipulating apoptosis regulators such as PUMA can potentiate the anticancer effects of kinase inhibitors.

PUMA contributes to the anti-tumor activity of UCN-01 in a xenograft model

To determine whether the PUMA-dependent apoptotic effect of UCN-01 contributes to its anti-tumor activity *in vivo*, *p21*-KO and *p21*-KO/*PUMA*-KO HCT116 cells were injected into nude mice to establish xenograft tumors. Tumor-bearing mice were treated with 9 mg/kg of UCN-01 for 5 days as previously described (20), and tumor volumes were measured every 2–3 days for 3 weeks. The *p21*-KO tumors responded to the UCN-01 treatment with slower growth, and were roughly half the size of the untreated tumors on day 12 following treatment (Fig. 6A). In contrast, the *p21*-KO/*PUMA*-KO tumors were indistinguishable from the untreated mice, and did not respond to UCN-01 treatment (Fig. 6A). UCN-01 treatment suppressed AKT and FoxO3a phosphorylation (Fig. 6B), and induced PUMA expression in the *p21*-KO tumors (Fig. 6C). TUNEL and active caspase 3 staining revealed significant apoptosis induction in the *p21*-KO tumors, which was almost completely abolished in the *p21*-KO/*PUMA*-KO tumors (Fig. 6D and S5). These data clearly demonstrate the necessity of PUMA for the *in vivo* anti-tumor and apoptotic effects of UCN-01.

Discussion

Our results provide novel mechanistic insight into the anticancer mechanism of the broad-range kinase inhibitor UCN-01. Most of the previous studies have focused on the effect of UCN-01 on cell cycle checkpoints, which is widely believed to be its major mode of action in anticancer therapy. Several reports suggest UCN-01 can potentiate apoptosis via the mitochondrial pathway (24–26). However, the exact mechanism underlying this activity is unclear, and the role of apoptosis in the therapeutic response to UCN-01 remains conjecture. Our results demonstrate for the first time that UCN-01 treatment leads to PUMA induction by FoxO3a

following AKT inhibition. Induction of PUMA accounts for most, if not all, of *in vitro* and *in vivo* activities of UCN-01 against colon cancer cells, and such a requirement for PUMA was not affected by the genetic background and p53 status. In addition to PUMA, other BH3-only proteins, such as Bim, may also contribute to the effect of UCN-01 on other tumor types (6). It is notable that UCN-01 induces apoptosis in solid tumor cells typically at micromolar concentrations, which are significantly higher than those required for checkpoint inhibition (27,28). The UCN-01 dose used in our study (1.0 μM) is similar to those used in the previous apoptosis studies (5,29,30). Pharmacokinetic analyses showed that UCN-01 plasma concentrations can be as high as 30 μM (31,32), suggesting that apoptosis-inducing concentrations are achievable.

UCN-01 is commonly used as a chemosensitizer in combination with other anticancer agents. It has additive or even synergistic effects on apoptosis induced by a variety of commonly used chemotherapeutic drugs, such as 5-fluorouracil (33), topotecan (34), tamoxifen (35), and mitomycin C (36). UCN-01 can also potentiate a number of non-genotoxic agents, such as the CDK inhibitor roscovitine (37), the mTOR inhibitor rapamycin (38), the PKC activator PMA (25), and the NF- κB inhibitor BAY 11-7082 (39). Research into its mechanism of action revealed activation of the ERK pathway by UCN-01 (40), supporting a rationale of inhibiting the proliferative ERK signal to enhance the activity of UCN-01. It is exciting that a number of ERK inhibiting agents were found to potentiate UCN-01-induced apoptosis, such as the MEK inhibitor PD184352 (41), the farnesyltransferase inhibitor L744832 (42), and the Hsp90 antagonist 17-AAG (43). These studies highlight the multitudinous ways UCN-01 can be used in chemotherapy, as well as the importance of investigating UCN-01 modes of action to design rational combinations. Our results suggest enhanced PUMA induction may be a novel mechanism of chemosensitization by UCN-01. The expression of PUMA is controlled by several proapoptotic transcription factors in a cell type- and stimuli-dependent manner (7). Combinations of different classes of PUMA-inducing agents may allow simultaneous PUMA induction via multiple pathways, thereby lowering the threshold proapoptotic activity required for apoptosis induction.

Targeting cancer-specific molecular changes has brought new hopes to therapeutic intervention of cancer. Most of the targeted drugs proven to be clinically useful are inhibitors of the kinase signaling pathways that are required for tumor cell growth. In addition to STS and UCN-01, PUMA can also be induced by other kinase inhibitors, such as the MEK inhibitor U0126 (44), phosphoinositide-3 kinase (PI3K) inhibitors wortmanin and LY294002 (12,45), the human epidermal growth factor receptor (HER)/vascular endothelial growth factor receptor (VEGFR) inhibitor BMS-690514 (46), and the farnesyltransferase inhibitor BMS-214662 (47). It has recently been shown that PUMA can be induced by the clinically used EGFR inhibitors, gefitinib and erlotinib, in head and neck cancer cells, and its expression correlated with therapeutic response to the EGFR inhibitors (48). Therefore, PUMA may play a broad functional role in determining response to targeted therapies, and the induction of PUMA may serve as a marker for predicting therapeutic response to these agents.

Maintenance of tumor phenotypes is dependent upon the suppression of apoptosis by certain pro-survival proteins, because neoplastic transformation would normally trigger an apoptotic response (49). The ability to reinstate the induction of apoptosis in cancer cells is an attractive approach for anticancer therapy. It is perhaps not surprising that apoptosis has emerged as a critical therapeutic endpoint of kinase inhibitors (1). The induction of PUMA by kinase inhibitors primarily occurs via p53-independent mechanisms. Such induction would bypass the p53 network that is required for the efficacy of common chemotherapeutics. Another way of circumventing the p53 pathway would be to use compounds that directly target the apoptotic machinery. For example, agents that mimic the BH3 domains of the proapoptotic Bcl-2 family members, such as ABT-263 and GX15-070, are currently being tested in clinical trials (50).

The effectiveness of Ad-PUMA and GX15-070 in enhancing UCN-01-induced apoptosis supports a rationale for combining kinase inhibitors and apoptosis targeting agents.

In conclusion, we demonstrated that PUMA is a critical mediator of therapeutic response to UCN-01, and possibly other kinase inhibitors, in colon cancer cells. Our study may serve as a proof-of-principle for targeting PUMA to improve therapeutic effects of kinase inhibitors, for using PUMA expression as a biomarker for predicting therapeutic response, and for rational design of new therapeutic regimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CDK	cyclin-dependent kinases
ChIP	Chromatin immunoprecipitation
CHK1	checkpoint kinase 1
EGFR	epidermal growth factor receptor
FoxO3a	Forkhead Box O3a
5-FU	5-Fluorouracil
KO	knockout
PKC	protein kinase C
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
RT-PCR	reverse transcriptase-PCR
shRNA	small hairpin RNA
siRNA	small interfering RNA
STS	staurosporine
VEGFR	vascular endothelial growth factor receptor

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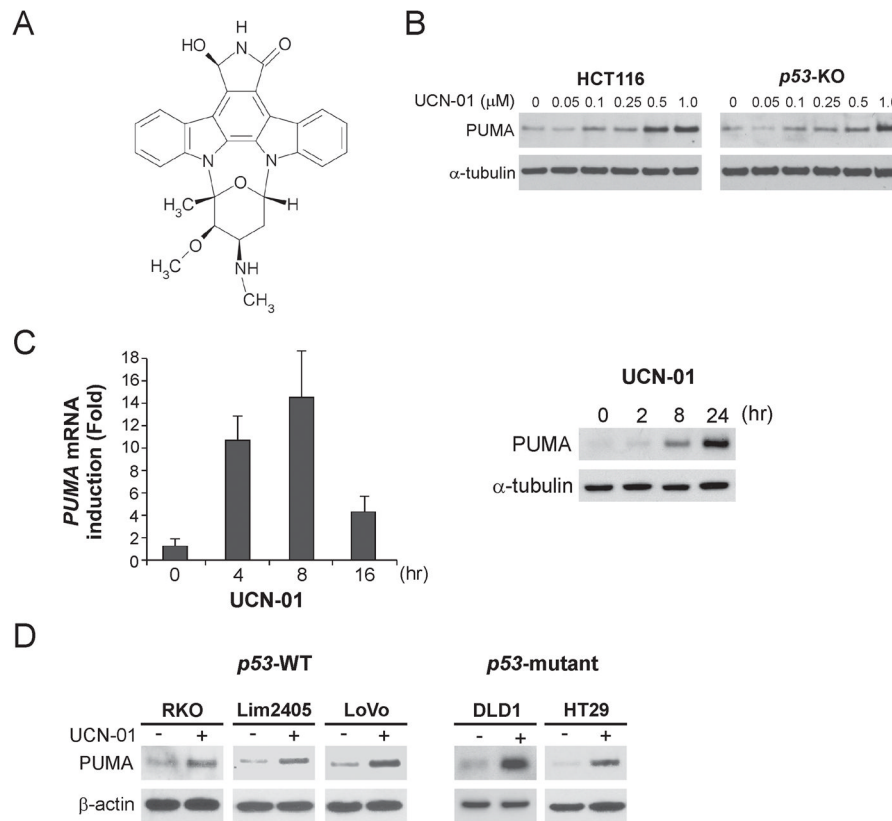


Figure 1. p53-independent PUMA induction by UCN-01 in colon cancer cells

A. Chemical structure of UCN-01. **B.** WT and *p53*-knockout (*p53*-KO) HCT116 colon cancer cells were treated with increasing doses of UCN-01 for 24 hr. PUMA expression was analyzed by Western blotting. **C.** Left: *p53*-KO HCT116 cells were treated with 1 μM UCN-01 and mRNA extracted at indicated time points. Reverse transcription followed by real-time PCR was completed as described in Materials and Methods. Right: Following treatment of *p53*-KO HCT116 cells with 1 μM UCN-01, PUMA protein expression at the indicated times was analyzed by Western blotting. **D.** Five colon cancer cell lines with different *p53* status were treated with UCN-01 for 24 hr. PUMA expression was analyzed by Western blotting.

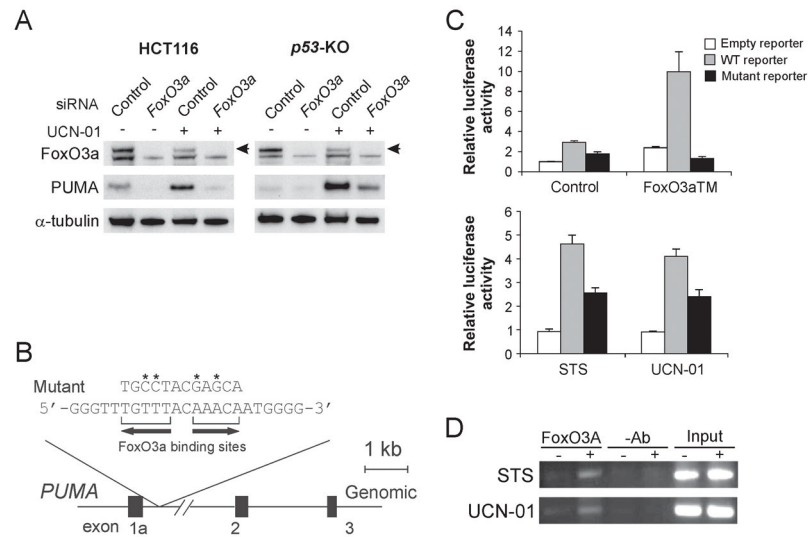


Figure 2. FoxO3a directly activates *PUMA* transcription following UCN-01 treatment
A. *p53*-KO HCT116 cells were transfected with either a control scrambled siRNA or a *FoxO3a* siRNA for 24 hr, and then treated with 1 μ M UCN-01 for 24 hr. FoxO3a and PUMA expression were probed by Western blotting. Arrow denotes FoxO3a-specific band. **B.** Schematic representation of the genomic structure of *PUMA* highlighting the two FoxO3a binding sites (AAACA) within the first intron. Asterisks indicate binding site mutations. **C.** Upper: *p53*-KO HCT116 cells were transfected with a reporter plasmid containing the WT or mutant FoxO3a binding sites in the *PUMA* promoter, along with a FoxO3a triple mutant (FoxO3aTM) expression construct or the control empty vector. The reporter activities were measured by luciferase assay on the next day. Lower: *p53*-KO HCT116 cells were transfected overnight with the reporter containing the WT or mutant FoxO3a binding sites, and then treated with 60 nM staurosporine (STS) or 0.5 μ M UCN-01 for 16 hr. Luciferase activity was quantified. **D.** Chromatin immunoprecipitation (ChIP) was performed on fixed *p53*-KO HCT116 cells following 8 hr 60 nM STS or 1 μ M UCN-01 treatment. An antibody specific for FoxO3a or no antibody was used to show specificity. PCR was carried out using primers surrounding the FoxO3a binding sites in the *PUMA* promoter.

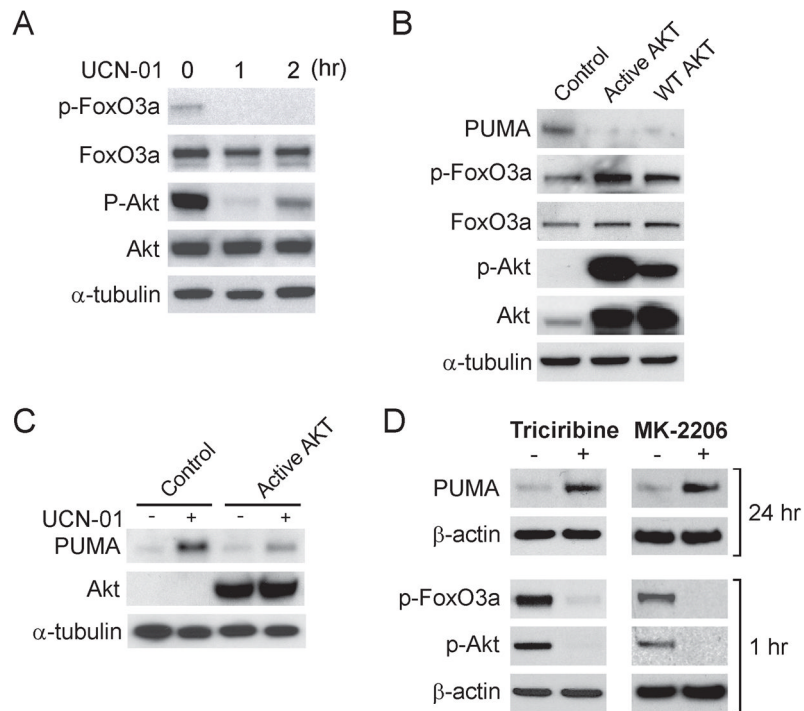


Figure 3. Induction of PUMA by UCN-01 is mediated through AKT inhibition

A. *p53*-KO HCT116 cells were treated with 1 μ M UCN-01 for 1 or 2 hr. Western blotting was used to analyze phospho-FoxO3a (p-FoxO3a), total FoxO3a, phospho-AKT (p-AKT), and total AKT. **B.** *p53*-KO cells were transfected for 24 hr with WT, constitutively active mutant AKT, or control empty vector. Indicated proteins were probed by Western blotting. **C.** *p53*-KO cells were transfected for 24 hr with constitutively active mutant AKT or control empty vector, and then were treated with 1 μ M UCN-01 for 24 hr. PUMA and AKT expression were probed by Western blotting. **D.** *p53*-KO cells were treated with the AKT inhibitor triciribine (1 μ M) or MK-2206 (25 μ M) for 1 hr (for analysis of p-FoxO3a and p-AKT) or 24 hr (for analysis of PUMA), and indicated proteins were analyzed by Western blotting.

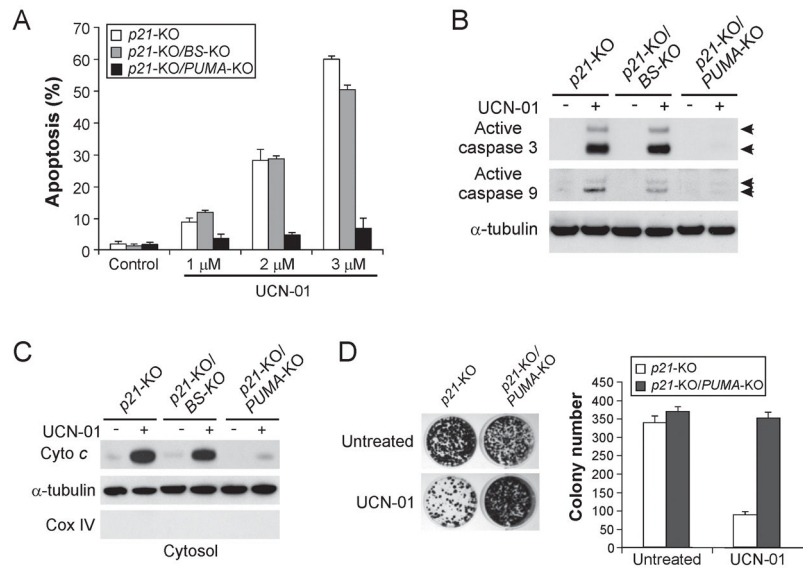


Figure 4. PUMA mediates the apoptotic and anticancer activities of UCN-01 through the mitochondrial pathway

HCT116 cells with different genotypes, including *p21*-KO, *p21*-KO/*p53* binding site-KO (*BS*-KO), and *p21*-KO/*PUMA*-KO, were treated with 0–3 μM UCN-01 for 48 hr. Apoptosis was analyzed using several methods. **A.** Apoptosis was measured by nuclear fragmentation assay following staining with Hoechst 33258. **B.** Activation of caspase 3 and caspase 9 in the cells treated with 3 μM UCN-01 was analyzed by Western blotting. **C.** Cytosolic fractions were isolated from the cells treated with 3 μM UCN-01 and probed for cytochrome *c* by Western blotting. Cytochrome oxidase subunit IV (Cox IV), which is a mitochondrial marker, was analyzed as the control for fractionation. α-tubulin was used as a control for loading. **D.** Colony formation assay was performed by seeding a equal number of UCN-01 (2 μM)-treated *p21*-KO and *p21*-KO/*PUMA*-KO 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. The results were averages of 3 experiments ± standard deviation.

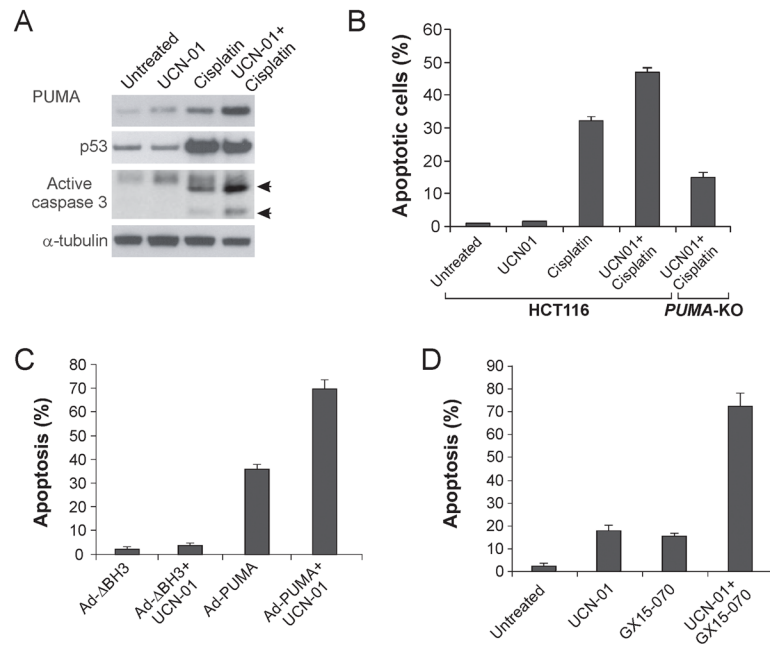


Figure 5. PUMA is required for the chemosensitization effects of UCN-01 and enhances its anticancer activity

A. WT HCT116 cells were treated with 0.5 μ M UCN-01, 50 μ M cisplatin, or their combination for 24 hr. PUMA, p53, and active caspase 3 were analyzed by Western blotting. **B.** WT and PUMA-KO HCT116 cells were treated with 0.5 μ M UCN-01, 50 μ M cisplatin, or their combination for 48 hr. Apoptosis was determined by nuclear staining with Hoechst 33258. **C.** p21-KO HCT116 cells were treated with 0.5 μ M UCN-01, alone or in combination with an adenovirus (10 MOI) expressing WT PUMA (Ad-PUMA) or mutant PUMA with BH3 domain deletion (Ad- Δ BH3). Apoptosis was determined by nuclear staining 48 hr after treatment. **D.** p21-KO HCT116 cells were treated with 2 μ M UCN-01, alone or in combination with 1 μ M of the BH3 mimetic GX15-070. Apoptosis was determined by nuclear staining 48 hr after treatment. The results were averages of three independent experiments \pm standard deviation.

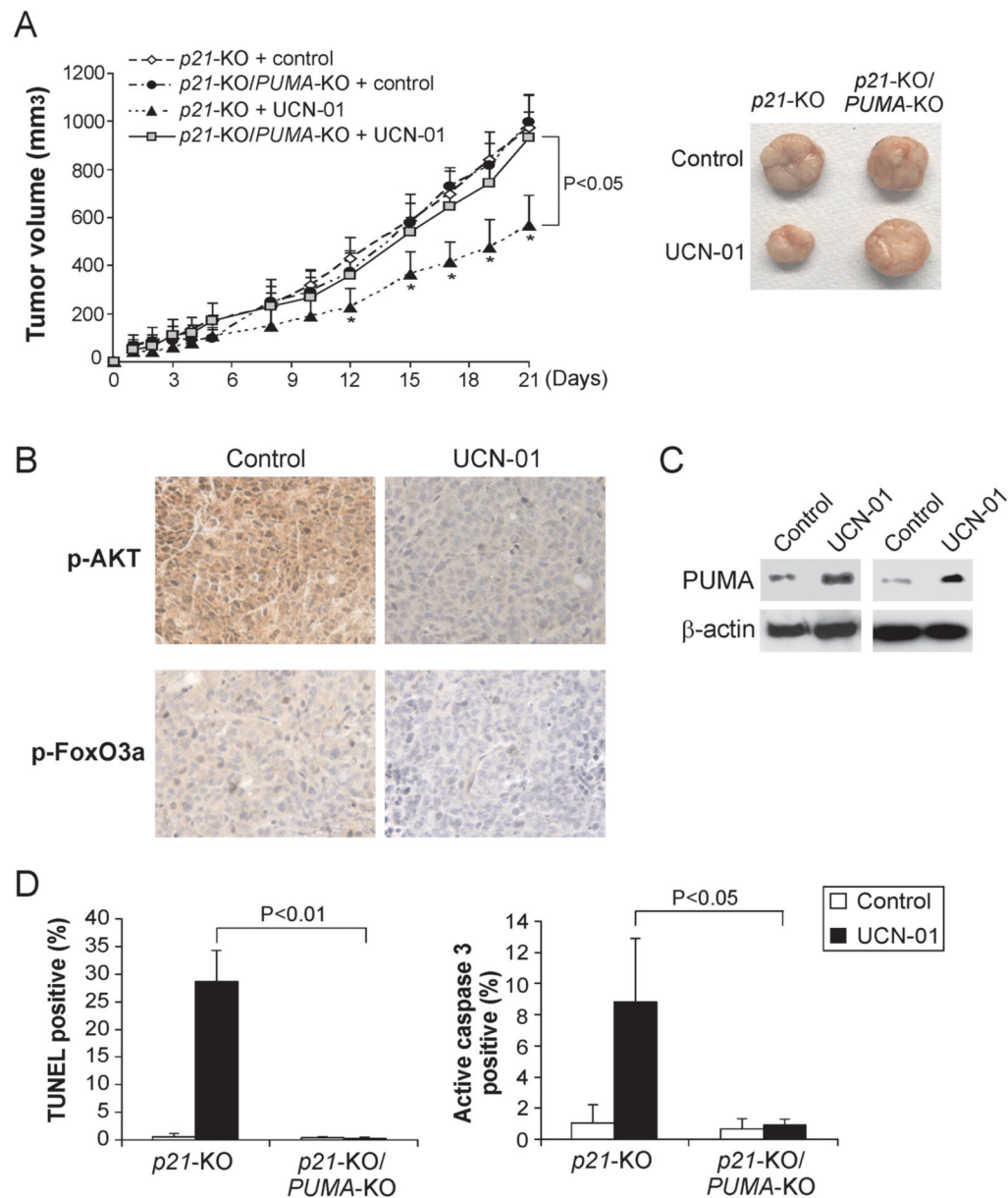


Figure 6. PUMA mediates the anti-tumor effect of UCN-01 in a xenograft model

A. Nude mice were injected s.c. with 4×10^6 *p21*-KO or *p21*-KO/*PUMA*-KO HCT116 cells. After one week, mice were treated with 9 mg/kg of UCN-01 or the control sodium citrate buffer by i.p. injection for 5 consecutive days. Left: Tumor volume was calculated and plotted. Right: Representative tumors at the end of experiment. **B.** Paraffin-embedded tumor sections were stained for p-AKT and p-FoxO3a according to Materials and Methods. **C.** PUMA expression in representative tumors with or without UCN-01 treatment was analyzed by Western blotting. **D.** Left: Following staining tumor sections for TUNEL according to Materials and Methods, TUNEL-positive cells were counted and plotted. Right: Tumor sections were stained for active caspase 3 according to Materials and Methods. Active caspase 3-positive cells were counted and plotted.