Hsp90 Inhibitors Promote p53-Dependent Apoptosis through PUMA and Bax

Kan He¹,³, Xingnan Zheng¹,³, Lin Zhang²,³, and Jian Yu¹,³

Abstract

Hsp90 is widely overexpressed in cancer cells and believed to be essential for the maintenance of malignant phenotypes. Targeting Hsp90 by small molecules has shown promise in solid and hematologic malignancies, which likely involves degradation of client oncoproteins in a cell-type–specific manner. In this study, we found that structurally unrelated Hsp90 inhibitors induce DNA damage and apoptosis via p53-dependent induction of PUMA, which indirectly triggers Bax activation and mitochondrial dysfunction in colon cancer cells. Deficiency in PUMA, BAX, or p53, at lesser extent, abrogated 17-allylamino-17-demethoxygeldanamycin (17-AAG)-induced apoptosis and mitochondrial dysfunction, and enhanced clonogenic cell survival. Furthermore, suppression of p53-dependent p21 induction or enhanced p53 activation synergized with 17-AAG to induce PUMA-dependent apoptosis. Finally, PUMA was found to mediate apoptotic and therapeutic responses to the 17-AAG analog 17-DMAG in xenografts. These results show an important role of the p53/PUMA/Bax axis in Hsp90 inhibitor–induced killing of p53 wild-type cells, and have important implications for their clinical applications.

Introduction

Hsp90 is one of the most abundant molecular chaperones in eukaryotes and regulates many cellular processes including signal transduction, protein degradation, protein folding, and maturation of client proteins (1). More than 200 proteins have been reported to be Hsp90 clients, including many oncopgenic proteins, such as mutant p53, Raf-1, Akt (1, 2), and others associated with hallmarks of cancer (3). Interestingly, Hsp90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells than their normal counterparts (4), and seems to be required for malignant transformation (5), and a selective therapeutic target in cancer cells (1, 2).

Hsp90 targeting has attracted considerable attention since the late 1990s. Currently, more than 17 Hsp90 inhibitors have entered clinical trials as potential anticancer agents (1, 5). These inhibitors, such as the natural product geldanamycin and its less toxic analog 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibit the molecular chaperone function of Hsp90 by binding to its ATP/ADP pocket and causing destabilization of its complexes with client proteins (2). Treatment with geldanamycin and 17-AAG can induce apoptosis, inhibit metastasis or angiogenesis in both cell and animal models (1, 5, 6). 17-AAG is one of the very first Hsp90 inhibitors to complete phase II clinical trials (1, 5). Other Hsp90 inhibitors with improved pharmaceutical properties, such as 17-DMAG and NVP-AUY922, can promote apoptosis in preclinical models and are currently in phase I/II clinical trials (1, 7). However, the mechanisms underlying Hsp90 inhibition-induced cell killing are currently not well understood, and may vary by cell type. Several mechanisms have been suggested, including Akt downregulation (8), inhibition of NF-κB activation due to inhibitor of IκB kinase destabilization (9), endoplasmic reticulum (ER) stress (10), and more recently p53 activation (11, 12).

The Bcl-2 family proteins are the central regulators of mitochondria-mediated apoptosis (13–15). The BH3-only subfamily members are proximal signaling molecules that respond to distinct as well as overlapping signals, and consist of at least 10 members. Several of BH3-only proteins, such as PUMA and Bim, activate Bax/Bak following the neutralization of all known antiapoptotic Bcl-2 family members (13–15) to promote mitochondrial dysfunction and caspase activation (16–18). PUMA is a critical mediator of p53-dependent and -independent apoptosis in multiple tissues and cell types (18). Transcription of PUMA is mainly dependent on p53 activity in response to DNA-damaging agents such as γ-irradiation, common
chemotherapeutic drugs (18, 19), and even some kinase inhibitors (20). Upon exposure to nongenotoxic stimuli, PUMA is induced by other transcriptional factors such as p73 (21, 22), NF-xB (23), and FoxO3a (24, 25). Bim regulates apoptosis in immune cells, normal and malignant hematopoietic cells, and some epithelial cells generally following nongenotoxic stresses (13).

In this study, we investigated the mechanisms of apoptosis induced by Hsp90 inhibitors in colon cancer cells. We found that p53-mediated induction of PUMA and Bax is required for the apoptotic responses to Hsp90 inhibitors via the mitochondrial pathway in vitro and in vivo. In contrast, p53-mediated p21 induction suppresses 17-AAG-induced apoptosis. Enhanced p53 activation or p21 inhibition sensitized p53 wild-type (WT) colon cancer cells to PUMA-dependent apoptosis following treatment with Hsp90 inhibitors. These results show an important role of the p53/PUMA/Bax axis in the therapeutic responses to Hsp90 inhibitors in p53 WT cancers, and have important implications for their future development and applications.

Materials and Methods

Cell culture and treatment

The human colorectal cancer cell lines, including HCT116, RKO, LoVo (WT p53), HT29, and DLD1 (mutant p53), were obtained from the American Type Culture Collection. Isogenic HCT116 knockout cell lines including p53-KO (26), PUMA-KO (27), BAX-KO (28), p21-KO (29), p21/PUMA double KO (DKO; ref. 27), and PUMA-KO DLD1 cells (30) have been described. We examine loss of expression of targeted proteins by Western blotting and conduct mycoplasma testing by PCR during culture routinely. No additional authentication was done by the authors. Details on cell culture and drug treatments are found in the Supplementary Materials and Methods.

Western blotting

Western blotting was conducted as previously described (16). More details on antibodies are found in the Supplementary Materials and Methods.

Real-time reverse transcriptase PCR

Total RNA was isolated from untreated or drug-treated cells using the Mini RNA Isolation II Kit (Zymo Research) according to the manufacturer’s protocol. Of note, 1 μg of total RNA was used to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was done by the authors. Details on cell culture and drug treatments are found in the Supplementary Materials and Methods.

siRNA knockdown

Transfection was conducted 24 hours before 17-AAG treatment using 400 pmol of siRNA in one well of a 12-well plate (20). All siRNA, including FoxO3a (ON-TARGET-plus J-003007-10), PUMA (ACGTGGAGCACTCCATTTdTdT, and ACCTCAACGCACTGATACGAdTdT; ref. 31), and Bim (GACCGAGAAGGUAGACAAUdTdT; ref. 32) were synthesized by Dharmacon (Lafayette), and siRNAs were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Luciferase assays

PUMA luciferase reporter constructs (Fragments A-E) have been described previously (22). For reporter assays, cells were transfected with PUMA reporter along with the transfection control β-galactosidase reporter pCMVβ (Promega). Cell lysates were collected and luciferase activities were measured as previously described (33). All reporter experiments were carried out in triplicate and repeated three times.

Analysis of apoptosis and cell death

Apoptosis was analyzed by nuclear staining with Hoechst 33258 (Invitrogen), and Annexin V/propidium iodide (Invitrogen) staining followed by flow cytometry as described (30). For colony formation assays, the same number of cells were treated and plated in 12-well plates at appropriate dilutions, and allowed to grow for 10 to 14 days before staining with crystal violet (Sigma). For detection of mitochondrial membrane potential change, the treated cells were stained by JC-1 (30001, Biotium) for 15 minutes according to the manufacturer’s instruction, and then analyzed by flow cytometry.

Analysis of cytochrome c release and Bax multimerization, conformational change, and interacting proteins

Cytoplasmic and mitochondrial fractions were separated by Mitochondrial Fractionation Kit (Active Motif) according to the manufacturer’s instructions. Cytochrome c in both cytoplasmic and mitochondrial fractions was detected by Western blotting. To detect Bax multimerization, purified mitochondrial fractions were cross-linked with 1 mmol/L of Dithiobis (succinimidyl) propionate (DSP; Pierce) as described (34, 35), followed by Western blotting under nondenaturing conditions. Methods on detection of Bax conformational change (27) and interacting protein (16) have been described and details are found in the Supplementary Materials and Methods.

Xenograft studies

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Pittsburgh, PA). 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 × 10⁶ WT or PUMA-KO HCT116 cells. After implantation, tumors were allowed to grow 7 days before treatment was initiated. Mice were randomized into two groups (n = 6 per group) receiving either vehicle (saline) or 17-DMAG (15 mg/kg/d) on days 1 to 4 and 7 to 11, respectively (36). Detailed methods on...
tumor measurements, harvests and histologic analysis are found in the Supplementary Materials and Methods and as described (24, 31).

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 mean ± one SD are displayed in the figures.

Results
p53-dependent induction of PUMA and Bax by 17-AAG in colon cancer cells
To determine a potential role of the Bcl-2 family in Hsp90 inhibitor–induced apoptosis, we first examined protein levels of its major members following 17-AAG treatment in HCT116 colon cancer cells (Fig. 1A). Among them, PUMA, Bim, and Bax were significantly induced, and induction was detected as early as 24 hours after...
treatment (Fig. 1A). The levels of PUMA, BIM, and BAX transcripts were also elevated (Fig. 1B). The expression of other Bcl-2 family members such as Bid, Bcl-2, and Bcl-xL remained unchanged, whereas that of Noxa and Mcl-1 decreased (Fig. 1A). We then treated parental, and p53-KO HCT116 or RKO cells with 17-AAG to determine whether p53 is involved in the induction of PUMA, Bim, and Bax. Induction of PUMA and Bax, but not Bim, was largely blocked in p53-KO cells, compared with parental cells (Fig. 1C). Furthermore, we used a series of PUMA promoter luciferase reporters (containing /C24 2 kb upstream from the transcriptional initiation site; ref. 22) to determine whether p53 directly activates PUMA transcription. We found that the reporters containing the two p53-binding sites (Frag A and E) had much higher activities after 17-AAG treatment in p53 WT cells than in p53-KO HCT116 cells (Fig. 1D). Moreover, the induction of PUMA and other p53 targets Bax and p21 paralleled that of p53, p35S15 phosphorylation, and γ-H2AX, a marker of DNA double-strand breaks (Fig. 1E). Consistent with reports in other systems (37, 38), 17-AAG treatment decreased AKT and p-ERK (Supplementary Fig. S1). These findings show that 17-AAG treatment leads to p53-dependent induction of PUMA and Bax, besides modulation of survival pathways.

**PUMA mediates Hsp90 inhibitor–induced apoptosis**

To examine a potential role of PUMA in 17-AAG–induced apoptosis, we compared the responses of parental HCT116 cells with isogenic PUMA-KO cells (27). 17-AAG treatment induced apoptosis in HCT116 cells as early as 24 hours, which increased to more than 30% at 72 hours, whereas apoptosis and activation of caspase-3 and -9 were abolished in PUMA-KO cells (Fig. 2A). Annexin V/propidium iodide staining confirmed PUMA depletion by siRNA. D, WT and PUMA-KO HCT116 cells were treated with 0.25 μmol/L 17-DMAG and 0.5 μmol/L NVP-AUY922 for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. *: P < 0.001, WT versus PUMA-KO.

He et al.

**Figure 2.** PUMA is required for Hsp90 inhibitor–induced apoptosis. A, WT and PUMA-KO HCT116 cells were treated with 1 μmol/L 17-AAG. Left, apoptosis at the indicated times was analyzed for by counting condensed and fragmented nuclei. **: P < 0.05; ***: P < 0.001, WT versus PUMA-KO. Right, active caspase-3 and caspase-9 at 72 hours were analyzed by Western blotting. B, colony formation assay was done by seeding an equal number of WT and PUMA-KO HCT116 cells treated with 1 μmol/L 17-AAG for 48 hours in 12-well plates, and the attached cells were stained with crystal violet after 14 days. Representative pictures of colonies (left) and quantification of colony numbers (right) are shown. **: P < 0.001, WT versus PUMA-KO. C, LoVo and RKO cells were transfected with either a scrambled siRNA or PUMA siRNA for 24 hours and then treated with 1 μmol/L 17-AAG for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. **: P < 0.001, si-PUMA versus scrambled. Bottom, Western blotting confirmed PUMA depletion by siRNA. D, WT and PUMA-KO HCT116 cells were treated with 0.25 μmol/L 17-DMAG and 0.5 μmol/L NVP-AUY922 for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. **: P < 0.001, WT versus PUMA-KO.
lines, LoVo and RKO (Fig. 2C). In contrast, BIM knockdown did not inhibit 17-AAG-induced apoptosis (Supplementary Figs. S2C and S2D). Furthermore, 17-DMAG and NVP-AUY922 induced significant apoptosis in HCT 116 cells, which was largely blocked in PUMA-KO cells (Fig. 2D and Supplementary Fig. S3). Collectively, these results indicate that PUMA is required for Hsp90 inhibitor-induced apoptosis in colon cancer cells with WT p53.

**PUMA mediates 17-AAG–induced apoptosis via the mitochondrial pathway and Bax activation**

17-AAG–induced apoptosis in HCT 116 was associated with mitochondrial membrane depolarization and release of cytochrome c and SMAC, which was suppressed in PUMA-KO cells (Fig. 3A). 17-AAG treatment also induced PUMA-dependent Bax conformational changes and oligomerization (Fig. 3B and C). BAX-KO HCT116 cells were found to be resistant to 17-AAG–induced apoptosis, and activation of caspase-3 and -9 (Fig. 3D), consistent with PUMA acting upstream of Bax (13–15). We further probed interactions of Bax with several Bcl-2 family members. 17-AAG treatment induced dissociation of Bax from Bcl-xL and Bcl-2, which was suppressed in PUMA KO cells (Fig. 3E). In contrast, Bim binding to Bax was largely unaffected by PUMA status or 17-AAG treatment. No interaction was detected between Bax and Mcl-1, PUMA or Noxa. Interestingly, levels of Mcl-1 and Noxa decreased significantly upon 17-AAG treatment in HCT 116 cells, which...
was reduced in PUMA KO cells (Figs. 1A and Fig. 3E). These results show that PUMA functions upstream of Bax to induce Bax activation and mitochondrial dysfunction in 17-AAG–induced apoptosis. PUMA likely activates Bax indirectly by replacing it from Bcl-xL or Bcl-2 (16, 39), and altered interactions among Bcl-2 family might affect their stabilities.

Enhanced p53 activity synergizes with 17-AAG to induce PUMA-dependent apoptosis

Chemosensitization effects of 17-AAG have been extensively documented (1, 7). We found that HCT116 cells were relatively resistant to cisplatin-induced apoptosis or PUMA expression, whereas p53 stabilization and serine 15 (S15) phosphorylation were readily detected (Fig. 5A and B). The combination of 17-AAG with cisplatin synergistically induced apoptosis, caspase activation, and PUMA expression in HCT 116 cells (Fig. 5A and B). The combination of 17-AAG with nutlin-3, a p53 activator that disrupts MDM2/MDMX and p53 interactions, also synergistically induced apoptosis and PUMA induction (Fig. 5C and D). Remarkably, the combination treatment-induced apoptosis was almost completely abolished in PUMA-KO cells (Fig. 5A and C). PUMA induction was reduced in p53-KO cells compared with WT HCT 116 cells (Fig. 5B and D). Loss of p21 induction likely explains why p53-KO cells were more sensitive than PUMA-KO to apoptosis induced by these combinations (Fig. 5A and B and Supplementary Fig. S5). In addition, p53-KO cells were also partially resistant to apoptosis induced by 17-DMAG and NVP-AUY922, and showed significantly reduced induction of PUMA and Bax, but not Bim (Supplementary Fig. S5). These data show a general role of the p53/PUMA/Bax axis in Hsp90 inhibitor–induced apoptosis and chemosensitization in colon cancer cells.

Antitumor activities of Hsp90 inhibitors are mediated by PUMA-dependent apoptosis in vivo

To determine whether PUMA-mediated apoptosis plays a critical role in the antitumor activities of Hsp90...
inhibitors in vivo, we established WT and PUMA-KO HCT116 xenograft tumors in nude mice. Tumor-bearing mice were then treated with 15 mg/kg 17-DMAG, which is water soluble, or the vehicle intraperitoneally, and tumor volumes were monitored every 2 days for 3 weeks. The WT tumors, but not PUMA-KO HCT116 tumors, responded well to 17-DMAG treatment and reached roughly 1/4 the size of the tumors in vehicle-treated mice on day 21 (Fig. 6A). PUMA, Bax, Bim, as well as p53 phosphorylation (S15) were significantly induced by 17-DMAG treatment in WT tumors (Fig. 6B), consistent with our in vitro data. TUNEL and active caspase-3 staining revealed marked apoptosis in these tumors, which decreased by more than 55% in the PUMA-KO tumors treated identically (Fig. 6C and D). These data showed an important role of the p53/PUMA/Bax axis in the antitumor activities of 17-DMAG in vivo.

Discussion

Hsp90 helps mammalian cells and lower eukaryotes to cope with environmental stresses (44). Cancer cells are constantly under a variety of stresses (3) and Hsp90 is believed to help buffer such stresses for their survival, while different client proteins might be involved depending on cellular context (1, 7). Hsp90 inhibitors induce apoptosis in a variety of preclinical models through degradation of oncogenic client proteins including mutant p53, Raf-1, Akt, Her-2, and ALK fusion proteins (1, 7, 45). Using multiple cell lines and structurally unrelated compounds, we show a role of the p53/PUMA/Bax axis in Hsp90 inhibitor-induced apoptosis and chemosensitization in colon cancer cells using cell culture and xenograft models. p53-dependent PUMA expression directly activates Bax and the mitochondrial apoptotic pathway. Modulation of other Bcl-2 family members and survival pathways (37, 38), and ER stress (Supplementary Fig. S6) can also contribute to apoptosis or PUMA induction. However, the cell-killing mechanisms by Hsp90 inhibitors are likely different in p53 WT and mutant cancer cells where mutant p53 is targeted for degradation (46–48).

Several recent studies showed that functional p53 is required for the induction of apoptosis by 17-AAG (11, 12), while the mechanism has not been well defined. We discovered distinct roles of p53-mediated PUMA and p21 induction in this process. In contrast with p53-mediated induction of PUMA/Bax, induction of p21 protected against 17-AAG–induced apoptosis, consistent with the known antiapoptotic function of p21 in p53-dependent apoptosis (40, 41). The induction of γ-H2AX further suggests a classical p53-dependent DNA damage response associated with double-strand breaks (Fig. 6E; refs. 40, 41, 49), whereas the precise cause of DNA damage upon Hsp90 inhibition remains to be defined. 17-AAG is commonly used as a chemosensitizer and enhances cell killing when combined with a variety of cytotoxic agents, such as cisplatin, UCN-01, taxol, gemcitabine, cytarabine, or proteasome inhibitors and HDAC inhibitors (7). Our results provide mechanistic insights for such a synergy in p53 WT cells. For examples, cisplatin or nutlin-3 enhances 17-

Figure 5. Enhanced p53 activity synergizes with 17-AAG to induce PUMA and apoptosis. A, WT, PUMA-KO, and p53-KO HCT116 cells were treated with 1 μmol/L 17-AAG, 10 μmol/L cisplatin, or their combination for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. B, Western blotting of active caspase-3, PUMA, p53, and p-p53 (S15) for samples in A. C, WT, PUMA-KO, and p53-KO HCT116 cells were treated with 1 μmol/L 17-AAG or 10 μmol/L nutlin-3 or their combination for 48 hours. Apoptosis was analyzed by counting condensed and fragmented nuclei. *, P < 0.01, WT versus p53-KO; **, P < 0.001, WT versus PUMA-KO. D, Western blotting of active caspase-3, PUMA, phosphorylation of p53 on serine 15 for samples in C.
AAG–induced cell killing via enhanced p53 activity and PUMA/Bax induction. On the other hand, UCN-01 enhances 17-AAG–induced cell killing by blocking p21 expression. Therefore, selective modulation of p53–mediated apoptosis or cell-cycle arrest can be exploited to enhance cancer cell killing.

The results of Hsp90 inhibitors in clinical trials are mixed (1, 5), likely reflecting lack of patient stratification or biomarkers. Improved response rates were observed in patients with HER2-positive metastatic breast cancer treated with 17-AAG and trastuzumab (50), as well as in ALK-rearranged non–small cell lung cancer patients treated with Hsp90 inhibitor IPI-504 (51). This has been attributed to the degradation of the Hsp90 clients as driver oncoproteins. Our study together with others (11, 12) would strongly suggest that this class of agents works differently in cancers with WT p53, and examining the modulation of PUMA, Bax, Bim, and p21 might help better distinguish responders from nonresponders in these tumors. No mutation in the PUMA gene has been reported in human cancer, whereas reduced PUMA expression has been reported in cutaneous melanoma and Burkitt lymphomas (18). Therefore reduced PUMA expression can potentially contribute to resistance to Hsp90 inhibitors.

Toxicity to normal tissues is also a concern of Hsp90 inhibitors. It is tempting to speculate that blocking p53-dependent apoptosis might selectively reduce normal tissue toxicity without compromising efficacy in tumors harboring mutant p53.

In conclusion, we have shown that the p53/PUMA/Bax axis plays an important role in the therapeutic and apoptotic response to Hsp90 inhibitors in p53WT cancer cells. Loss of PUMA, BAX, or p53 leads to resistance at varying degrees, whereas loss of p21 leads to...
sensitization. These findings provide novel mechanistic insights for Hsp90 inhibitor–induced apoptosis, and can have important implications for their future development and application.

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

Authors' Contributions
Conceptualization and design: K. He, J. Yu
Development of methodology: K. He, J. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. He, J. Yu
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): K. He, X. Zheng, L. Zhang, J. Yu
Writing, review, and/or revision of the manuscript: K. He, X. Zheng, J. Yu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. He, X. Zheng, L. Zhang, J. Yu
Study supervision: J. Yu

References

Acknowledgments
The authors thank Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University) for p53-KO, p21-KO HCT116 cells, and p53-KO RKO cells, and other members of Yu and Zhang laboratories for helpful discussions.

Grant Support
This work was supported by NIH grant CA129829, American Cancer Society grant RGS-10-124-01-CCE, FAMRI (J. Yu), and by NIH grants CA106348, CA121105, and American Cancer Society grant RSG-07-156-01-CNE (L. Zhang). This project used shared facilities that were supported, in part, by UPCI CCSG award P30CA47904. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Molecular Cancer Therapeutics

Hsp90 Inhibitors Promote p53-Dependent Apoptosis through PUMA and Bax

Kan He, Xingnan Zheng, Lin Zhang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0284

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/08/21/1535-7163.MCT-13-0284.DC1

Cited articles
This article cites 51 articles, 27 of which you can access for free at:
http://mct.aacrjournals.org/content/12/11/2559.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/12/11/2559.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/12/11/2559.
Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.