HSP90 Supports Tumor Growth and Angiogenesis through PRKD2 Protein Stabilization

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Abstract

The kinase PRKD2 (protein kinase D) is a crucial regulator of tumor cell-endothelial cell communication in gastrointestinal tumors and glioblastomas, but its mechanistic contributions to malignant development are not understood. Here, we report that the oncogenic chaperone HSP90 binds to and stabilizes PRKD2 in human cancer cells. Pharmacologic inhibition of HSP90 with structurally divergent small molecules currently in clinical development triggered proteasome-dependent degradation of PRKD2, augmenting apoptosis in human cancer cells of various tissue origins. Conversely, ectopic expression of PRKD2 protected cancer cells from the apoptotic effects of HSP90 abrogation, restoring blood vessel formation in two preclinical models of solid tumors. Mechanistic studies revealed that PRKD2 is essential for hypoxia-induced accumulation of hypoxia-inducible factor-1α (HIF1α) and activation of NF-κB in tumor cells. Notably, ectopic expression of PRKD2 was able to partially restore HIF1α and secreted VEGF-A levels in hypoxic cancer cells treated with HSP90 inhibitors. Taken together, our findings indicate that signals from hypoxia and HSP90 pathways are interconnected and funneled by PRKD2 into the NF-κB/VEGF-A signaling axis to promote tumor angiogenesis and tumor growth. Cancer Res; 74(23); 7125–36. ©2014 AACR.

Introduction

Cancer development is a multistep process characterized by a multitude of genetic and epigenetic changes that induce resistance to proapoptotic stimuli, sustain angiogenesis, and confer insensitivity to antitumor signals and immune surveillance (1).

Rapid tumor growth often results in hypoxia, which triggers the stabilization of the transcription factor hypoxia-inducible factor-1 (HIF1), an oxygen sensor that controls the expression of multiple target genes implicated in angiogenesis, metabolism, and cell survival (2, 3). A prominent target of HIF1α is VEGF-A, which induces tumor angiogenesis by stimulating proliferation, survival, and migration of endothelial cells (4).

HIF1α has been reported to physically interact with HSP90 (5, 6), which can be targeted by small-molecule inhibitors of the chaperone, a growing class of clinically utilized antitumorogenic agents. HSP90 is a highly conserved and ubiquitously expressed molecular chaperone involved in the correct folding and final maturation of a plethora of proteins, so-called HSP90 clients, in an effort to maintain cellular homeostasis (7, 8). There are more than 200 HSP90 clients known, including protein kinases, transcription factors, and steroid hormone receptors (9–11). HSP90 is recruited to its kinase clients through interactions with kinase-specific co-chaperone CDC37 (12, 13), which stabilizes the HSP90/kinase (14). In tumor cells, HSP90 aids in folding dysregulated oncoproteins helping to sustain their aberrant activity. Amongst the most known client kinases of HSP90 are SRC (15), AKT (16), PDK-1 (17), and PKC (18). The latter was shown to directly activate protein kinase D (PRKD) family members via phosphorylation at two critical serine residues within the activation loop of the kinase catalytic domain (19). Recently, an affinity-based proteomic screen conducted to identify cancer-specific networks coordinated by HSP90 revealed PRKD2 as a potential client for the chaperone in chronic myelogenous leukemia (CML) cells (20). The serine-threonine kinase PRKD2 and its sister isoforms PRKD1 and PRKD3 belong to the calcium/calmodulin-dependent protein kinase superfamily (21) and are activated by various stimuli, including phorbol esters, reactive oxygen species, receptor tyrosine kinases, and hypoxia (22–24). PRKD2 expression and activity correlate positively with the state of dedifferentiation in lymphoma (25) and were demonstrated to be involved in myeloid leukemia by activating NF-κB.
transcription factors (26). Furthermore, PRKD2 is involved in migration, invasion, and growth of glioblastoma and pancreatic cancer cells (27–29). We have recently identified PRKD2 as a crucial mediator of hypoxia-induced VEGF-A expression and secretion in pancreatic cancer cells (24).

The aim of this study was to interrogate the contribution of PRKD2 to HSP90-mediated tumor growth and tumor angiogenesis. In addition, the involvement of PRKD2 in the regulation of hypoxia-mediated HIF1α stabilization, NF-κB activation, and VEGF-A production in the context of pharmacologic inhibition of HSP90 represented a major focus of our work. We identified PRKD2 as a novel client of HSP90 and revealed its requirement for tumor viability and tumor angiogenesis during abrogation of chaperone activity in vitro and in vivo. The fact that HSP90 regulates the stability of PRKD2, which acts as a two-pronged protein-mediating tumor blood vessel formation via hypoxia-induced HIF1α stabilization, VEGF-A production and tumor vascularization on one hand and cancer cell viability on the other, makes HSP90 inhibition a strategy to target two cancer characteristics with one drug.

Our work shows that PRKD2 represents a crucial molecule that seems to orchestrate hypoxia/HIF1α and chaperone’s molecular signals in epithelial tumors through the activation of NF-κB and their target gene VEGF. Furthermore, our data indicate that HSP90 inhibitors (PU-H71 and STA-9090) currently undergoing clinical evaluation in patients might be used to target cancer growth and blood vessel formation particularly in hypoxic tumors with high expression of PRKD2. Given current efforts to develop PRKD2 kinase inhibitors, we envision the combined use of HSP90 and PRKD2 kinase inhibitors to achieve synergistic effects.

Materials and Methods
For details, see Supplementary Data.

Cell lines and inhibitors
Cancer cell lines originating from ATCC were cultured in early passages in DMEM (Invitrogen) supplemented with 10% FCS (PAA), 1% penicillin/streptomycin, and 5 μg/ml Plasmocin (InvivoGen). HCT-116 colon cancer cells were maintained in McCoy media supplemented with 10% FCS. Cell lines were authenticated using Multiplex Cell Authentication standard procedures. The 5-μm sections were processed and stained with antibodies directed against PRKD2 (1:250; Abcam #ab12520); pan-cytokeratin (1:80; Dako, clone AE1/AE3); desmin (1:80; Dako, clone D35); von Willebrand Factor VIII (1:100; Biocare Medical, #CP039B); and Ki-67 (1:100; Dako, clone MIB-1). Apoptotic cells were detected by TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) using the In Situ Cell Death Detection Kit, POD (Roche, # 11684817910) and quantified by counting >700 cells from at least four microscopic fields.

IHC of CAM and mouse tumors
Formalin-fixed tumors were embedded in paraffin using standard procedures. The 5-μm sections were processed and stained with antibodies directed against PRKD2 (1:250; Abcam #ab12520); pan-cytokeratin (1:80; Dako, clone AE1/AE3); desmin (1:80; Dako, clone D35); von Willebrand Factor VIII (1:100; Biocare Medical, #CP039B); and Ki-67 (1:100; Dako, clone MIB-1). Apoptotic cells were detected by TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) using the In Situ Cell Death Detection Kit, POD (Roche, # 11684817910) and quantified by counting >700 cells from at least four microscopic fields.

Mouse xenotransplantation experiments
All animal experiments were conducted according to German Animal Welfare and research protocols were approved by the Animal Care and Use Committee at the Regierungspräsidium Tübingen, Germany (TV-1153). MDA-MB231 breast cancer and HCT-116 colon cancer cells (5 × 10^6 each) were subcutaneously inoculated at the left and right dorsal sides of 6-week-old female athymic mice (NMRI-(nu/nu; Janvier Labs). Each experimental group consisting of 9 animals received either 75 mg per kg body weight PU-H71 intraperitoneally three times/week or PBS as vehicle. Tumor size was monitored and measured for the next 3 weeks. After tumor retrieval, tumor volume was calculated according to the formula 0.5 × L × W × T (L, length; W, width; T, thickness). Tumors were further processed for IHC.

Statistical analysis
Analyses were performed with GraphPad Prims 5.0. Statistical significance was assessed by an unpaired Student t test. P < 0.05 was considered significant.

Results
Using an affinity-based proteomic assay followed by chemical precipitation and Western blotting validation, Moulick and colleagues (20) identified PRKD2 as a putative HSP90 client in...
K562 CML cells. To assess whether HSP90 is able to bind to PRKD2 in solid tumors, coimmunoprecipitation experiments with lung cancer (A549), breast cancer (MDA-MB-231), and pancreatic cancer (PaTu2) cells were performed (Fig. 1A). Although PRKD2 interacted with HSP90 in all three cancer cell lines (Fig. 1A), an interaction between PRKD2 and HSP27 or HSP70 chaperones could not be observed (data not shown).

To investigate whether the stability of PRKD2 requires HSP90, we performed knockdown experiments using shRNAs targeting HSP90α (shHSP90α) or HSP90β (shHSP90β), respectively. shRNA-mediated abrogation of both HSP90 isoforms resulted in a decrease of PRKD2 protein levels in A549, MDA-MB-231,
and PaTu2 cell lines (Fig. 1B and C) and this was associated with induction of apoptosis as revealed by enhanced PARP cleavage in Western blot analysis (Fig. 1B and C) or TUNEL assay (Supplemental Fig. S1A). Altogether, these data infer PRKD2 as a putative novel HSP90 client in epithelial tumor cells and suggest PRKD2 depletion via HSP90 inhibition as a potential strategy to target cancer cells.

HSP90 is highly expressed in many tumors and allows the activation of tumor-specific signaling pathways and buffering stress conditions in the tumor microenvironment (31). Therefore, several ATP-competitive HSP90 inhibitors targeting a wide range of malignant tumors are currently under clinical investigation (1, 20, 32). To investigate whether PRKD2 stability is affected after pharmacologic HSP90 inhibition, eight human cancer cell lines representing six different tumor types (breast cancer, pancreatic cancer, lung cancer, colon cancer, acute myeloid leukemia, and glioblastoma) were incubated for 24 hours with increasing concentrations of two different compounds: PU-H71, an optimized water soluble member of the purine class of HSP90 inhibitors (20) and STA-9090, a resorcino1-containing triazole molecule with a novel chemical structure, both unrelated to the geldanamycin class of HSP90 inhibitors (1). Both inhibitors caused dose-dependent degradation of PRKD2 in all tumor cell lines (Fig. 1D and Supplemental Fig. S1B). HSP90 inhibition-mediated PRKD2 degradation was associated with increased apoptosis as revealed by augmented PARP and caspase-9 cleavage in all tumor cell lines (Fig. 1D and Supplemental Fig. S1B). The enhanced cleaved caspase-9 indicates apoptosis induction via the mitochondrial pathway. To prove that HSP90 inhibition-mediated depletion of PRKD2 contributes to the induction of cell death in tumor cells, we first sought to investigate whether downregulation of PRKD2 preceded the induction of apoptosis. Pancreatic, breast, and lung cancer cell lines were incubated with PU-H71 for 4, 8, 12, 16, 20, and 24 hours and PRKD2 and cleaved PARP protein levels were assessed by Western blotting (Fig. 1E). Degradation of PRKD2 commenced after 4 hours in PaTu2, 8 hours in MDA-MB-231, and 12 hours in A549 and was followed by PARP cleavage at around 12 hours in all tumor cell lines, indicating causality between the reduction of PRKD2 protein levels and induction of apoptosis (Fig. 1E). To further substantiate that PRKD2 is crucial for killing cancer cells after HSP90 inhibition, we ectopically expressed a GFP-PRKD2 construct in three cancer cell lines (MDA-MB-231, A549, and PaTu2), treated them with PU-H71, and analyzed PRKD2 levels and PARP cleavage by Western blotting. Both endogenous and overexpressed PRKD2 were subject to degradation; however, the higher remaining PRKD2 protein levels partially rescued cell viability after HSP90 inhibition (Fig. 2A). We confirmed these results involving additional approaches such as determination of apoptosis by annexin V staining in MDA-MB-231 cells and MTT assay for PaTu2 and MDA-MB-231 cancer cells after incubation with PU-H71 for 24 hours. Enforced expression of PRKD2 resulted in partial rescue of cell viability (Fig. 2B and Supplementary Fig. S2A and S2B). Conversely, overexpression of a kinase-inactive mutant of PRKD2 (GFP-PRKD2-KD) did not prevent cell death triggered by pharmacologic abrogation of HSP90 activity in MDA-MB-231 and PaTu2 cancer cells, suggesting the involvement of PRKD2 kinase activity in tumor cell viability (Fig. 2C). Furthermore, the examination of the abundance of PRKD2 protein in control or PU-H71–treated A549 and MDA-MB-231 cells after incubation with 5 µmol/L cycloheximide (CHX) showed that HSP90 inhibition accelerated the PRKD2 protein decay after protein translation blockade by CHX (Supplemental Fig. S2C). We next characterized the mechanism of PRKD2 degradation after treatment with PU-H71. To assess whether PRKD2 is degraded via the lysosomal pathway, we treated MDA-MB-231 breast cancer and A549 lung cancer cells with the lysosome inhibitor NH4Cl before PU-H71 incubation. Western blot analysis testing the abundance of PRKD2 protein in the detergent-soluble and detergent-insoluble fractions showed that preincubation with NH4Cl did not result in an increase of PRKD2 levels compared with the HSP90 inhibitor treatment alone, indicating that PRKD2 is not degraded via the lysosomal pathway (Supplementary Fig. S2D). In contrast, pretreatment of A549 and MDA-MB-231 cell lines with two different proteasome inhibitors, bortezomib or MG-132, followed by incubation with PU-H71 rescued PRKD2 levels and resulted in redistribution of PRKD2 to the detergent-insoluble fraction (Fig. 2D and Supplementary Fig. S2E). Consistent with its degradation via the proteasomal pathway, PRKD2 was extensively ubiquitinated in 293T cells transiently overexpressing PRKD2 after treatment with PU-H71 in combination with bortezomib (Fig. 2E). Together, these findings indicated that degradation of PRKD2 upon HSP90 inhibition occurs via the proteasomal pathway. Furthermore, we analyzed whether the effect of HSP90 inhibition might be caused by depletion of other proteins such as serine/threonine kinases AKT1 and RAF1 that have been reported to be HSP90 clients. Pharmacologic inhibition of HSP90 in A549, MDA-MB-231, and HCT-116 cancer cells triggered the abrogation of AKT1 and RAF1 protein expression (Supplementary Fig. S3A and S3B). Interestingly, shRNA-mediated deletion of AKT1 or RAF1 was associated with increased apoptosis in HCT-116 but not in A549 and MDA-MB-231 cancer cells (Supplementary Fig. S3C and S3D). As previously shown, the ectopic expression of either AKT1 or RAF1 was not sufficient to restore the cell viability after HSP90 inhibition (Supplementary Fig. S3E and S3F and 33). Although all these results argue against AKT1 and RAF1 to be responsible for the apoptotic effect of PU-H71 across different tissues, it is of course possible that other clients (33) contribute to the observed effects in addition to PRKD2.

We previously reported a crucial role for PRKD2 in tumor angiogenesis and cancer cell proliferation (24, 27). We therefore sought to investigate whether PRKD2 depletion via HSP90 inhibition might impair tumor growth and blood vessel formation using a CAM xenotransplantation assay. MDA-MB-231 breast cancer and HCT-116 colon cancer cells stably expressing PRKD2 or empty vector were xenografted on the surface of chicken CAM 8 days after egg fertilization. The in vivo efficacy of PU-H71 has been previously tested (33). PU-H71 was ectopically applied 24 and 48 hours after implantation and tumor growth was monitored. Four days after implantation, tumors were excised, photographed, and analyzed by IHC. Treatment with PU-H71 of cancer cell lines expressing empty vector resulted in a significant decrease in
Figure 2. Destabilization of PRKD2 is essential for HSP90 inhibition-triggered apoptosis in tumor cells. A, lysates of cancer cell lines transiently transfected with empty vector (e.v.) or GFP-PRKD2-wild-type (PRKD2-wt) and incubated with PU-H71 for 24 hours were subjected to Western blot analysis with PRKD2 and cleaved PARP antibodies. Cleaved PARP bands were quantified by densitometric analysis using ImageJ program (right). B, MDA-MB-231 cancer cells transiently transfected with empty vector (e.v.) or PRKD2-wild-type (PRKD2-wt) and incubated with PU-H71 for 24 hours were subjected to Annexin V/propidium iodide staining. The bar graphs represent the mean of Annexin V+/PI− cells from three independent experiments. C, cancer cell lines transfected with a GFP-tagged kinase dead PRKD2 (PRKD2-KD) mutant and incubated with PU-H71 for 24 hours were subjected to Western blotting. Membranes were incubated with PRKD2 and cleaved PARP antibodies. Cleaved PARP bands were quantified by densitometric analysis using ImageJ program (right). D, soluble and insoluble protein fractions of breast and lung cancer cell lines pretreated with bortezomib for 2 hours before incubation with PU-H71 were subjected to Western blotting. Membranes were incubated with PRKD2 antibody. E, 293T cells transfected with GFP-PRKD2 were treated as described in D and immunoprecipitation analysis was performed with anti-GFP antibody. Membranes were incubated with anti-ubiquitin and PRKD2 antibodies.
tumor size (Fig. 3A and B). IHC analysis showed pronounced PRKD2 degradation upon HSP90 inhibition, which was associated with a significantly reduced proliferation rate as measured by Ki-67 staining, and increased apoptosis as determined by TUNEL analysis (Fig. 3C–E and Supplementary Fig. S4A and S4B). Examination of tumor-driven vascularization in xenografts revealed a marked reduction of blood vessel density, as determined by desmin and von Willebrand Factor (vWF) staining upon PU-H71 treatment compared with tumors treated with vehicle (Fig. 3C, F and G). Overexpression of PRKD2 was able to revert all PU-H71-induced effects as demonstrated by restored tumor formation (Fig. 3A and B), enhanced tumor proliferation (Fig. 3C and D and Supplementary Fig. S4A), impaired apoptosis (Fig. 3C and E and Supplementary Fig. S4B), and restored blood vessel formation (Fig. 3C, F and G).

To further substantiate the data obtained in the CAM model, we examined the effects of HSP90 inhibition-mediated PRKD2 degradation in an additional in vivo model. HCT-116 colon carcinoma and MDA-MB-231 breast cancer cells stably expressing PRKD2 (PRKD2-wt) or empty vector (e.v.) were injected subcutaneously into both flanks of nude mice. One week later when tumors were palpable, mice received either 75 mg PU-H71 per kg body weight or vehicle (PBS) intraperitoneally three times per week. After 3 weeks, mice were sacrificed and tumors were analyzed. In line with the CAM experiments, pharmacologic inhibition of HSP90 resulted in substantially decreased tumor growth, increased apoptosis, and impaired angiogenesis in tumors treated with PU-H71 compared with vehicle (Fig. 3C, F and G).
Figure 4. HSP90 inhibition decreases tumor growth and tumor blood vessel formation in nude mice in a PRKD2-dependent manner. A, PRKD2 expression in colon and breast cancer cells lines stably transduced with PRKD2 is presented. B, one week following subcutaneous tumor transplantation, mice were injected with PU-H71 or PBS as control. Three weeks later, animals were sacrificed and tumors were analyzed. Photographs of five representative tumors per experimental group and cell line are depicted. C, the volume of explanted tumors is shown. Graphs represent mean ± SEM of at least 14 tumors per experimental group and cell line as indicated in B. D, IHC analysis of xenografted HCT-116 tumors with antibodies against von vWF and TUNEL are displayed. E, vWF labeling was quantified by subtracting the background staining using Optima software. F, quantification of HCT-116 TUNEL-positive tumor cells is presented. Error bars represent mean ± SEM of at least four microscopic fields with 600 cells. Scale bar, 125 μm.

tumors expressing control vector (Fig. 4A–F and Supplementary Figs. S5A, S5B, and S6A and S6B). Conversely, administration of PU-H71 to mice that received cancer cells overexpressing PRKD2 showed little effect. Tumors from PBS-treated mice and tumors overexpressing PRKD2 from PU-H71–treated mice were associated with less TUNEL-positive cells (Fig. 4D and F and Supplementary Fig. S5B), increased vWF expression (Fig. 4D and E and Supplementary Fig. S5A), augmented VEGF expression (Supplementary Fig. S5A), and higher number of Ki-67–positive tumor cells as compared with tumors transduced with empty vector and treated with PU-H71 (Supplementary Fig. S6A and S6B). These data are in line with our previous finding that PRKD2 plays a major role in tumor growth and tumor angiogenesis and suggest that these properties can be counteracted by HSP90 inhibition-mediated PRKD2 depletion.

VEGF-A is one of the most potent mediators of the formation of blood vessels both under physiologic and pathologic conditions. We have previously reported that PRKD2 ablation impairs hypoxia-induced VEGF-A expression and secretion in pancreatic cancer cells (24). Because hypoxic upregulation of VEGF-A occurs mainly via the stabilization of HIF1α, we sought to investigate whether PRKD2 might regulate VEGF-A via this sensor protein. Specific PRKD2 suppression via RNAi
in pancreatic (PaTu2) and lung (A549) cancer cells abrogated hypoxia-induced accumulation of HIF1α protein (Fig. 5A and B). Accumulation of hypoxia-induced HIF1α was also prevented when A549 and MDA-MB-231 cancer cells were treated with the PRKD inhibitor Gö6976 (Supplementary Fig. S7A). In addition, depletion of PRKD2 in tumor cells was associated with a significant reduction of transcriptional activation of the HIF-response element (HRE), an HIF1α-docking site present in promoters that contain the RCGTG sequence (Fig. 5C). HIF1α has been reported to be an HSP90 client (6, 34). In line with this, shRNA-mediated depletion of HSP90 and pharmacologic HSP90 inhibition in breast cancer cells resulted in impaired

Figure 5. PRKD2 mediates hypoxia-induced accumulation and promoter activity of HIF1α. A, lung, breast, and pancreatic cancer cell lines were stably transduced with PRKD2-specific miRNA (miR-PRKD2) or a noncoding miRNA (miR-Scr). Western blot analysis was conducted with antibodies against PRKD1, PRKD2, and PRKD3. B, pancreatic and lung cancer cell lines stably transduced with miR-PRKD2 or miR-Scr were incubated for 24 hours under low oxygen atmosphere. Cell extracts were subjected to Western blot analysis with PRKD2 and HIF1α antibodies. C, breast and lung cancer cell lines with abrogated PRKD2 were transiently transfected with 3xHRE-luc and pTK-Renilla. Four hours after transfection, cells were incubated under normoxic or hypoxic conditions and then cell lysates were subjected to luciferase assay. Bars are the mean ± SEM of at least three independent experiments. D, breast cancer cells transduced with a nontargeting control shRNA or HSP90α and HSP90β-specific shRNAs were incubated under hypoxia or normoxia for 24 hours and HIF1α and VEGF-A levels were determined using Western blot analysis. E, supernatants of MDA-MB-231 cells with suppressed HSP90α/β and incubated in low oxygen were subjected to VEGF-A–specific ELISA. F, lysates of MDA-MB-231 cancer cells incubated under low oxygen or normoxia in the presence or absence of PU-H71 inhibitor were subjected to Western blot analysis with HIF1α and VEGF-A antibodies. G, supernatants of MDA-MB-231 cells incubated in hypoxic or normoxic conditions in the presence or absence of PU-H71 were subjected to VEGF-A–specific ELISA. Bars represent the mean ± SEM of two independent experiments in triplicate. No, normoxia; Hy, hypoxia.
hypoaxia-induced HIF1α accumulation (Fig. 5D and F). In both cases, abrogation of HIF1α protein accumulation was associated with decreased hypoaxia-induced intracellular and secreted VEGF-A levels (Fig. 5D–G).

We next asked whether PRKD2 is involved in hypoaxia-induced stabilization of HIF1α during HSP90 inhibition. Therefore, cancer cell lines stably transduced with PRKD2 or empty vector were incubated in low-oxygen atmosphere in the presence or absence of HSP90 inhibitor. As expected, treatment of tumor cells containing empty vector with PU-H71 impaired hypoaxia-stabilized HIF1α levels (Fig. 6A and B). Overexpression of PRKD2 was able to partially rescue the hypoaxia-induced accumulation of HIF1α protein (Fig. 6A and B) and HRE promoter activity (Fig. 6C), resulting in restored VEGF-A levels secreted by MDA-MB-231 and HCT-116 cells (Fig. 6D). Together, these data suggest that hypoaxia-induced stabilization of HIF1α protein is mediated by HSP90 directly and through PRKD2, supporting a concept where PRKD2 links chaperone and hypoaxia signaling pathways. VEGF-A can be secreted by tumor cells upon activation of HIF1α and NF-κB transcription factors (35). Furthermore, low-oxygen environment was reported to promote not only the accumulation of HIF1α, but also to activate NF-κB transcription factors via TAK1/IKK signaling (36). HSP90 was shown to interact with the kinase domain of IKKβ/IKKγ, and inhibition of HSP90 by geldanamycin prevented TNF-induced activation of IKK and NF-κB (37). We therefore wanted to know whether NF-κB signaling might be connected to the hypoaxia response regulated by PRKD2 and HSP90. Our experiments show that NF-κB promoter activity is increased upon incubation of MDA-MB-231 cells in low-oxygen atmosphere (Fig. 6E and F). HSP90 inhibition resulted in impaired hypoaxia-induced NF-κB promoter and reduced binding activity (Fig. 6E and data not shown). Similarly, shRNA-mediated suppression of PRKD2 resulted in decreased luciferase production of the NF-κB reporter (Fig. 6F). We previously demonstrated that hypoaxia-induced VEGF-A promoter activity, and intracellular and secreted VEGF-A levels are also impaired upon PRKD2 knockdown in cancer cells (24). Because hypoaxia and HSP90 mediate their signals through the IKK complex toward NF-κB, which is also activated by PRKD2 (26, 46), we asked whether PRKD2 plays any role in this scenario. A triple active mutant of PRKD2 (PRKD2-244/706/710E, PRKD2-3SE) was sufficient to enhance both, the NF-κB and VEGF-A promoter activity (Fig. 6G and H). Conversely, coexpression of a dominant negative IκBα (TD-IκBα) mutant was associated with the blockade of hypoaxia and hypoaxia/PRKD2-3SE-induced NF-κB and VEGF-A transcriptional activity (Fig. 6G and H). Altogether, these data suggest that activation of NF-κB by PRKD2 involves the phosphorylation and proteasomal degradation of IκBα. Notably, the activation of VEGF-A promoter by hypoaxia and/or active PRKD2 is suppressed upon blockade of NF-κB signaling, which would suggest a hypoaxia → PRKD2 → NF-κB → VEGF-A signaling axis. However, enforced expression of PRKD2 was able to marginally restore the hypoaxia-induced NF-κB promoter activity affected by the inhibition of HSP90 (Supplementary Fig. S7B). Taken together, these results favor PRKD2 as a kinase acting in both the NF-κB and HIF1α pathways, thereby connecting hypoaxia signals and HSP90 chaperone function to promote tumor growth and tumor angiogenesis.

Discussion

HSP90 serves as an ATP-dependent stabilizer of diverse signaling proteins, including many kinases that are involved in cell proliferation and survival. Chaperone inhibitors were recently shown to effectively inhibit tumor cell growth and angiogenesis in hematologic and solid-organ malignancies. However, it remains elusive whether cancer cell killing or disruption of vascular network supplying tumor cells is mediated by depletion of a single molecule or simultaneous degradation of multiple client proteins that are overexpressed and/or mutated in cancer (38).

In this study, we have identified PRKD2 as a novel client of the HSP90 chaperone. We could demonstrate that PRKD2 interacts with HSP90 in several cancer cell lines. Depletion of PRKD2 protein following pharmacologic inhibition of HSP90 was associated with tumor cell death in vitro in various human cancer cell lines, as well as in two in vivo xenograft models. These data not only confirm the role of PRKD2 as an anti-apoptotic signaling molecule (39, 40), but also implicate PRKD2 in the cell death evoked by HSP90 inhibition. Our earlier findings demonstrated that PRKD2 is a crucial mediator of tumor angiogenesis involving upregulation and secretion of VEGF-A (24). This prompted us to investigate whether HSP90 might contribute to tumor angiogenesis through PRKD2 protein stabilization. We demonstrated that pharmacologic inhibition of HSP90 impaired blood vessel formation in vivo. The fact that PRKD2 overexpression restored vascularization and cell viability after HSP90 inhibition points to the involvement of PRKD2 in these HSP90 inhibitor-induced effects. Our data support PRKD2 degradation through HSP90 inhibition as a putative strategy to hit two important cancer characteristics, angiogenesis and cell viability, with one drug.

HSP90 inhibitors have been reported to indirectly regulate HIF1α (41–44). Furthermore, the HSP90 inhibitor geldanamycin reduced hypoaxia-mediated HIF1α activation, indicating that chaperone activity is needed for this activation (6). The fact that HSP90 interacts both with HIF1α (6) and PRKD2 (this study) prompted us to evaluate the contribution of PRKD2 with respect to HIF1α stabilization in hypoaxic tumors. We found that abrogation of PRKD2 in cancer cells prevented hypoaxia-mediated HIF1α accumulation and HIF1α promoter activity. In line with several reports, reduced HSP90 expression and/or activity resulted in impaired hypoaxia-triggered HIF1α accumulation and decreased VEGF-A expression. Notably, ectopically expressed PRKD2 was able to partially restore HIF1α protein levels, HIF1α transcriptional activity, and secreted VEGF-A levels after pharmacologic HSP90 inhibition. Together, these findings suggest that PRKD2 is required for hypoaxia-induced HIF1α accumulation and that HSP90-supported angiogenesis is modulated by PRKD2 in hypoaxic tumors by regulating HIF1α protein levels and subsequent VEGF-A secretion.

VEGF-A can be produced by tumor cells upon activation of HIF1α and NF-κB (35). Hypoxia drives the accumulation of...
HIF1α but also activates NF-κB transcription factors (36). Furthermore, inhibition of HSP90 was shown to promote apoptosis through suppression of AKT/NF-κB signaling (45). Thus, NF-κB represents a downstream effector of two major signaling routes: the hypoxia-induced HIF1α and HSP90 pathways. Because PRKD2 was reported to mediate stress-induced NF-κB activation and cell survival (46), we reasoned that PRKD2 acts upstream of NF-κB and might be a possible candidate for regulating this pathway.
Figure 7. PRKD2 modulates HSP90-driven tumor growth and tumor angiogenic by regulating hypoxia-mediated HIF1α accumulation and inducing VEGF-A secretion via activation of NF-κB. A, stabilization of PRKD2 by HSP90 contributes to enhanced HIF1α accumulation in low oxygen environment. In this scenario, activation of NF-κB and its target VEGF-A is associated with augmented tumor growth and increased blood vessel formation. B, degradation of PRKD2 following HSP90 inhibition affects HIF1α/VEGF-A and/or HIF1α/NF-κB/VEGF-A signaling pathways and triggers enhanced cancer cell apoptosis and impaired tumor vascularization. Dotted bold lines, basal signaling in hypoxic tumors; dotted thin lines, impaired signaling.

important molecule involved in hypoxia/HIF1α and HSP90 signaling down to NF-κB and subsequently to VEGF-A expression/secretion. We found that hypoxia-induced NF-κB activation was blocked by HSP90 inhibition and shRNA-mediated suppression of PRKD2. The finding that PRKD2 was able to just marginally restore hypoxia-induced NF-κB promoter activity affected by the inhibition of HSP90 suggests that other factors might be required as well for the HSP90 angiogenic signals through the NF-κB pathway.

In conclusion, our data suggest a central role for PRKD2 to enhance HIF1α accumulation in low-oxygen environment. Stabilization of PRKD2 by HSP90 also results in the activation of NF-κB and its target VEGF-A, which promotes cancer cell growth and increases blood vessel formation in hypoxic tumors (Fig. 7). Whether and how PRKD2 activates NF-κB/VEGF-A via upregulation of HIF1α in hypoxic tumors or whether and how it contributes to the parallel activation of distinct HIF1α/VEGF-A or NF-κB/VEGF-A pathways remains to be elucidated. This study may also have clinical implications because several HSP90 and PRKD2 inhibitors are currently in clinical trials or under development. The combination of HSP90 and PRKD2 inhibitors might have synergistic effects in patients with hypoxic tumors expressing high levels of PRKD2.

Disclosure of Potential Conflicts of Interest
G. Chiosis is director of Samus Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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