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Original Paper

C-Terminal HSP90 Inhibitors Block the HIF-1 Hypoxic Response by Degrading HIF-1α through the Oxygen-Dependent Degradation Pathway

Nalin Kataria[a] Chloe-Anne Martinez[a] Bernadette Kerr[a] Samantha S. Zaiter[b] Monica Morgan[a] Shelli R. McAlpine[b] Kristina M. Cook[a]

[a]School of Medicine and Health, Faculty of Medicine and Health, Charles Perkins Centre, University of Sydney, Sydney, Australia, [b]School of Chemistry, University of New South Wales, Sydney, Australia

Key Words
Hypoxia • HSP90 • HIF • Cancer • Heat shock

Abstract
Background/Aims: Hypoxia Inducible Factor-1α (HIF-1α) is involved in cancer progression and is stabilized by the chaperone HSP90 (Heat Shock Protein 90), preventing degradation. Previously identified HSP90 inhibitors bind to the N-terminal pocket of HSP90, which blocks binding to HIF-1α and induces HIF-1α degradation. N-terminal inhibitors have failed in the clinic as single therapy treatments partially because they induce a heat shock response. SM molecules are HSP90 inhibitors that bind to the C-terminus of HSP90 and do not induce a heat shock response. The effects of these C-terminal inhibitors on HIF-1α are unreported. ***Methods:*** HCT116, MDA-MB-231, PC3, and HEK293T cells were treated with HSP90 inhibitors. qRT-PCR and western blotting was performed to assess mRNA and protein levels of HIF-1α, HSP- and RACK1-related genes. siRNA was used to knockdown RACK1, while MG262 was used to inhibit proteasome activity. Dimethyloxalylglycine (DMOG) was used to inhibit activity of the prolyl hydroxylases (PHDs). Anti-angiogenic activity of HSP90 inhibitors was assessed using a HUVEC tubule formation assay. ***Results:*** We show that SM compounds decrease HIF-1α target expression at the mRNA and protein level under hypoxia in colorectal, breast and prostate cancer cells, leading to cell death, without inducing a heat shock response. Surprisingly, we found that when the C-terminal of HSP90 is inhibited, HIF-1α degradation occurs through the proteasome and prolyl hydroxylases in an oxygen-dependent manner even in very low levels of oxygen (tumor hypoxia levels). RACK1 was not required for proteasomal degradation of HIF-1α. ***Conclusion:*** Our results suggest that by targeting the C-terminus of HSP90 we can exploit the prolyl hydroxylase and proteasome pathway to induce HIF-1α degradation in hypoxic tumors.

Kristina M. Cook
Faculty of Medicine and Health, Charles Perkins Centre, University of Sydney
Sydney, NSW, 2006 (Australia)
Tel. +61 286274858, E-Mail kristina.cook@sydney.edu.au

Introduction

Hypoxia is common in tumors and it activates a transcription factor known as Hypoxia Inducible Factor-1 (HIF-1). HIF-1 controls the expression of genes involved in glycolysis, angiogenesis and cell survival, and its presence is associated with a poor cancer prognosis [1]. Given that HIF-1 is upregulated in the majority of solid tumors, there are ongoing drug development efforts to inhibit its activity. Previous work has focused on inhibiting the HIF-1α subunit from binding to partners such as p300 [2-6], HIF-1β [7], or binding to the DNA itself [8].

HIF-1 is a heterodimer composed of α and β subunits. Activity of the HIF-1 transcriptional complex is regulated post-translationally through the HIF-1α subunit in an oxygen-dependent process [9, 10]. In the presence of oxygen, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) which enables binding by the von Hippel-Lindau protein (pVHL) and E3 ubiquitin ligase complex, leading to proteasomal degradation of HIF-1α. In the absence of oxygen, HIF-1α evades the degradation pathway, allowing it to translocate to the nucleus to form the transcriptionally active HIF-1 complex.

HIF-1α stability and degradation is also regulated by the chaperone Heat Shock Protein 90 (HSP90). HSP90 has also been under intense scrutiny as a cancer target because it is upregulated in many cancers and increases the activity of other transcription factors involved in cancer [11]. HSP90 drugs can inhibit many cancer pathways including those involving HIF. Newly synthesized HIF-1α is stabilized by HSP90, preventing its degradation [12]. N-terminal HSP90 inhibitors such as 17-AAG (17-N-allylamino-17-demethoxygeldanamycin, also known as tanespimycin) and related analogues bind in the ATP binding pocket on the amino terminus (N-terminus) of HSP90 [13]. Upon 17-AAG binding, HSP90 disassociates from HIF-1α, which then induces pVHL-independent degradation of HIF-1α through RACK1 [14-16]. While N-terminal HSP90 inhibitors effectively block the HIF-1 hypoxic response [14, 17, 18], they induce a survival mechanism in cancer cells known as a heat shock response (HSR) which leads to increased chemoresistance and promotes cell survival [19].

C-terminus inhibitors of HSP90 (SM molecules) [20] act in a selective manner, and do not induce a heat shock response in normoxia [21, 22]. Although the SM molecules are effective at inhibiting HSP90 function and blocking co-chaperones that bind to the C-terminus [23, 24], there is currently no evidence that targeting the C-terminus will impact the hypoxic response. Using the newly identified SM molecules, we aimed to determine whether modulating the C-terminus of HSP90 would inhibit HIF-1 and the downstream gene expression in hypoxia. This is the first study examining the effect of C-terminal HSP90 inhibitors on the activity of HIF-1α.

Materials and Methods

Cell culture

HCT116 cells were maintained in McCoy's 5A medium (Gibco), supplemented with 10% fetal bovine serum (FBS), 50 μg/ml Streptomycin, 50 IU/ml Penicillin and 1x GlutaMAX. HEK293T and MDA-MB-231 cells were maintained in DMEM with high glucose (4500 mg/l) and supplemented with 10% fetal bovine serum (FBS), 50 μg/ml Streptomycin, 50 IU/ml Penicillin and 1x GlutaMAX. PC3 cells were maintained in DMEM/F12, supplemented with 10% fetal bovine serum (FBS), 50 μg/ml Streptomycin, 50 IU/ml Penicillin and 1x GlutaMAX. For non-hypoxic experiments, cells were incubated in humidified air supplemented with 5% CO_2 at 37°C (18.6% O_2 v/v). Cell lines were a gift from Professor Philip Hogg or purchased from ATCC, Bethesda, MD. Cell lines are tested four times per year for mycoplasma contamination.

Cell viability assays

For 72 hr viability assays, HCT116 cells were seeded into 96-well plates in 100 μl of medium at a concentration of 2×10^3 cells/well (for 18 hr viability assays 5.1×10^4 cells/well were used). After overnight incubation at 37 °C, medium was removed and replaced with 100 μl of fresh medium (with 0.5% fetal bovine

serum) containing drug or vehicle control (1% DMSO). Reduced serum is used to mimic the hypoxic tumor environment where there is both reduced oxygen and reduced nutrient levels. Plates were placed in either a normoxic (18.6% v/v oxygen) or hypoxic (0.5% v/v oxygen) incubator for 18 or 72 hrs. Cell viability was measured by adding 20 µl CellTiter-Blue cell viability reagent (Promega) to each well, after which the cells were returned to the 37 °C incubator until sufficient color change. Fluorescence intensity was measured using 570 nm excitation and 600 nm emission on a Tecan Infinite M1000 Pro.

siRNA transfection

HCT116 cells were plated in 12 well plates at 3×10^5 in 1.2 ml of McCoy's media with 10% FBS and Glutamax. HEK293T cells were plated in 12 well plates at 3.4×10^5 in 1.2 ml of DMEM media with 10% FBS and Glutamax. HiPerfect (Qiagen) was used to transfect cells with RACK1 siRNA (sc-36354, Santa Cruz Biotechnology) or AllStars Negative control siRNA (SI03650318, Qiagen) using a reverse transfection protocol according to the manufacturer. After incubating for 30 hours at 37 °C, media was removed and replaced with 1 ml fresh medium (with 0.5% fetal bovine serum) containing drug or vehicle control (1% DMSO). Plates were placed in either a normoxic or hypoxic incubator for 18 hrs before harvesting cells for qPCR or western blot.

Western blots

For non-siRNA experiments, HCT116 cells were seeded into 12 well plates at 6×10^5 cells/well. PC3 cells were seeded into 12 well plates at 7×10^4 cells/well to 2.7×10^5 cells/well and MDA-MB-231 were seeded into 12 well plates at 9×10^4 cells/well to 3.4×10^5 cells/well depending on the experiment and length of incubation. The following day, cells were treated with medium containing 0.5% FBS and SM compounds or vehicle control (DMSO). Plates were placed in incubators under normoxic or hypoxic conditions for 6 – 18 hrs. Cells were lysed using RIPA buffer containing protease and phosphatase inhibitors (Roche). Lysates were sonicated, mixed with 4x LDS sample buffer (ThermoFisher Scientific) containing DTT and heated to 70 °C for 10 min.

SDS-solubilized protein samples were resolved using the Novex NuPage SDS-PAGE gel system (ThermoFisher Scientific; 4-12% Bis-Tris gels) and transferred via semi-dry electrophoresis to 0.45 µm PVDF membranes (Millipore). Membranes were blocked for 1 h in 5% non-fat dry milk in TBST and then incubated overnight at 4°C in one of the following antibodies: anti-GLUT1 (ab115370, 1:1000, Abcam), anti-HIF1α (NB100-479, 1:500, Novus), anti-Histone H3 (ab1791, 1:2000, Abcam), anti-beta Actin (1:2000, ab8226, Abcam), anti-HK2 (TA325030, 1:500, Origene), anti-LDHA (3582T, 1:1000, Cell Signaling Technologies), anti-HSP90 (NB120-1429, 1:1000, Novus) and anti-HSP70 (4873, 1:1000, Cell Signaling Technologies). All blots used Histone H3 or β-actin as a housekeeping protein to ensure equal loading. Following washes, blot was incubated for 1 hour room temperature in a 1:1000 dilution of corresponding secondary antibodies (Dako). Bound antibodies were visualized using the BioRad Chemidoc Touch System. Densitometry was calculated using BioRad ImageLab software as per instructions from the manufacturer.

Treatment with proteasome or PHD inhibitors

HCT116 cells were seeded into 12-well plates at 3×10^5 in 1 ml of McCoy's media with 10% FBS and Glutamax. After overnight incubation at 37 °C, medium was removed and replaced with 1 ml of fresh medium (with 0.5% fetal bovine serum) containing SM compounds and drug (1 mM DMOG for PHD inhibition or 1 mM MG-262 for proteasome inhibition) or vehicle control (1-2% DMSO). Plates were placed in either a normoxic (18.6% oxygen) or hypoxic (0.5% oxygen) incubators for 6 hrs before harvesting as described for mRNA or protein analysis. DMOG (dimethyloxalylglycine) was purchased from Sigma Aldrich and MG-262 was purchased from Calbiochem.

Nuclear and cytoplasmic protein separation

Nuclear and cytoplasmic fractions were separated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (ThermoFisher Scientific) according to the manufacturer's instructions.

Gene expression

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA quality was assessed using an RNA bleach gel according to [25]. The quantity of isolated RNA was measured using a Nanodrop (260nm). Total

RNA (2 µg) was used as a template for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For qRT-PCR, pre-designed TaqMan Gene Expression Assays were used with TaqMan Gene Expression Master Mix (Life Technologies, Thermo-Fisher). 20 ng of cDNA was used per well/reaction. qRT-PCR protocol followed according to manufacturer on a QuantStudio 7 Flex machine. Samples were normalized using 18s RNA and fold changes calculated based on normoxic or hypoxic control. Fold-change in mRNA levels was calculated using the $\Delta\Delta C_t$ method. Statistical analysis was conducted using an Ordinary One Way ANOVA in Graphpad Prism with multiple comparisons. TaqMan Primers (Assay ID) used for qPCR (ThermoFisher Scientific) were as follows: CA9 (carbonic anhydrase IX) (Hs00154208_m1), SLC2A1 (GLUT1) (Hs00892681_m1), LDHA (Hs01378790_g1), HSP90AA1 (HSP90α) (Hs00743767_sH), RACK1 (Hs00272002_m1), VEGFA (Hs00900055_m1), HK2 (Hs00606086_m1) and 18s (Hs03003631_g1).

Endothelial cell tube formation assay

HUVEC cells were grown in EGM-2 media (Lonza, Walkersville USA) according to the supplied protocol. For tube formation, 96 well plates were treated with 38ul EHS Matrix Extract (Sigma-Aldrich) for 60 min at 37° C. HUVECs (24, 000 cells per well) were plated on top and treated with 25 µM 17-AAG, 10 µM 17-AAG, 25 µm SM122, 25 µM SM253 or 25 µM SM258. DMSO was used as a control. Plates were incubated for 18 hours and imaged using phase contrast microscopy. The program ImageJ with the Angiogenesis Analyzer (National Institutes of Health) was used to analyze ≥2 representative images of tubule formation from a 10× magnification from a 96-well plate using a similar protocol from [26]. The angiogenesis analyzer is has been shown to accurately quantify the morphological features of the capillary-like network formed by endothelial cells [26]. The program was set to calculate total segment length, total area of meshes and the number of nodes. Three iterations were used with the minimum object size set to 50 pixels. The master segment threshold was set to 30 pixels, with the minimum branch size at 25 pixels, artifactual loop size set to 1000 pixels and the isolated element threshold set to 100 pixels.

SM Compounds solid-phase peptide synthesis

Stepwise solid phase peptide synthesis (SPPS) was performed in a polypropylene solid-phase extraction cartridge fitted with a 20 µm polyethylene frit purchased from Applied Separations (Allentown, PA). All chemicals were purchased from commercial suppliers (Chem-Impex International and Sigma Aldrich) and used without further purification.

Coupling reaction and FMOC removal

Peptide coupling was carried out by following the published procedure for SPPS [27]. Prior to each coupling reaction, 2-chlorotrityl chloride resin pre-loaded with the first amino acid was swelled in *N,N*-dimethylformamide (DMF) for 30 mins, then the DMF was drained. Couplings were performed in DMF at a concentration of 0.1 - 0.3 M. Fmoc-protected amino acid (3 equiv.) and either 1-Hydroxybenzotriazole hydrate (HOBt) or 1-Hydroxy-7-azabenzotriazole HOAt (3 equiv.) were mixed with the resin. *N, N′*-Diisopropylcarbodiimide (DIC) (6 equiv.) was then added to activate the reaction. Coupling reaction was run for a minimum of 3 hours while shaking (Labquake tube shaker, Thermo Fisher Scientific) at room temperature. A negative ninhydrin test was used to confirm reaction completion. Once completed, the reaction mixture was drained, and the resin was subjected to *Fmoc Removal*.

The Fmoc protecting group was removed using the following washes: DMF (3 x 1 min), 20% piperidine in DMF (1 x 5 min), 20% piperidine in DMF (1 x 10 min), DMF (2 x 1 min) isopropanol (*i*PrOH, 1 x 1 min), DMF (1 x 1 min), *i*PrOH (1 x 1 min) and DMF (3 x 1 min). The resin was then ready for the next coupling reaction.

Resin cleavage of linear peptide

Once the desired peptide was generated, the final Fmoc protecting group was removed following *Fmoc Removal* procedure with the following additional washes: DMF (3 x 1 min), *i*PrOH (3 x 1 min) and MeOH (3 x 1 min). The resin-bound peptide was then dried *in vacuo* overnight. The resin was then cleaved from the linear peptide using TFE and CH_2Cl_2 (1:1 v/v) at a concentration of 10 mL/g resin. The reaction mixture was stirred at room temperature for 24 hours before filtering the resin. The filtrate was concentrated and washed at least 10 times with CH_2Cl_2 to remove residual entrapped TFE. The product was then dried *in vacuo* overnight to produce the linear peptide.

Macrocyclisation

Macrocyclisation of the linear peptide was achieved using a cocktail of 3 coupling reagents: HATU (1 eq.), TBTU (0.8 equiv.) and DMTMM (0.8 equiv.). The reaction was performed under nitrogen and in dilute conditions using anhydrous dichloromethane (CH_2Cl_2, 0.001 M). The linear peptide and coupling reagents were dissolved separately in CH_2Cl_2, where 20% of the final volume was used to dissolve the linear peptide and the other 80% dissolved the coupling reagents. Diisopropyl ethyl amine (DIPEA, 4 equiv.) was added to each solution. The linear peptide solution was then added drop-wise to the coupling reagents solution *via* a syringe pump over approximately 2 hours. The reaction was stirred overnight and monitored *via* LC/MS. Upon completion, the reaction mixture was concentrated down under reduced pressure. Upon completion, the reaction mixture was worked up with 10% (v/v) HCl solution. The organic layer was then re-extracted with saturated $NaHCO_3$ aqueous solution. The organic layers were combined and dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The resulting crude product was purified via flash column chromatography on silica gel using a methanol-ethyl acetate-hexane gradient system followed by purification with reverse phase HPLC. Final product was lyophilized to obtain the pure product as a white-off white powder.

Results

SM Compounds inhibit HIF-1α stabilization in hypoxia

Since it is established that HSP90 N-terminal inhibitors decrease HIF-1α in both normoxia and hypoxia [14, 18, 28, 29], we compared these molecules to the impact of SM compounds, which bind to the C-terminus of HSP90 (Fig. 1a). Using HCT116 (colorectal), PC3 (prostate) and MDA-MB-231 (breast), we exposed cells to hypoxia for 6 hrs. Under hypoxia alone there is a substantial increase in HIF-1α protein, as compared to normoxia (Fig. 1b, 1c and 1d). When cells were incubated in hypoxia in the presence of 25 μM SM122, SM253 or SM258, nuclear HIF-1α protein decreased by ~38% - 70% in HCT116 and MDA-MB-231 cells. Only 25 μM SM122 led to a decrease in HIF-1α in PC3 cells. The decrease in nuclear HIF-1α decrease is particularly noticeable in all cell lines treated with SM122, SM253 and 17-AAG.

The HSP90 inhibitors were also compared to chetomin, which blocks the interaction between HIF-1α and p300 by a zinc ejection mechanism [2]. Chetomin also reduced the levels of HIF-1α in HCT116 cells (Fig. 1b).

SM compounds decrease HIF-1 target gene expression at the mRNA and protein level

To determine if the SM compounds decrease HIF-1 target gene expression, we exposed cell lines to SM compounds in hypoxia for 18 hrs, which is the approximate time scale for maximum HIF-1 target mRNA expression, though this can vary depending on the target [30]. Cells treated with SM compounds showed varying degrees of inhibition of HIF-1 target gene expression in hypoxia which correlates to their respective protein levels (Fig. 1e-g) and mRNA levels (Fig. 1h).

HIF-1 controls the expression of glycolytic pathway genes including the glucose transporter SLC2A1 (GLUT1), lactate dehydrogenase A (LDHA) and hexokinase 2 (HK2). We examined the protein expression of multiple glycolytic genes within HCT116 cells upon exposure to hypoxia and HSP90 inhibitors. Comparing the compounds' impact on protein expression of GLUT1, SM122 decreased GLUT1 protein by ~60% of hypoxic levels, similar to levels seen in normoxia (Fig. 1e). In contrast, 17-AAG, slightly increased GLUT1 protein levels in hypoxia (10 μM) (Fig. 1e). Interestingly, SM122 was more effective at reducing GLUT1 than chetomin (Fig. 1e). HSP90 affects many cell pathways and there may be additive effects involving the HIF pathway and others when inhibiting HSP90. SM253 and SM258 both reduce GLUT1 but cells must be treated at higher concentrations than SM122. These data indicate that SM molecules produce a functional reduction in the glucose transporter.

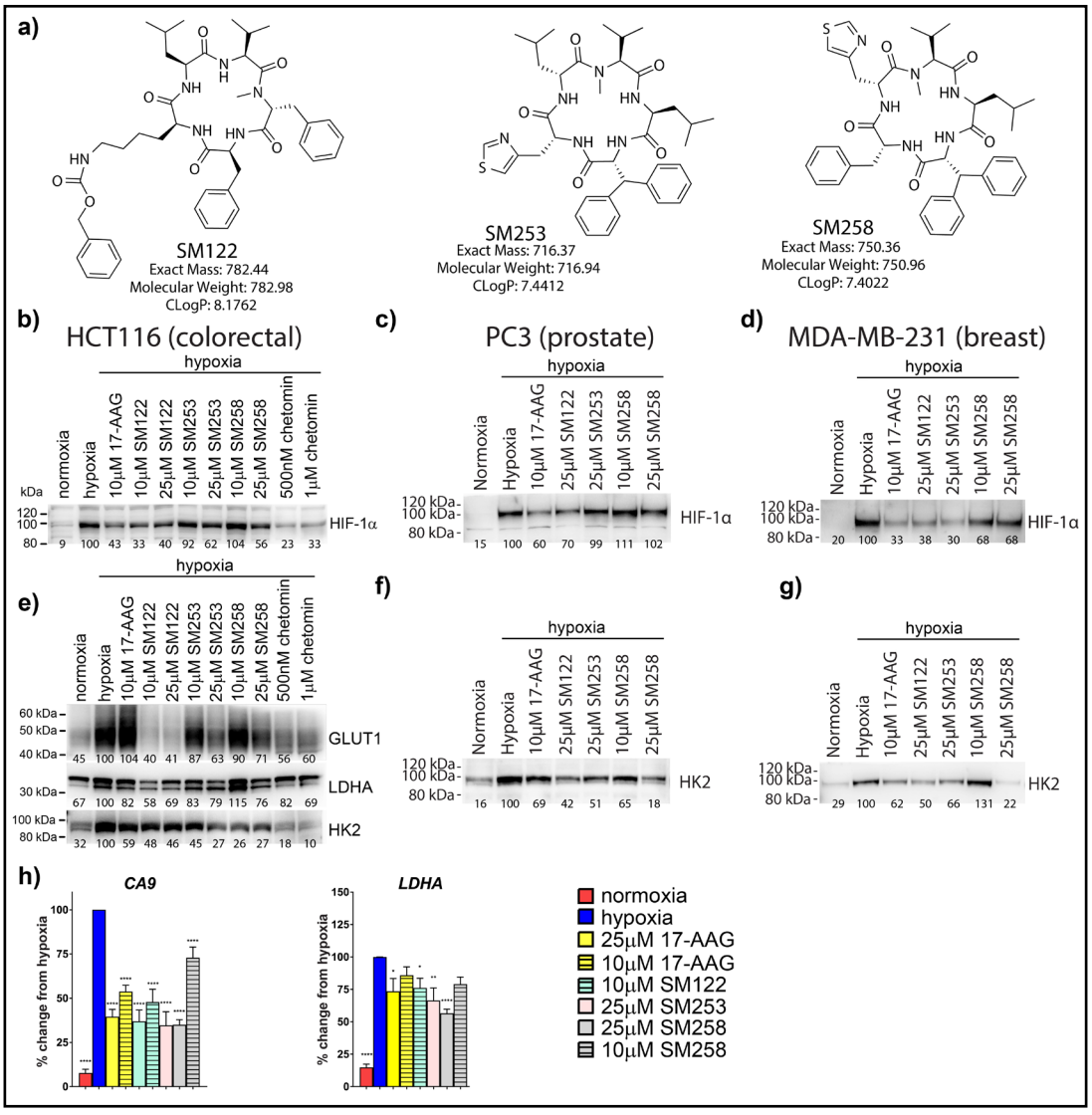

Fig. 1. C-terminal HSP90 inhibitors, SM compounds, inhibit HIF-1α stabilization under hypoxia and decrease HIF-1 target gene expression in cancer cells. a) Chemical structures of SM122, SM253 and SM258. b) HCT116, (c) PC3 or (d) MDA-MB-231 cells were treated with SM compounds and exposed to hypoxia for 6 hrs prior to making nuclear cell lysates for western blotting. e) HCT116 cells were incubated ± SM compounds or 17-AAG in hypoxia for 18 hrs. Total protein was isolated, run on SDS-PAGE and transferred to western blot to assess expression of the HIF target genes, GLUT1, LDHA and HK2. f) Western blot of HK2 expression following treatment of PC3 cells with HSP90 inhibitors. g) Western blot of HK2 expression following treatment of MDA-MB-231 cells with HSP90 inhibitors. Representative blots shown with densitometry measured using digital imaging and BioRad ImageLab software. The numbers shown below blots are the direct densitometric measurements. The hypoxic control is normalized to 100 in each blot. Each blot was n ≥ 3. h) mRNA expression of HIF-1 target genes CA9 and LDHA. HCT116 cells were incubated ± SM compounds or 17-AAG in hypoxia for 18 hours. Total RNA was isolated, converted to cDNA and used for qPCR measurement (calculated using the ΔΔCt method). Results expressed as % of hypoxic control. Mean ± SEM of independent experiments run in duplicate (n ≥ 3). Statistical analysis conducted using an ordinary one-way ANOVA with multiple comparisons to hypoxia in Graphpad Prism. * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001. Histone H3 loading controls for Figs. 1b-1g are located in the online supplemental material (see www.cellphysiolbiochem.com).

All the SM compounds (at 25 μM) reduced LDHA protein levels compared to the hypoxic control (Fig. 1e). We also assessed LDHA mRNA levels in HCT116 cells and found that the SM compounds led to a similar reduction in LDHA mRNA levels as with LDHA protein levels (Fig. 1h).

To assess the effects of SM compounds on downstream HIF target gene expression in multiple cell lines, we measured the expression of hexokinase2 (HK2), in HCT116, PC3 and MDA-MB-231 cells. HK2 is another enzyme involved in glycolysis and cellular adaptation to hypoxia through the activation of HIF-1 [31]. Protein levels of HK2 were reduced by 17-AAG and all SM compounds when compared to the hypoxia control. SM122 was the most uniformly effective compound and treatments of 25 μM decreased HK2 protein levels by ~50% in HCT116 (Fig. 1e), ~58% in PC3 (Fig. 1f) and ~50% in MDA-MB-231 cells (Fig. 1g). SM122 and SM253 were also both effective at 25 μM treatment, decreasing HK2 in HCT116 (Fig. 1e), PC3 (Fig. 1f) and MDA-MB-231 cells (Fig. 1g). These results indicate C-terminal SM compounds are effective at reducing glycolytic enzymes that are commonly upregulated by hypoxia in cancers.

HIF-1α controls genes outside the glycolytic pathway including carbonic anhydrase IX (CA9), which is involved in tumor acidosis and is used as a marker of tumor hypoxia. When HCT116 cells are treated with 17-AAG or SM compounds under hypoxia, there is a substantial reduction in CA9 mRNA, indicating expression of genes outside of glycolysis are inhibited (Fig. 1h).

SM Compounds do not induce a heat shock response in hypoxia

N-terminal HSP90 inhibitors induce a stress response called the heat shock response (HSR). The HSR is thought to be one of the reasons for N-terminal inhibitors failing in the clinic [19]. The HSR increases levels of HSP90, the protein targeted by the N-terminal drugs, and activates mechanisms that block apoptosis and increases chemo-resistance [27]. SM HSP90 inhibitors do not induce the HSR in normoxia thus greatly increasing their cellular efficacy [21]. The HSR and the impact of SM compounds on HSP mRNA and protein expression under hypoxic conditions is unknown.

To test if a heat shock response is activated by N-terminal and C-terminal HSP90 inhibitors in hypoxia, we measured HSP related proteins following an 18 hr drug treatment in HCT116 cells. Treatments using 10 μM 17-AAG produced high levels of HSP70 protein, and a slight increase in HSP90 protein, while the SM compounds did not (Fig. 2a, b, c and d). We also measured expression of HSP90AA1 mRNA under normoxia and hypoxia in the presence of HSP90 inhibitors (Fig. 2e and 2f). Only 17-AAG increased the expression of HSP90AA1 while the C-terminal SM compounds did not change expression or slightly decreased it. We also compared the heat shock response from the HSP90 inhibitors to chetomin. At 1 μM chetomin, there was an increase in the protein expression of HSP70. This is not entirely unexpected given that the heat shock response can be induced by cell stress and chetomin is associated with cytotoxicity [2, 6].

Blocking the HSP90: HIF-1α interaction reduces cell viability

To test the cytotoxic effect of SM compounds, we measured HCT116 cell viability at 18 and 72 hrs and compared to the N-terminal HSP90 inhibitor 17-AAG. Most cells were still viable at concentrations up to 25 μM for most of the tested drugs at 18 hrs (Fig. 2g), where HIF-1 inhibition was documented (Fig. 1). However, after extended periods (72 hrs) (Fig. 2h), cell viability decreased under both normoxic and hypoxic conditions. The observed cytotoxicity was similar for cells treated under both normoxic and hypoxic conditions. This is not unexpected given that HSP90 is a chaperone protein involved in many cell functions and the effect of HSP90 inhibition is not solely through the HIF pathway. The HSP90 inhibitors were also compared to chetomin which is known to have cytotoxic and necrotic effects [2, 6, 32], possibly independent from its effects on HIF-1α. Chetomin was more cytotoxic than the SM compounds and at concentrations below HIF-1 inhibitory doses, while the SM compounds show cytotoxicity at the concentrations that inhibit HIF-1 activity.

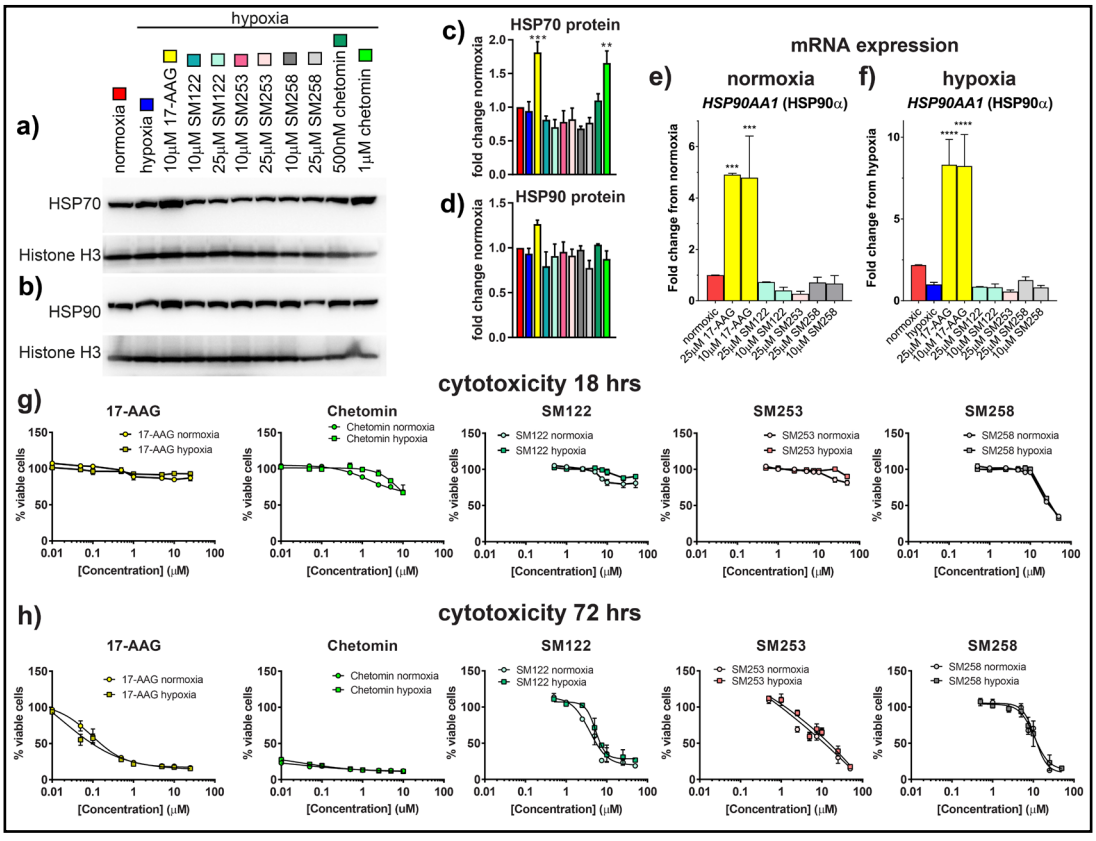

Fig. 2. The N-terminal HSP90 inhibitor 17-AAG increases the heat shock response, while C-terminal SM compounds do not. HCT116 cells were incubated ± SM compounds, 17-AAG or chetomin in normoxia or hypoxia for 18 hrs (a-g) or 72 hrs (h) to measure the heat shock response and cytotoxicity. a) Representative blot shown for HSP70 (a) or HSP90 (b) induction in hypoxia by HSP90 inhibitors. Densitometry graphs of western blots are normalized to normoxia and shown for HSP70 (c) (n=3) and HSP90 (d) (n=4). e) mRNA HSP90AA1 expression by HSP90 inhibitors in normoxia. f) mRNA HSP90AA1 induction by HSP90 inhibitors in hypoxia (n ≥ 3, run in duplicate for (e) and (f)). mRNA data normalized to 18s RNA. Data analyzed using an ordinary one-way ANOVA with multiple comparisons to normoxia (e) or hypoxia (f) in Graphpad Prism. Statistically significant results are indicated as follows: ** = p <0.01 vs normoxia, *** = p <0.001 vs normoxia, and **** = p <0.0001 vs normoxia. g) Cytotoxicity of HSP90 inhibitors and chetomin was measured using Celltiter Blue Assay following 18hrs (g) or 72 hrs (h) of drug treatment (n ≥ 3, run in duplicate). DMSO controls are equal to 100% in each graph. Data was fit using non-linear regression in GraphPad Prism (Sigmoidal, 4PL, x is log(concentration)).

SM compounds degrade HIF-1α through the proteasome

The mechanism of HIF-1α degradation when hypoxic cells are treated with SM compounds was identified by examining the proteasome pathway. The proteasome is a common degradation pathway for HIF-1α and is used in both oxygen-dependent and oxygen-independent pathways [10, 14, 15, 16].

To test if SM compounds were degrading HIF-1α through the proteasome, we treated HCT116 cells with MG262 (a proteasome inhibitor), in combination with the SM compounds. Treating HCT116 cells with MG262 in hypoxia leads to an increase in HIF-1α accumulation in the cytoplasm, compared to both the normoxic and hypoxic DMSO controls (Fig. 3a and 3b). Treatment of the SM compounds alone produces degradation of cytoplasmic HIF-1α (Fig. 3a and b), with SM258 being slightly more effective, and SM122, SM253 and 17-AAG having a moderate effect. Nuclear HIF-1α was reduced by a similar percentage for all three SM compounds (Fig. 3a and 3c). Treating cells in combination with MG262 should restore HIF-1α

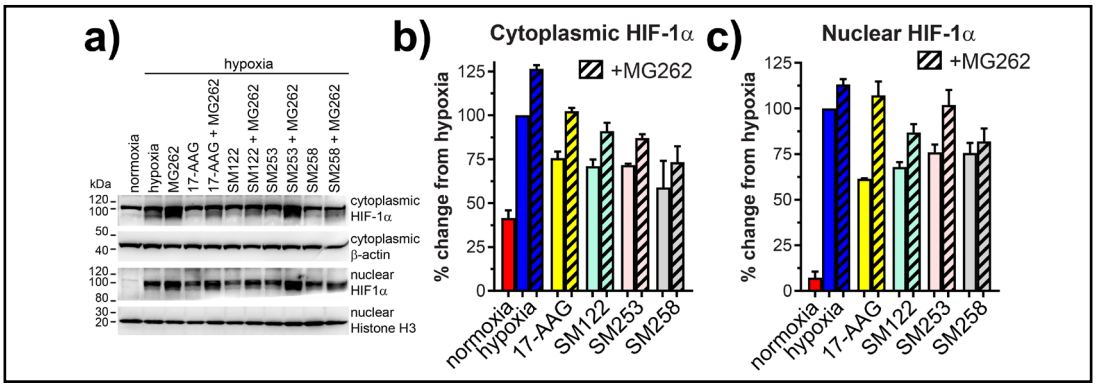

Fig. 3. SM compounds degrade HIF-1α via the proteasome in hypoxia. a) HCT116 cells were treated with 25 μM 17-AAG, 25 μM SM compounds and/or the proteasome inhibitor MG262 (1 μM) for 6 hrs in hypoxia. b) Densitometry of cytoplasmic HIF-1α protein levels from three independent experiments. c) Densitometry of nuclear HIF-1α protein levels from three independent experiments.

levels if HIF-1α is being degraded by SM compounds through the proteasome. As expected, co-treatment produced the anticipated increase in HIF-1α levels in both the cytoplasm and nucleus showing that the proteasome was required for HIF-1α degradation (Fig. 3a, b and c).

SM compounds do not require RACK1 for proteasomal degradation of HIF-1α

HIF-1α can be degraded by the proteasome using multiple pathways, including the oxygen-dependent pVHL/PHD pathway, and the oxygen-independent mechanism using RACK1 (receptor of activated protein kinase C) [16]. RACK1 binds to HIF-1α, recruiting Elongin C, which leads to the ubiquitination and proteasomal degradation of HIF-1α. RACK1-mediated proteasomal degradation of HIF-1α is independent of pVHL and oxygen levels [14, 16]. Given that HIF-1α degradation was occurring via the proteasome with C-terminal HSP90 inhibitors in hypoxia, it was important to determine if an oxygen-independent degradation mechanism, such as RACK1, was involved. Previous work has shown that RACK1 degrades HIF-1α in the presence of N-terminal HSP90 inhibitors and HIF-1 target gene expression was restored by a RACK1 loss-of-function in cells treated with 17-AAG, showing that HSP90 competes with RACK1 for binding to HIF-1α [16].

In order to test whether SM compounds require RACK1 for proteasomal-mediated degradation of HIF-1α, HCT116 cells were transfected with either RACK1 siRNA or negative control siRNA. RACK1-siRNA led to a ~92-93% decrease in RACK1 mRNA in normoxia and hypoxia as measured using qPCR (Fig. 4a) and an ~68% decrease in RACK1 protein as measured by western blot (Fig. 4b). These knockdown results appear to be similar to those achieved by Liu et al.

In contrast to the previous study [16], we found that RACK1 knockdown in HCT116 cells did not increase expression of the HIF-1 target genes when measuring CA9 (Fig. 4c) and LDHA (Fig. 4d) in normoxia or hypoxia. Importantly, RACK1 knockdown did not restore HIF-1 target gene expression in hypoxic cells treated with the HSP90 inhibitor SM258 (Fig. 4c and 4d). In contrast, we found that expression of the HIF-1 target genes, CA9 and LDHA, slightly decreased when HCT116 cells were treated in combination with SM258 and RACK1 siRNA (Fig. 4c and d). We examined the protein levels of LDHA in normoxia following RACK1 siRNA and did not find any change in LDHA protein levels (Fig. 4e). These results would seem to indicate that SM compounds do not require RACK1 for degradation of HIF-1α, though the proteasome is required. They also indicate that RACK1 is not required for HIF-1α degradation in HCT116 cells.

In an attempt to determine whether there are cell-type specific differences in RACK1 utilization, we repeated the RACK1 knockdown experiments in HEK293T cells as in the original study [16]. Liu et al. found that depletion of RACK1 in normoxic and hypoxic

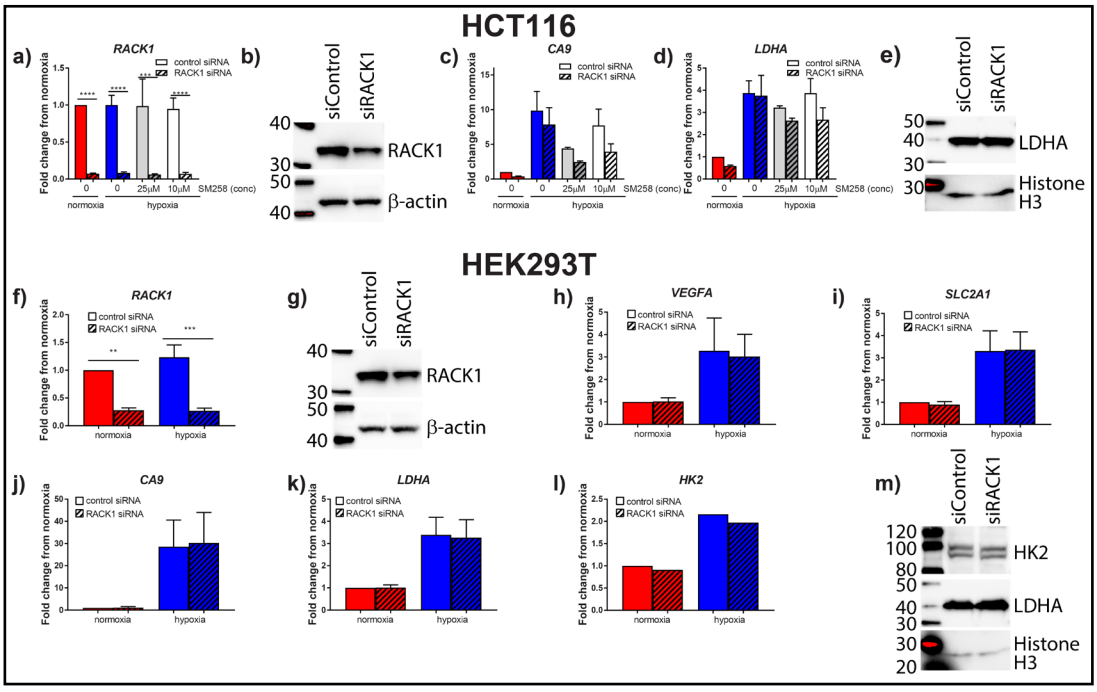

Fig. 4. HIF-1α activity is not affected by RACK1 levels. a) HCT116 cells were reverse transfected with RACK1 siRNA, which led to a 92-94% decrease in RACK1 mRNA expression. b) RACK1 protein levels decrease 68% following RACK1 siRNA treatment. c) RACK1 knockdown does not restore HIF-1 mRNA expression for either CA9 or LDHA (d) in HCT116 cells. e) RACK1 knockdown does not restore LDHA protein expression. f) HEK293T cells were reverse transfected with RACK1 siRNA, which led to a 72-79% decrease in RACK1 expression. g) RACK1 protein levels in HEK293T cells following RACK1 siRNA treatment. RACK1 knockdown does not restore HIF-1 gene expression for (h) VEGFA, (i) SLC2A1, (j) CA9, (k) LDHA, or (l) HK2 in HEK293T cells. m) Protein levels of HK2 or LDHA are not affected by RACK1 knockdown in normoxia. mRNA results are the mean ± SEM of independent experiments run in duplicate (n ≥ 3). mRNA data normalized to 18s RNA. Data analyzed using an ordinary one-way ANOVA with multiple comparisons to compare control siRNA to RACK1 siRNA in a given condition for each graph (Graphpad Prism). Statistically significant results are indicated as follows: ** = p <0.01 vs siRNA control, *** = p <0.001 vs siRNA control and **** = p <0.0001 vs siRNA control.

HEK293T cells led to increased levels of HIF-1α protein and increased expression of the HIF-1 target genes VEGF and SLC2A1 (GLUT1), due to decreased HIF-1α degradation [16].

In our study, RACK1 siRNA treatment led to a ~72% decrease in RACK1 mRNA in normoxia and a ~78% decrease in hypoxia. This resulted in a ~44% decrease in RACK1 protein in normoxic HEK293T cells (Fig. 4f and 4g).

RACK1 siRNA knockdown in HEK293T cells (Fig. 4f and 4g) did not affect expression of the HIF-1 target genes used by Liu et al. [16] in our experiments, including VEGFA (Fig. 4h) or GLUT1 (SLC2A1) (Fig. 4i). We also measured additional HIF-1 target genes to see if they might be affected. RACK1 siRNA did not alter normoxic or hypoxic mRNA expression of CA9 (Fig. 4j), LDHA (Fig. 4k) or HK2 (Fig. 4l) in HEK293T cells. HK2 and LDHA protein levels did not change in normoxic HEK293T cells when RACK1 was knocked down (Fig. 4m). Thus, the presence or absence of RACK1 does not appear to impact HIF-1 activity in our experiments using either HCT116 or HEK293T cells.

SM Compounds degrade HIF-1α through the proteasome using oxygen, PHDs and pVHL

Under normoxic conditions, HIF-1α is mainly degraded by the proteasome in an oxygen-dependent manner. Prolyl hydroxylase enzymes (PHDs) hydroxylate HIF-1α using oxygen as a substrate, which enables binding of pVHL and recruitment of the E3 ubiquitin ligase

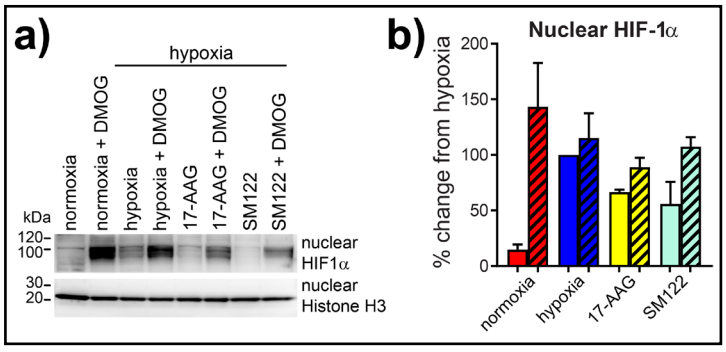

Fig. 5. SM compounds degrade HIF-1α through the proteasome using PHDs. a) HCT116 cells were treated with 25 μM 17-AAG or SM122 and/or 1 mM DMOG, which inhibits the PHD prolyl hydroxylases, for 6 hrs in hypoxia. b) Densitometry of nuclear HIF-1α protein levels from three independent experiments. Mean ± SEM.

complex, leading to proteasomal degradation of HIF-1α. Since RACK1 was not involved in HIF-1α degradation, it is possible that at low oxygen levels the PHDs may be degrading HIF-1α through the proteasome.

To test if the PHDs were involved in degradation of HIF-1α by the SM compounds, we used a PHD inhibitor, dimethyloxalylglycine (DMOG) under hypoxia. Surprisingly, treating HCT116 cells with DMOG in hypoxia leads to a slight increase in HIF-1α accumulation in the nucleus (Fig. 5a, lane 4). This indicates that PHD-mediated hydroxylation is still occurring even at very low levels of oxygen (0.5% v/v). Treating cells with 17-AAG (25 μM) or SM122 (25 μM), decreases the amount of HIF-1α under hypoxia. In contrast, combining either drug with DMOG increases the amount of HIF-1α that accumulates in the nucleus under hypoxia (Fig. 5a and 5b). This suggests that PHD activity is required for HIF-1α to be degraded by the proteasome in the presence of SM122.

SM compounds inhibit angiogenesis

N-terminal HSP90 inhibitors, such as 17-AAG, possess anti-angiogenic activity largely by inhibiting HIF-1α stabilization which controls expression of pro-angiogenesis genes [9]. HSP90 also interacts with several other pro-angiogenic proteins, and prevents their stabilization, thereby contributing additional anti-angiogenic activity. To test the anti-angiogenic effects of the SM compounds, an endothelial cell tube formation assay was used. This assay mimics multiple steps in angiogenesis, including cell adhesion, migration, and tubule formation and is highly predictive of *in vivo* anti-angiogenic activity. After coating plates with a basement membrane extract, HUVECs were added with either 17-AAG or the SM compounds. Tubule formation was imaged 18 hrs later and evaluated using the Angiogenesis Analyzer available for the program ImageJ similar to [26]. Representative images are shown in Fig. 6a and quantified in Fig. 6b. In the presence of vehicle only (1% DMSO), an extended network of tubules formed.

25 μM SM122 was the most effective drug at inhibiting tubule formation, and it showed the largest decrease in total tubule network length, number of meshes and total mesh area (Fig. 6a and 6b). 25 μM SM253 also limited tubule network length, reducing the number of meshes and decreasing the mesh area. 25 μM SM258 did not appear to impact on tubule formation. When comparing these results to 17-AAG, 10 μM slightly decreased total tubule length, number of meshes, and total mesh area. Our results indicate that HSP90 compounds 17-AAG, SM122 and SM253 possess anti-angiogenic activity in endothelial tubule formation assays, consistent with inhibiting HIF-1α from interacting with HSP90. Both HIF and HSP90 play a role in angiogenic processes [9] and inhibiting this interaction decreases endothelial tubule formation.

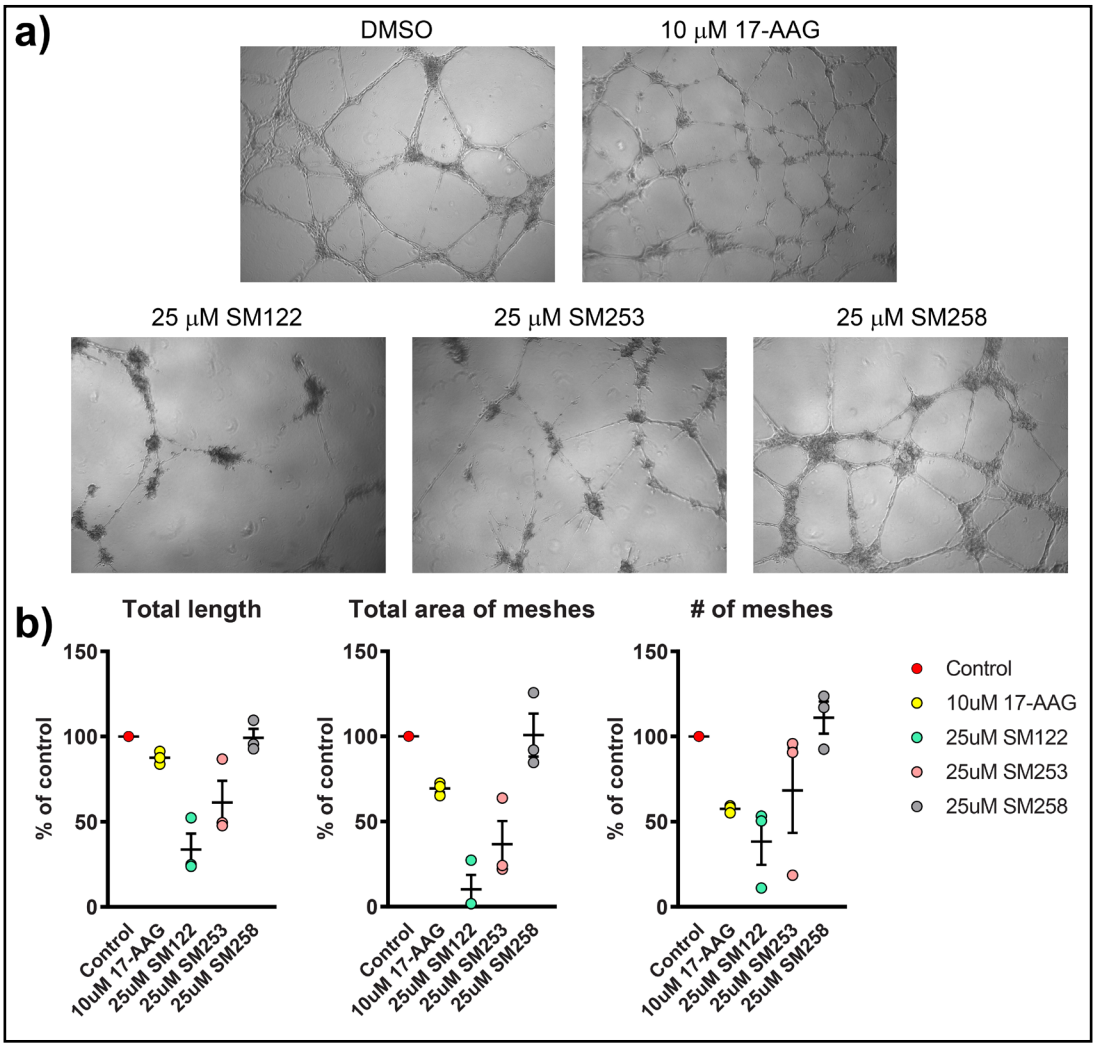

Fig. 6. SM compounds decrease endothelial tube formation. a) Representative images of HUVEC cells from three independent experiments where cells were treated with N-terminal HSP90 inhibitor 17-AAG, or one of the C-terminal inhibitors, SM122, SM252, or SM258 and plated on EHS matrix. Images taken after 18 hours at 10× magnification. b) Tubule formation was quantified using ImageJ with the add-on Angiogenesis Analyzer (NIH) (n = 3). Mean ± SEM shown with individual values.

Discussion

This is the first comprehensive study of how a new series of C-terminal HSP90 inhibitors, SM122, SM253 and SM258, impact the HIF hypoxic response. These molecules lead to HIF-1α degradation and decrease downstream HIF-1 target gene expression, particularly GLUT1 expression. GLUT1 (SLC2A1) imports glucose and increased expression of the protein is associated with decreased cancer patient survival [33]. GLUT1 itself has been investigated as a cancer drug target in order to block glucose uptake and limit glycolysis. Treating colorectal cancer cells with SM molecules reduced GLUT1 protein expression (Fig. 1), with SM122 being the most effective. Hexokinase 2 protein was reduced in colorectal, breast and prostate cancer cells treated with SM compounds. The SM compounds were generally effective HIF-1α inhibitors, while also possessing anti-cancer effects, which was likely produced via their impact on other HSP90 pathways.

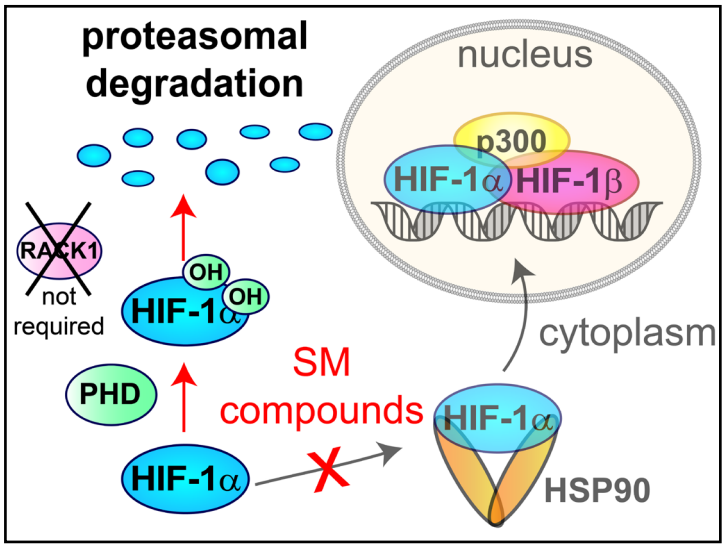

Fig. 7. Proposed model of HIF-1α degradation by SM compounds in hypoxia. SM compounds prevent HIF-1α:HSP90 binding which allows the PHDs to hydroxylate HIF-1α, even under low oxygen conditions, targeting HIF-1α for proteasomal degradation.

Our tested cell lines did not always respond in the same manner to SM compounds. Cancer cell lines are known to show diverse responses to cytotoxic drugs [33], and this is not unexpected with HSP90 targeted drugs, given that the driving mutations will vary. This may explain why particular SM compounds were more potent in different cell lines (Fig. 1). Furthermore, when the activity of individual SM compounds are compared within the same cell line, they typically showed somewhat similar potency, though not always. Compounds have unique structures, and although they have been shown to bind to the same binding site on HSP90 [23, 24], they are allosteric inhibitors, and therefore modulate HSP90 activity and the client and co-chaperones of HSP90 in a unique manner.

The C-terminal SM compounds do not induce a heat shock response in either normoxia or hypoxia (Fig. 3). This is interesting given that the heat shock response from N-terminal inhibitors directly counteracts the efficacy of the HSP90 inhibitors and is thought to be a primary factor in the failure of HSP90 inhibitors in the clinic [19, 35]. Minimizing the heat shock response by targeting the expressed HSPs with siRNAs increases the potency of the N-terminal HSP90 inhibitors [36-38]. This indicates the N-terminal HSP90 inhibitors would be more effective drugs if they did not induce the heat shock response. The SM series overcomes this hurdle. The SM compounds also inhibit endothelial tube formation, indicating they possess anti-angiogenic activity, in addition to their HIF inhibitory properties.

Finally, we determined that the SM compounds degrade HIF-1α in hypoxia through the proteasome in an oxygen-dependent manner (Fig. 7). We found that the PHDs and canonical oxygen degradation pathway was still relevant for HIF-1α degradation even in hypoxic conditions as low as 0.5% v/v oxygen (4 mmHg).

These results are in contrast to work previously performed using the N-terminal HSP90 inhibitor, 17-AAG, in HEK293T cells [16]. Specifically, Liu et al., found that in HEK293T cells, RACK1 siRNA increased HIF-1α target gene expression in normoxia. In contrast, we found that in HCT116 cells HIF-1 target gene expression for CA9 and LDHA did not change following RACK1 siRNA. We also found that RACK1 siRNA treatment did not increase HIF-1 target gene expression in HEK293T cells, indicating that RACK1 was not essential to HIF-1α degradation in either normoxia or hypoxia using our experimental conditions. RACK1 siRNA combined with SM258 led to a slight decrease in HIF-1 target gene expression. Our knockdown is effective at the RACK1 mRNA and protein level and our blots look similar to the results obtained by Liu et al. [16], so the reason for the discrepancy between Liu and our results is not clear.

There are multiple HIF-1α degradation pathways and it is possible that a cell uses a combination of degradation mechanisms, with some more or less important depending on the environment. It's also possible that RACK1 may only play a minor role in HIF-1α degradation and its role may only be seen when a knockout model is used versus a knockdown. However,

the knockdown levels we achieved appear to be highly similar to Liu et al. [16]. There are possibly off target effects from the siRNAs used, but this will require further investigation with knockout models or other techniques.

While somewhat surprising that the PHDs remain active under low oxygen conditions, the genes encoding the three PHD enzymes (EGLN1, EGLN2 and EGLN3) are expressed by HIF-1 itself [38], meaning that the amount of available PHD enzymes increases in hypoxia and act as a feedback loop to limit an excessive hypoxic response. Additionally, PHD activity has been observed at oxygen levels as low as 0.2% [39], which is lower than the conditions we used.

We, and others, have found that targeting HIF-1α through its cofactors and binding partners can be an effective strategy for blocking HIF-1 activity and reducing both tumor growth and angiogenesis [2-7].

Conclusion

Based on our results, HSP90 inhibitors are another strategy for blocking HIF-1 activity. Specifically, C-terminal inhibitors, the SM compounds, are a promising anti-cancer approach for targeting HSP90 and impacting HIF-1α function.

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Authors' contributions: KC and SM conceived and designed the study. NK, CM, BK, MM and KMC performed and analyzed the experiments. SM designed the compounds and SSZ made the compounds. KMC wrote the paper with SM and all authors approved the final draft.

Disclosure Statement

The authors have no conflicts of interest to declare and no ethical conflicts to disclose.

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