

Inhibition of heat shock protein 90 attenuates post-angioplasty intimal hyperplasia

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Abstract. Intimal hyperplasia (IH) is a pathologic process that leads to restenosis after treatment for peripheral arterial disease. Heat shock protein 90 (HSP90) is a molecular chaperone that regulates protein maturation. Activation of HSP90 results in increased cell migration and proliferation. 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) are low toxicity Food and Drug Association approved HSP90 inhibitors. The current study hypothesized that HSP90 inhibition was predicted to reduce vascular smooth muscle cell (VSMC) migration and proliferation. In addition, localized HSP90 inhibition may inhibit post-angioplasty IH formation. For proliferation, VSMCs were treated with serum-free media (SFM), 17-DMAG or 17-AAG. The selected proliferative agents were SFM, platelet derived growth factor (PDGF) or fibronectin. After three days, proliferation was measured. For migration, VSMCs were treated with SFM, 17-AAG or 17-DMAG with SFM, PDGF or fibronectin as chemoattractants. Balloon injury to the carotid artery was performed in rats. The groups included in the present study were the control, saline control, 17-DMAG in 20% pluronic gel delivered topically to the adventitia or intraluminal delivery of 17-DMAG. After 14 days, arteries were fixed and sectioned for morphometric analysis. Data was analyzed using ANOVA or a student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference. The results revealed that 17-AAG and 17-DMAG had no effect on cell viability. PDGF and fibronectin also increased VSMC proliferation and migration.

Furthermore, both 17-AAG and 17-DMAG decreased cell migration and proliferation in all agonists. Topical adventitial treatment with 17-DMAG after balloon arterial injury reduced IH. HSP90 inhibitors suppressed VSMC proliferation and migration without affecting cell viability. Topical treatment with a HSP90 inhibitor (DMAG) decreased IH formation after arterial injury. It was concluded that 17-DMAG may be utilized as an effective therapy to prevent restenosis after revascularization.

Introduction

Peripheral arterial disease (PAD) remains a major clinical problem that can lead to disability, limb loss and is potentially fatal in the elderly (1,2). Growth factors and extracellular matrix proteins are important mediators in the pathogenesis of PAD and the development of restenosis secondary to intimal hyperplasia (IH) after balloon angioplasty (3,4). IH is a complex process that begins by platelet activation (5,6). Platelets then bind to the area of vascular injury releasing many agents including thrombospondin-1 (TSP-1) and platelet derived growth factor (PDGF) (5), which in turn cause vascular smooth muscle (VSMC) migration into the area of injury where they begin to proliferate and produce extracellular matrix (5). All of these processes clearly contribute to IH by regulating the arterial response to injury. Recently heat shock protein 90 (HSP90) has also been implicated in pathologic vascular remodeling (7).

HSP90 is a molecular chaperone that binds many signaling proteins regulating their final maturation (8). HSP90 is ubiquitously expressed and is important for normal cell function (8). However, aberrant activation of HSP90 can result in increased cell migration and proliferation (8). Inhibition of HSP90 has been examined in states of aberrant cell growth such as cancer (8). Several different HSPs have been detected in atherosclerosis, and HSP90, in particular, is strongly expressed in atherosclerotic plaque (9). However, the role HSP90 plays in IH after balloon angioplasty is unknown. At least one previous study has shown that blocking HSP90 inhibits VSMC proliferation and migration (9). As the development of IH depends on VSMC migration and proliferation, inhibition of HSP90 may be an efficacious approach to this

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important clinical problem. The quintessential HSP90 inhibitor is the natural product geldanamycin; however, geldanamycin exhibits a relatively high toxicity (10,11). Several derivatives of geldanamycin have been created that have significantly less toxicity and are in clinical trials for cancer therapy, two of these are 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (10,12).

In the present study, we sought to understand the contribution of HSP90 in VSMC physiologic processes and IH formation after arterial balloon injury. We hypothesized that HSP90 inhibition would reduce agonist-induced VSMC proliferation and migration, and that localized HSP90 inhibition would inhibit post-angioplasty IH formation.

Materials and methods

Materials. Smooth muscle cell growth and basal medium were purchased from Cell Applications, Inc. (San Diego, CA). Trypsin and trypsin neutralizing solution were purchased from Cell Applications Inc. (San Diego, CA). 17-AAG and 17-DMAG were purchased from Tocris Bioscience (Bristol, UK). Fibronectin and PDGF were purchased from R&D Systems (Minneapolis, MN).

Cell culture. Human aortic VSMCs (Cell Applications, Inc., San Diego, CA, USA) were used in early passage (P3-P5). Cells were made quiescent by incubation in serum free media (SFM) for 48 h at 37°C in a tissue culture incubator (5% CO₂).

Cell viability. Cell viability was determined with the Trypan Blue exclusion assay (Thermo Fisher Scientific, Waltham, MA) using the Countess Cell counter (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. All experiments were performed in triplicate studying 17-AAG or 17-DMAG at three different concentrations (100 nM, 10 or 30 μ M). The doses chosen have been previously shown to be effective (13).

Migration assay. Chemotaxis was assessed in quiescent human VSMCs incubated in a 96-well FluoroBlok migration plate (One Riverfront Plaza, Corning, NY) according to the manufacturer's instructions with 50,000 calcein AM labeled cells per well. Experiments were performed in triplicate. Cells were exposed to the chemoattractant agents: SFM (negative control), PDGF 20 ng/ml (positive control) or Fibronectin 20 μ g/ml (positive control), which were placed in the bottom chamber. VSMCs treated with 17-AAG (100 nM, 10 or 30 μ M) or 17-DMAG (100 nM, 10 μ M or 30 μ M) for 2 h at 37°C in a tissue culture incubator (5% CO₂) in addition to SFM (negative control) and PDGF or fibronectin (positive control) were placed in the top chamber. Migrated cells were measured on a fluorescent plate reader with excitation of 480 nm and emission of 520 nm. Data is expressed as relative fluorescence units (RFU).

Proliferation assay. Cell proliferation was assessed in growth-arrested VSMCs incubated in a 96 well plate with 5,000 cells/well. Experiments were performed in triplicate. Quiescent cells were exposed to serum free media (SFM), PDGF (20 ng/ml) or Fibronectin (20 μ g/ml), 17-AAG (100 nM,

10 or 30 μ M) or 17-DMAG (100 nM, 10 or 30 μ M). VSMCs were incubated for 72 h at 37°C in a tissue culture incubator (5% CO₂). Proliferation was measured using a MTS tetrazolium absorbance assay (Cell Titer 96 Aqueous Cell Proliferation Assay, Promega, Madison, WI) according to manufacturer's instructions.

Carotid artery balloon injury model. This study was approved by the Syracuse VA IACUC and the animal care complied with the Guide for the Care and Use of Laboratory Animals. Sprague-Dawley rats at 10-12 weeks of age (Harlan Laboratories, Indianapolis, IN) were randomized into four groups (8-10 animals per group)-control, saline control, intraluminal 17-DMAG or topical 17-DMAG. 17-DMAG was selected as the HSP90 inhibitor for the *in vivo* work because of ease of use (water soluble) and equivalent lack of toxicity to cells and inhibition of VSMC proliferation *in vitro*. Rats were anesthetized using inhaled isoflurane at 5% and then maintained at 2% for the surgery. The left common carotid artery (CCA) and its bifurcation were exposed via carotid artery cutdown. A 2F Fogarty catheter (Edwards Lifesciences, Irvine, CA) was placed into the CCA via the external carotid artery and the balloon was inflated (5 atmospheres for 5 min). Then a polyethylene (PE) catheter attached to a pump was placed into the vessel and the vessel gently distended with either saline (0.9%, PH=8.5) with flow rate 10 ml/min in saline control and topical treatment groups or 17-DMAG (500 nM) suspended in saline for 20 min (intraluminal treatment group). The catheter was withdrawn, and the external carotid artery ligated. For the topical group, 17-DMAG (500 nM) in 20% pluronic gel (F-127, Anaspec, Ferment, CA) was placed on top of the vessel prior to closing the incision. We chose 17-DMAG for the *in vivo* part of the study over 17-AAG as it is more water soluble and has better bioavailability than 17-AAG (12). Thereby, delivery formulations of 17-DMAG do not require organic solubilizing agents which have their own limitations. After 14 days, the animals were euthanized and bilateral CCAs were perfusion fixed, harvested, sectioned, stained with hematoxylin and eosin and morphometric analysis was performed to assess severity of IH. Two groups have been assigned as control groups for this particular study, isolated angioplasty injury (control) and angioplasty plus intraluminal saline infusion (saline control). The reason behind using dual control groups is to exclude the assumption of concomitant saline injury to the vessel wall.

Morphometric analysis. The left CCA was fixed in paraffin, sectioned, stained with hematoxylin and eosin for cross-sectional morphometric analysis. The medial thickness (M) was quantified between the internal (IEL) and external elastic (EEL) laminae, i.e., $M=EEL-IEL$. The intimal thickness (I) was measured between the endothelial cell layer and the internal elastic lamina, i.e., $I=IEL-Luminal\ area$. The ratio of intimal area to medial area was calculated as the intimal thickness divided by the combined intimal and medial thickness (14).

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Data was tested via ANOVA with post-hoc testing with Fisher's PLSD in StatView (SAS Institute, Cary, NC) or a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

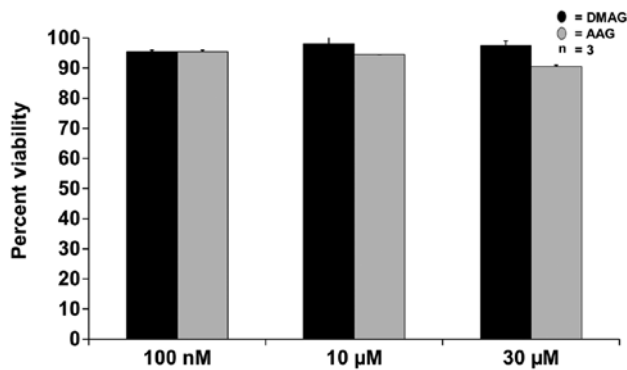


Figure 1. 17-AAG and 17-DMAG exert no toxic effects on VSMCs. VSMCs were treated with different concentrations of 17-AAG or 17-DMAG for 24 h. Viability was assessed using a trypan blue exclusion assay. All groups had a cell viability >90%. Data are presented as the mean \pm SEM (n=2). 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; VSMC, vascular smooth muscle cell.

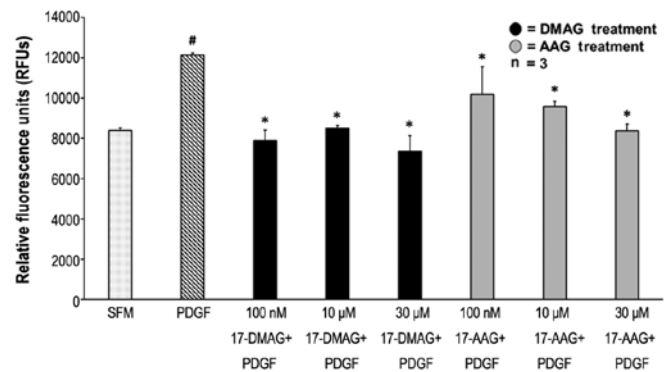


Figure 2. 17-AAG and 17-DMAG decreased VSMC migration to PDGF. VSMCs were treated with different concentrations of either 17-AAG or 17-DMAG in the presence of 20 ng/ml PDGF as a chemoattractant agent. SFM treatment was used as a negative control. Results were assessed after 120 min. *P<0.05 vs. PDGF; #P<0.05 vs. SFM. Data are presented as the mean \pm SEM (n=3). 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; VSMC, vascular smooth muscle cell; platelet derived growth factor; SFM, serum-free media.

Results

Cell viability. 17-AAG and 17-DMAG had no effect on VSMC viability. After 24-hour incubation, VSMC viable cell count did not significantly change in all tested concentrations (100 nM, 10 μM or 30 μM) of either drug. At 100 nM, the cells were 95.5% viable with both 17-AAG and 17-DMAG treatment. At 10 μM the viability was 94.5% for 17-AAG and 98% for 17-DMAG. At the highest tested dose-30 μM-cell viability was determined to be 90.5% for 17-AAG and 97.5% for 17-DMAG (Fig. 1).

Migration. 17-AAG and 17-DMAG decreased VSMC migration in response to PDGF. PDGF at a concentration of 20 ng/ml increased smooth muscle migration by 30% compared to SFM (P=0.0001). At the concentration of 100 nM, 17-AAG decreased migration by 16% (P=0.0048) while 17-DMAG decreased migration by 34.9% (P=0.0003). Ten μM 17-AAG and 17-DMAG decreased migration by 21.1% (P=0.003) and 29.9% (P=0.0002) respectively. At 30 μM, 17-AAG and 17-DMAG were found to decrease migration by 30.8% (P=0.0003) and 39.4% (P=0.0003) respectively. There was no difference in inhibition of VSMC migration between 17-AAG and 17-DMAG (Fig. 2).

17-AAG and 17-DMAG decreased VSMC migration in response to Fibronectin. Fibronectin at a concentration of 20 μg/ml increased smooth muscle cell migration by 16% compared to SFM (P=0.01). At a concentration of 100 nM, 17-AAG decreased migration by 19.21% (P=0.015) while 17-DMAG decreased migration by 13.78% (P=0.03). Ten μM 17-AAG and 17-DMAG decreased smooth muscle migration by 15.35 (P=0.01) and 21.3% (P=0.01) respectively. At 30 μM, 17-AAG and 17-DMAG were found to decrease migration by 17.3 (P=0.004) and 19.2% (P=0.01) respectively. There was no difference in inhibition of VSMC migration between 17-AAG and 17-DMAG (Fig. 3).

Proliferation. 17-AAG and 17-DMAG decreased VSMC proliferation in response to PDGF. PDGF at a concentration of 20 ng/ml increased smooth muscle cell proliferation by 15.42% compared to SFM (P=0.006). At a concentration of 100 nM,

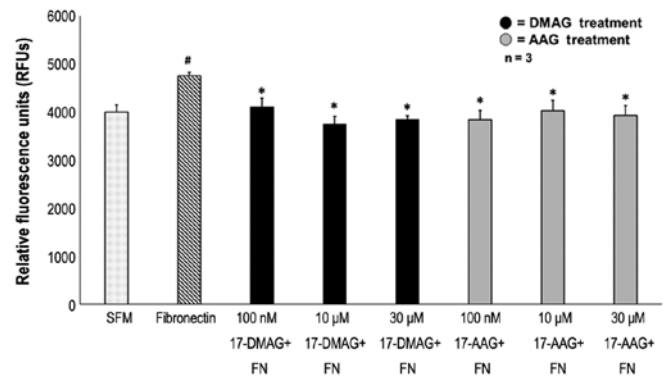


Figure 3. 17-AAG and 17-DMAG decreased VSMC migration to fibronectin. VSMCs were treated with different concentrations of 17-AAG or 17-DMAG in the presence of 20 μg/ml FN as a chemoattractant. SFM treatment was used as a negative control. Results were assessed after 3 h. *P<0.05 vs. FN; #P<0.05 vs. SFM. Data are presented as the mean \pm SEM (n=3). 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; VSMC, vascular smooth muscle cell; FN, fibronectin; SFM, serum-free media.

17-AAG decreased proliferation by 73.9% (P=0.001) while 17-DMAG decreased proliferation by 77.5% (P=0.0002). Ten μM 17-AAG and 17-DMAG decreased proliferation by 62.6% (P=0.01) and 72.8% (P=0.0002) respectively. At 30 μM, 17-AAG and 17-DMAG were found to decrease proliferation by 72.8% (P=0.0002) and 72.4% (P=0.0006) respectively. There was no difference in VSMC proliferation between 17-AAG and 17-DMAG by PDGF (Fig. 4).

17-AAG and 17-DMAG decreased VSMC proliferation in response to Fibronectin. Fibronectin at a concentration of 20 μg/ml increased smooth muscle proliferation by 25% absorbance at 490 nm from 0.18 to 0.24 (P<0.0001). At a concentration of 100 nM, 17-AAG decreased proliferation by 62.9% (decreased absorbance to 0.089, P=0.0001) while 17-DMAG decreased proliferation by 67.5% (decreased absorbance to 0.078, P=0.0001). At 10 μM 17-AAG and 17-DMAG decreased proliferation by 58.1% (P=0.0001) and 55.6%

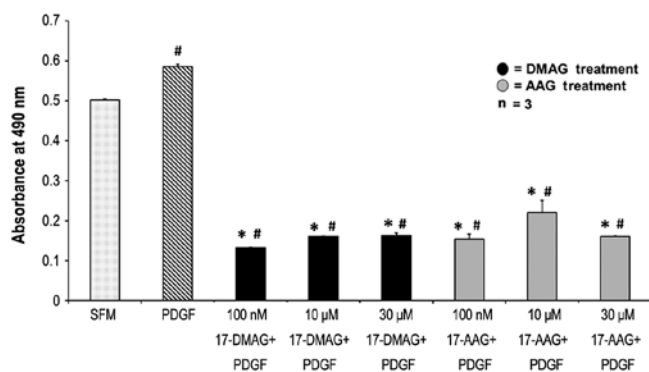


Figure 4. 17-AAG and 17-DMAG decreased VSMC proliferation to PDGF. VSMCs were treated with different concentrations of either 17-AAG or 17-DMAG in the presence of 20 ng/ml PDGF as a proliferative agent. SFM treatment was used as a negative control. Results were assessed after 72 h. * $P < 0.05$ vs. PDGF; # $P < 0.05$ vs. SFM. Data are presented as the mean \pm SEM (n=3). 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; VSMC, vascular smooth muscle cell; PDGF, platelet derived growth factor; SFM, serum-free media.

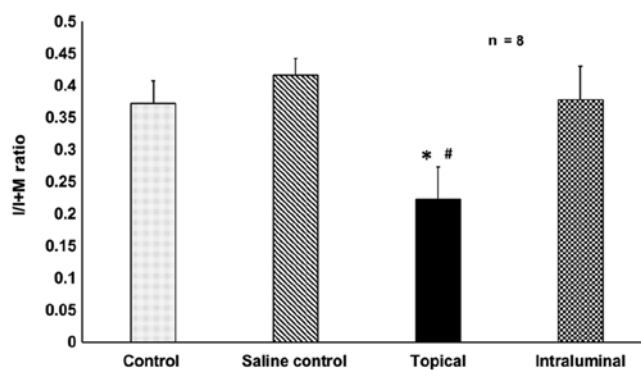


Figure 6. Topical HSP90 inhibition attenuates IH. Topical 17-DMAG treatment attenuated IH and decreased the I/M ratio compared with the untreated group. * $P < 0.05$ vs. the untreated group; # $P < 0.05$ vs. the saline control. Data are presented as the mean \pm SEM (n=7). HSP90, heat shock protein 90; IH, intimal hyperplasia; 7-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin.

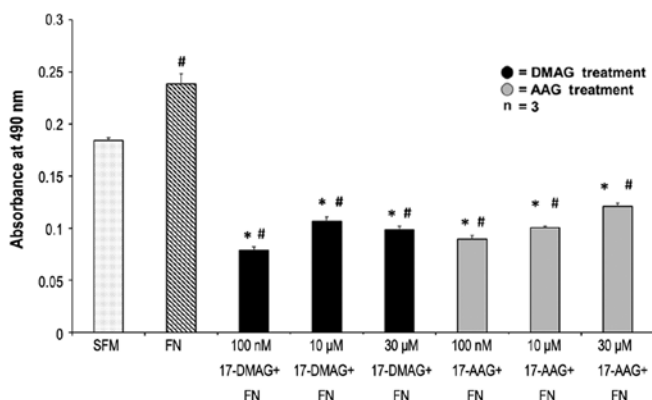


Figure 5. 17-AAG and 17-DMAG decreased VSMC proliferation to fibronectin. VSMCs were treated with different concentrations of 17-AAG or 17-DMAG in the presence of 20 μ g/ml FN as a proliferative agent. SFM treatment was used as a negative control. Results were assessed after 72 h. * $P < 0.05$ vs. FN; # $P < 0.05$ vs. SFM. Data are presented as the mean \pm SEM (n=3). 17-AAG, 17-N-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; VSMC, vascular smooth muscle cell; FN, fibronectin; SFM, serum-free media.

($P=0.0001$) respectively. At 30 μ M, 17-AAG and 17-DMAG were found to decrease proliferation by 49.6% ($P=0.0001$) and 59% ($P=0.0001$) respectively. There was no difference in the degree of inhibition of VSMC proliferation between 17-AAG and 17-DMAG (Fig. 5).

Morphometric analysis

Topical 17-DMAG reduces IH after arterial injury in Sprague-Dawley rats. Rats that were treated with adventitial 17-DMAG dissolved in 20% pluronic gel had 46.5% less IH when compared to the saline control group (0.22 ± 0.02 vs. 0.42 ± 0.05 , $P=0.001$). Intra-luminal delivery of 17-DMAG had no effect on IH, with the control group I/M ratio being 0.41 ± 0.22 and the intra-luminal group being 0.38 ± 0.05 . No difference was determined between control and saline control IH (Figs. 6 and 7).

Discussion

IH is a major cause of long-term failure after either open or endovascular revascularization (15). Despite all the technological advances in endovascular revascularization modalities, restenosis remains a significant risk, particularly within the first 12 months after angioplasty (16). A fuller understanding of the cellular processes behind VSMC migration and proliferation (key processes in IH development), is critical to developing new therapies to attenuate restenosis. The present study examined the role of HSP90 in IH after balloon arterial injury and the potential therapeutic role of HSP90 inhibitors, 17-AAG and 17-DMAG, in attenuating or even preventing IH. Our results demonstrate that 17-DMAG dissolved in 20% pluronic gel, applied topically on the adventitia of the common carotid artery of Sprague-Dawley rats reduced the amount of IH after injury. We further found that intra-luminal delivery of 17-DMAG dissolved in saline to the injured artery didn't attenuate the IH in the affected animals.

HSPs are a family of conserved intracellular proteins that are involved in proper protein folding which helps both in protein function and intracellular targeting (17). HSP90 is a common member of the HSP family that was found to be overexpressed in atherosclerotic plaque in patients and is associated with plaque instability (18). The essential function of HSP90 is believed to be the interaction with proteins known as clients (19). Many of the clients are protein kinases and transcription factors that regulate cell differentiation, proliferation and apoptosis (20,21).

Proliferation of VSMCs is crucial in the pathogenesis of IH and restenosis (9,22,23). After arterial injury, VSMCs, originating primarily from the adventitia, migrate into the area of vascular injury and proliferate, thus initiating the process of IH. HSP90 has been shown to be involved in the proliferation of many cell types including VSMCs (21,24-26). The current study demonstrates that HSP90 does play a broad role in VSMC proliferation, as 17-AAG and 17-DMAG were able to inhibit VSMC proliferation in response to two functionally different agonists, PDGF and fibronectin.

Geldanamycin, was the first HSP90 inhibitor to be developed; however, this inhibitor has poor solubility and is highly toxic to various human cell types (27). Two main geldanamycin

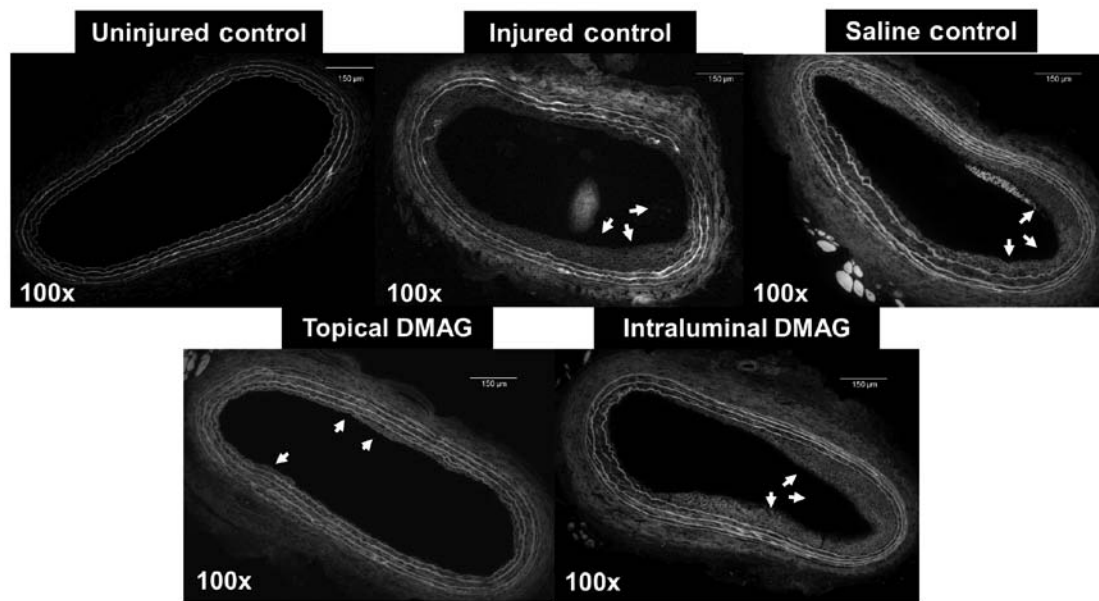


Figure 7. Representative CCA cross sections. The images present representative cross sections of the CCA, obtained via endogenous fluorescence of the blood vessels in an un-injured control, injured control, saline control, topical 17-DMAG and intraluminal 17-DMAG. Scale bar=150 μ m. CCA, common carotid artery; 7-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin.

derivatives-17-AAG and 17-DMAG-have less toxicity and have been tested in phase II/III clinical trials as novel antineoplastic agents (9). In the present study, we needed to determine if either geldanamycin derivative has the same cellular toxic effects as geldanamycin (11,27,28). Our data shows that VSMC viability was determined to be above 90% at any tested concentration of 17-AAG or 17-DMAG used. Both 17-AAG and 17-DMAG work by inhibiting the binding of ATP to HSP90, which is necessary for HSP90 function (18).

The current study further investigated whether HSP90 contributes to the development of IH after arterial injury in an animal model. At least one other study has demonstrated the HSP90 inhibition can attenuate IH (7). However in that study the authors used a peptide inhibitor which can be immunogenic in humans, our study used FDA approved HSP90 inhibitors that have been shown to be safe in humans (7,12,29). Given that our *in vitro* data demonstrated no differences in efficacy between 17-AAG and 17-DMAG, we chose to use 17-DMAG *in vivo* as this drug is more water soluble and therefore easier to use. We found topical (periadventitial) delivery of 17-DMAG significantly reduced the formation of IH. The specific downstream effects of HSP90 that account for its role in IH development are unknown. Recent studies showed that HSP90 inhibition downregulates cyclinD3, PCNA, and pRb, leading to cell cycle arrest which could contribute to the antiproliferative effect of either 17-DMAG or 17-AAG (27). These effects and their role in IH will need further study. Our findings indicate that HSP90 is a vital factor in the process of post-arterial injury, including VSMC migration, proliferation and IH.

The present study does have limitations that will need further study. Since HSP90 inhibition affects several intracellular proteins, kinases and intracellular signaling pathways at the same time, other pathophysiological processes such as apoptosis, angiogenesis or oxidative stress could also be affected by the drug used in the study and warrants further investigation to

accurately clarify the role of HSP90 in human arterial disease. Another limitation of our study is how to optimize the delivery route of the HSP90 inhibitor agent, as in our model only the periadventitial topical route was found to significantly decrease IH. This method may have been effective because of the longer residence time of the inhibitor with the vessel. Periadventitial delivery is relevant for open revascularization, but may not be practical for endovascular procedures, mitigating a need for further technological advancements to optimize the intraluminal 17-DMAG delivery method. The method used for intraluminal delivery in the current study may have been inadequate for absorbance by the vessel wall. Nanotechnology for the improved delivery of 17-DMAG could be one option. Endovascular adventitial delivery with an infusion device through the arterial wall (e.g., Bullfrog Micro-Infusion Device, Mercator MedSystems) would be an alternative method to endovascular delivery.

In conclusion, the present study demonstrates the significance of HSP90 in the development of IH. In addition, these studies showed no toxic effects of either 17-AAG or 17-DMAG on VSMC viability, indicating possible safety of these agents if used as anti-intimal hyperplastic agents in revascularization procedures. Interestingly, both agents were able to markedly reduce PDGF-induced and fibronectin-induced VSMC migration and proliferation. Topical periadventitial delivery of the HSP90 inhibitor was found to attenuate post-balloon injury IH. The current study provides a foundation for future explorations into potential therapeutic targets for reducing the damaging effects of IH.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MMK performed the experiments, analyzed the data and prepared the manuscript for submission in this work. FM and MD performed the experiments and analyzed data. DB performed all surgical procedures. VG assisted in experimental design, data interpretation and manuscript editing. KGM was the principal investigator of the grant, who designed the present study, interpreted the data, and wrote and edited the manuscript.

Ethics approval and consent to participate

All animal studies in this manuscript were approved by The Syracuse Department of Veterans Affairs Institutional Animal Care and Use Committee in accordance with AAALAC guidelines.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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