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14. ABSTRACT
Peripheral arterial disease (PAD) remains a major threat to life and limb and represents a disabling and potentially fatal condition in the aging military and Veteran population. Heat shock protein 90 (HSP90) is a molecular chaperone binds many signaling proteins regulating their final maturation. HSP90 is ubiquitously expressed and is important for normal cell function. However, aberrant activation of HSP90 can result in increased cell migration and proliferation. Inhibition of HSP90 has been in examined in states of aberrant cell growth such as cancer. Our long-term goal is to understand how HSP90 signaling pathways can be manipulated therapeutically to prevent IH in vivo. The objective of this proposal is to investigate the mechanisms by which localized HSP90 inhibition regulate IH development. Our central hypothesis is that localized HSP90 inhibition will effectively inhibit the formation of IH. The rationale for the proposed project is that understanding the underlying mechanisms of dyslipidemia on IH and establishing optimal HSP90 inhibitor delivery to reduce IH will result in novel and innovative approaches to prevent restenosis after angioplasty.

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Introduction

The research is relevant to military and Veterans' health because peripheral arterial disease and restenosis after balloon angioplasty to treat arterial blockages is a significant cause of disability and death in the military and Veteran population. Intimal hyperplasia is an important process in arterial restenosis development. These studies will (1) expand our understanding of the pathophysiology of intimal hyperplasia and the impact of HSP90 inhibitors on intimal hyperplasia and (2) establish the optimal method(s) of delivery of HSP90 inhibitors to decrease intimal hyperplasia. Thus, the findings from the proposed research are relevant to the Department of Defense's mission to decrease disability by providing safer, more effective care for military personnel and Veterans with peripheral arterial disease.

Keywords

Vascular disease, restenosis, atherosclerosis, aging, smooth muscle, HSP90, balloon angioplasty

Accomplishments

What were the major goals of the project?

1. Determine the effect of HSP90 inhibition on vascular smooth muscle cell migration and proliferation.
2. Examine the effect of HSP90 inhibition on the miR-17~92 cluster expression.
3. Evaluate the efficacy of HSP90 inhibition in preventing intimal hyperplasia after balloon injury.

What was accomplished under these goals?

Please see figures at the end of the appendix

1. Determine the effect of HSP90 inhibition on vascular smooth muscle cell migration and proliferation.

   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, 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 100nM, 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% (p=0.0001) respectively. At 30 µM, 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)

2. Examine the effect of HSP90 inhibition on the miR-17~92 cluster expression.

   There was no effect on the miR-17~92 cluster by HSP90 inhibition in vascular smooth muscle cells.

3. Evaluate the efficacy of HSP90 inhibition in preventing intimal hyperplasia after balloon injury.

   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).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Presentation at a scientific meeting

Publication in press

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

Impact

What was the impact on the development of the principal discipline(s) of the project?

The finding that HSP90 inhibition has a strong potential to treat recurring vascular disease.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

Changes/Problems

None

Products

Data was presented at the 32nd Eastern Vascular Society annual meeting Sep. 6-Sep 8 2018 in Washington, D.C. and published as abstract no. 3734 in J Vasc Surg 68 e20: 2018.

The publication is in the final editing stages for publication in Molecular Medicine Reports.

Participants & Other Collaborating Organizations

Kristopher Maier – no change

Vivian Gahtan – no change

David Bruch – no change

Special Reporting Requirements

None

Appendices

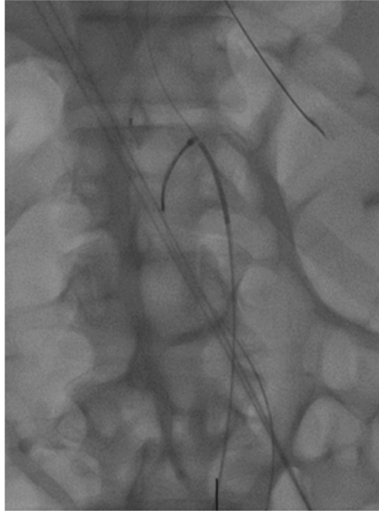

Fig 2. Cheese-wire fenestration.

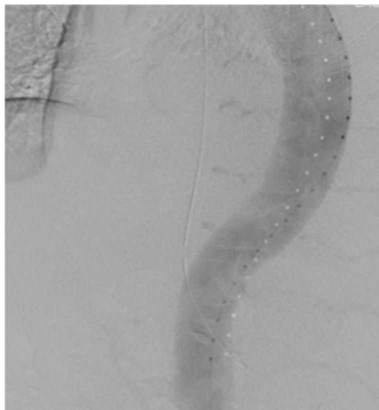

Fig 3. Aortogram showing repaired true lumen.

Methods: A 40-year-old man developed a type B aortic dissection with distal malperfusion. He underwent endovascular repair of the dissection with endograft with coverage of the left subclavian artery. Intravascular ultrasound examination was used to confirm true lumen placement of the wire throughout the aorta. Immediately after surgery, the patient's symptoms of malperfusion resolved.

Results: On postoperative day 1, the patient developed abdominal pain with an increasing lactate level and a computed tomography scan demonstrated compression of the true lumen distal to the stent graft. The patient was brought to the operating room and a cheese wire fenestration was performed distal to the stent graft down to the aortic bifurcation (Fig 2). Femoral thrombectomy was performed for removal of the intima. The patient's recovery was unremarkable.

Conclusions: True lumen compression distal to a stent graft reentry tear (Fig 1) can be salvaged by cheese wire fenestration (Fig 3). Larger studies are needed to determine long-term effectiveness.

Author Disclosures: D. J. O'Connor: Nothing to disclose; **S. Nielsen:** Nothing to disclose; **A. Ratnathicam:** Nothing to disclose; **K. Cook:** Nothing to disclose; **M. Wilderman:** Nothing to disclose; **G. Simonian:** Nothing to disclose; **M. Napolitano:** Nothing to disclose.

Inhibition of Heat Shock Protein-90 Attenuates Postangioplasty Intimal Hyperplasia

Mohammed M. Kassem, MD, Marry DaCosta, Furqan Muqri, MD, David Bruch, MS, Vivian Gahtan, MD, Kristopher G. Maier, PhD. SUNY Upstate Medical University, Syracuse, NY

Objectives: Peripheral arterial disease represents a disabling and potentially fatal condition in the aging population. Intimal hyperplasia (IH) is the major pathologic event that leads to restenosis after balloon angioplasty. IH is a complex process which starts immediately after endothelial injury. Growth factors and extracellular matrix proteins are released by platelets and induce vascular smooth muscle cell (VSMC) migration and proliferation. Heat shock protein 90 (HSP90) is a chaperone that binds many proteins regulating their maturation. The HSP90 inhibitors 17-AAG and 17-DMAG are low toxicity geldanamycin derivatives. We hypothesized that HSP90 inhibition would reduce agonist-induced VSMC proliferation. In addition, localized HSP90 inhibition would inhibit postangioplasty IH formation.

Methods: To assess VSMC proliferation, quiescent VSMCs were treated with SFM, 17-DMAG, or 17-AAG. The proliferative agents were SFM, platelet-derived growth factor (20 ng/mL), or fibronectin ($20\ \mu g/mL$). After 3 days, proliferation was determined with an MTS dye assay. Drug toxicity was assessed by trypan exclusion. Balloon injury to the common carotid artery was performed in Sprague-Dawley rats to induce IH. There were two groups, no treatment or 17-DMAG dissolved in 20% pluronic gel delivered to the adventitia of the common carotid artery. After 14 days, animals were euthanized, common carotid artery perfusion fixed, and sectioned. Sections were stained with hematoxylin and eosin for morphometric analysis. Data were analyzed by analysis of variance or the Student *t*-test. $P < .05$ was considered significant.

Results: Local adventitial treatment with DMAG after balloon arterial injury reduced IH (46.5% compared with injury alone). 17-AAG and 17-DMAG had no effect on cell viability ($\geq 90\%$). Platelet-derived growth factor and fibronectin increased VSMC proliferation. Both 17-AAG and 17-DMAG decreased proliferation to all agonists. There was no difference between 17-AAG and 17-DMAG in inhibiting VSMC proliferation.

Conclusions: HSP90 inhibitors suppressed chemoattractant induced VSMC proliferation without affecting cell viability. Local treatment with a HSP90 inhibitor (DMAG) decreased IH formation after arterial injury. Thus HSP90 may be a therapeutic target to prevent postangioplasty restenosis. However, the mechanism by which HSP90 decreases IH and affects VSMC proliferation warrants further investigation.

Author Disclosures: M. M. Kassem: Nothing to disclose; **M. DaCosta:** Nothing to disclose; **F. Muqri:** Nothing to disclose; **D. Bruch:** Nothing to disclose; **V. Gahtan:** Nothing to disclose; **K. G. Maier:** Nothing to disclose.

Basilic Vein Superficialization Is an Effective Alternative to Transposition in Patients Requiring Brachiobasilic Arteriovenous Fistula

Tarundeep Singh, Joe T. Huang, Frank T. Padberg Jr, Michael A. Curi, Timothy Wu. Rutgers New Jersey Medical School, Newark, NJ

Objectives: The goal of this study was to compare outcomes for techniques in the creation of brachiobasilic arteriovenous fistulas, namely in the second stage basilic vein transposition or the second stage basilic vein superficialization.

Title: **Inhibition of Heat Shock Protein 90 Attenuates Post-Angioplasty Intimal Hyperplasia**

Short Title: Inhibition of HSP90 Attenuates IH

Category: Original Research Article

Authors: Mohammed M. Kassem[1,2], Furqan Muqri[1,2], Mary DaCosta[3], David Bruch[1,2], Vivian Gahtan[1,2], Kristopher G. Maier[1,2].

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Keywords: HSP90, intimal hyperplasia, pluronic gel, balloon arterial injury, 17-AAG, 17-DMAG

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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-AAG and 17-DMAG, are low toxicity FDA approved HSP90 inhibitors. We hypothesized that HSP90 inhibition would reduce vascular smooth muscle cell (VSMC) migration and proliferation. In addition, localized HSP90 inhibition would inhibit post-angioplasty IH formation. For proliferation VSMCs were treated with serum-free media (SFM), 17-DMAG or 17-AAG. The proliferative agents were SFM, 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 were 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 by ANOVA or student's *t*-test. *p*-values< 0.05 were considered significant. 17-AAG and 17-DMAG had no effect on cell viability. PDGF and fibronectin increased VSMC proliferation and migration. Both 17-AAG and 17-DMAG decreased migration and proliferation to 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. Thus, 17-DMAG may be 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 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 current 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.

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 hours at 37° C in a tissue culture incubator (5% CO_2).

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 μM or 30 μM). 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 µg/ml (positive control), which were placed in the bottom chamber. VSMCs treated with 17-AAG (100nM, 10µM or 30µM) or 17-DMAG (100nM, 10µM or 30µM) for 2 hours at 37^{0}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 5000 cells/well. Experiments were performed in triplicate. Quiescent cells were exposed to serum free media (SFM), PDGF (20 ng/ml) or Fibronectin (20 µg/ml), 17-AAG (100 nM, 10 µM or 30µM) or 17-DMAG (100 nM, 10 µM or 30 µM). VSMCs were incubated for 72 hours at 37° C in a tissue culture incubator (5% CO_2).

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 minutes). 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 minutes (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, Fermont, 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 was tested by ANOVA with post hoc testing with Fisher's PLSD in StatView (SAS Institute, Cary, NC) or Student's *t*-test. A *p*-value < 0.05 was considered statistically significant.

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).

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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 current 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 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 our 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 current 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, Emeryville, CA) would be an alternative method to endovascular delivery.

In conclusion, the current 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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Author Contribution: Mohammed M. Kassem performed experiments, analyzed data and prepared the manuscript for submission in this work. Furqan Muqri and Mary DaCosta performed experiments and analyzed data. David Bruch performed all surgical procedures. Vivian Gahtan assisted in experimental design, data interpretation and manuscript editing. Kristopher G. Maier is the principal investigator of the grant, designed the study, interpreted the data and wrote and edited the manuscript.

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Figure Legends

Figure 1. 17-AAG and 17-DMAG Have No Toxicity to VSMCs

VSMCs were treated with different concentrations of 17-AAG or 17-DMAG for 24 hours. Viability was assessed with the trypan blue exclusion assay. All groups had a cell viability greater than 90%. n=2, data is presented as mean ± S.E.M.

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 Platelet derived growth factor (PDGF) as a chemoattractant agent. SFM treatment was used as the negative control. Results were assessed after 120 minutes. n=3, * = $p<0.05$ as compared to PDGF. **#** = $p<0.05$ as compared to SFM. Data is presented as mean ± S.E.M

Figure 3. 17-AAG and 17-DMAG Decreased VSMC Migration to Fibronectin

VSMCs were treated with different concentrations of either 17-AAG or 17-DMAG in the presence of 20 µg/ml fibronectin (FN) as a chemoattractant agent. SFM treatment was used as the negative control. Results were assessed after 3 hours. n=3, * = $p<0.05$ as compared to FN, **#** = $p<0.05$ as compared to SFM. Data is presented as mean ± S.E.M

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 Platelet derived growth factor (PDGF) as a proliferative agent. SFM treatment was used as the negative control. Results were assessed after 72 hours. n=3, * = $p<0.05$ as compared to PDGF, # = $p<0.05$ as compared to SFM. Data is presented as mean ± S.E.M

Figure 5. 17-AAG and 17-DMAG Decreased VSMC Proliferation to Fibronectin

VSMCs were treated with different concentrations of either 17-AAG or 17-DMAG in the presence of 20 µg/ml fibronectin (FN) as a proliferative agent. SFM treatment was used as the negative control. Results were assessed after 72 hours. n=3, * = $p<0.05$ as compared to FN, # = $p<0.05$ as compared to SFM. Data is presented as mean ± S.E.M

Figure 6. Topical HSP90 Inhibition Attenuates IH

The topical DMAG treatment attenuated IH and decreased I/M ratio in comparison to the untreated group. n=7, * = $p<0.05$ compared to the untreated group. **#** = $p<0.05$ as compared to saline control. Data is presented as mean ± S.E.M

Figure 7. Representative CCA Cross Sections

This slide shows representative cross sections of the CCA using endogenous fluorescence of the blood vessels in the Un-injured Control, Injured Control, Saline Control, Topical DMAG and Intraluminal DMAG. The white line represents a 150 µM distance.

Figure 1.

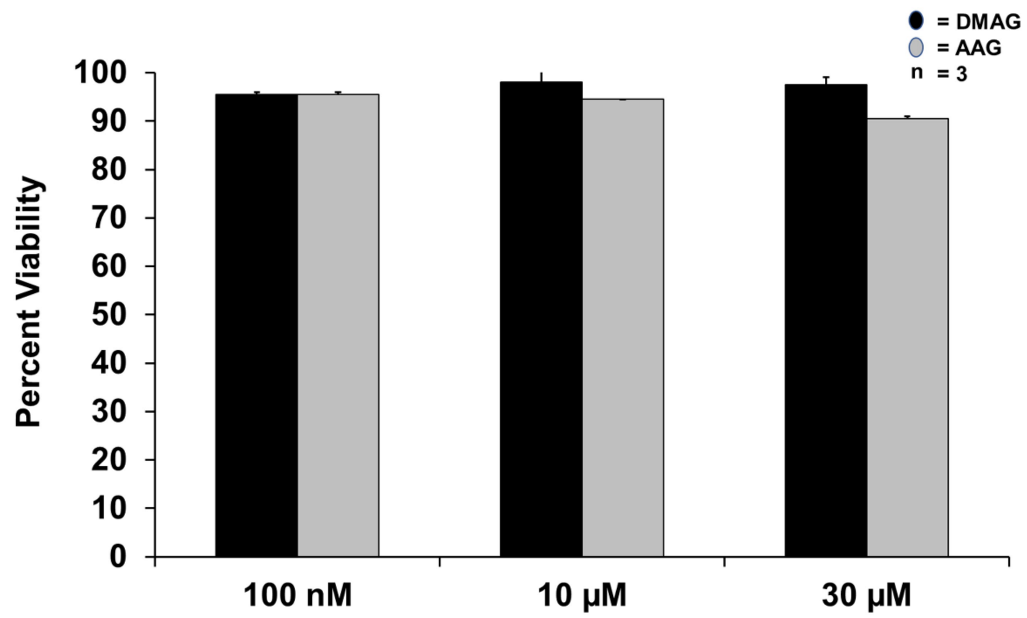

Figure 2.

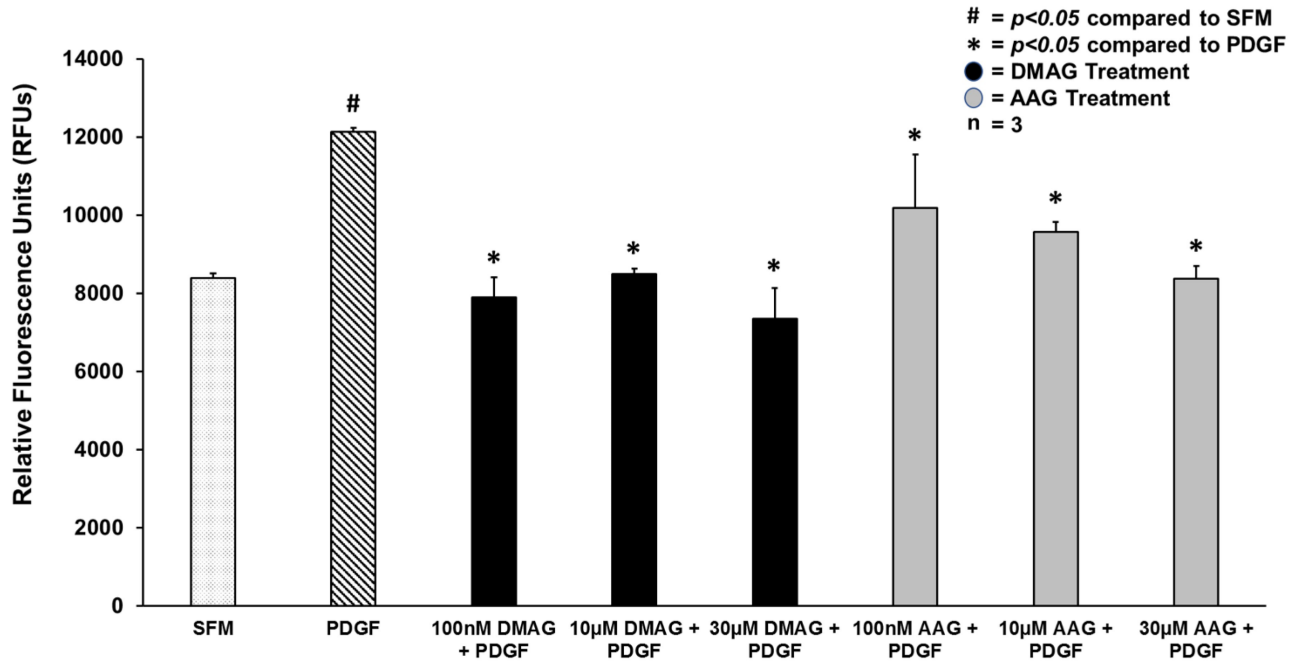

Figure 3.

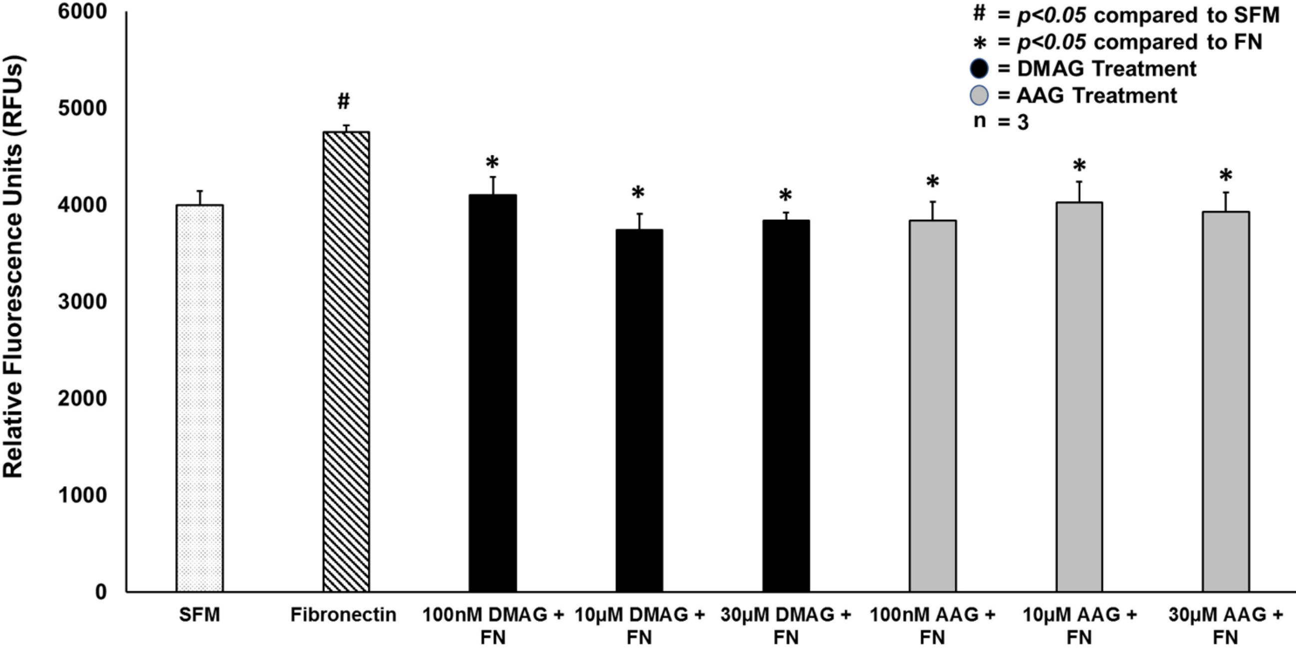

Figure 5.

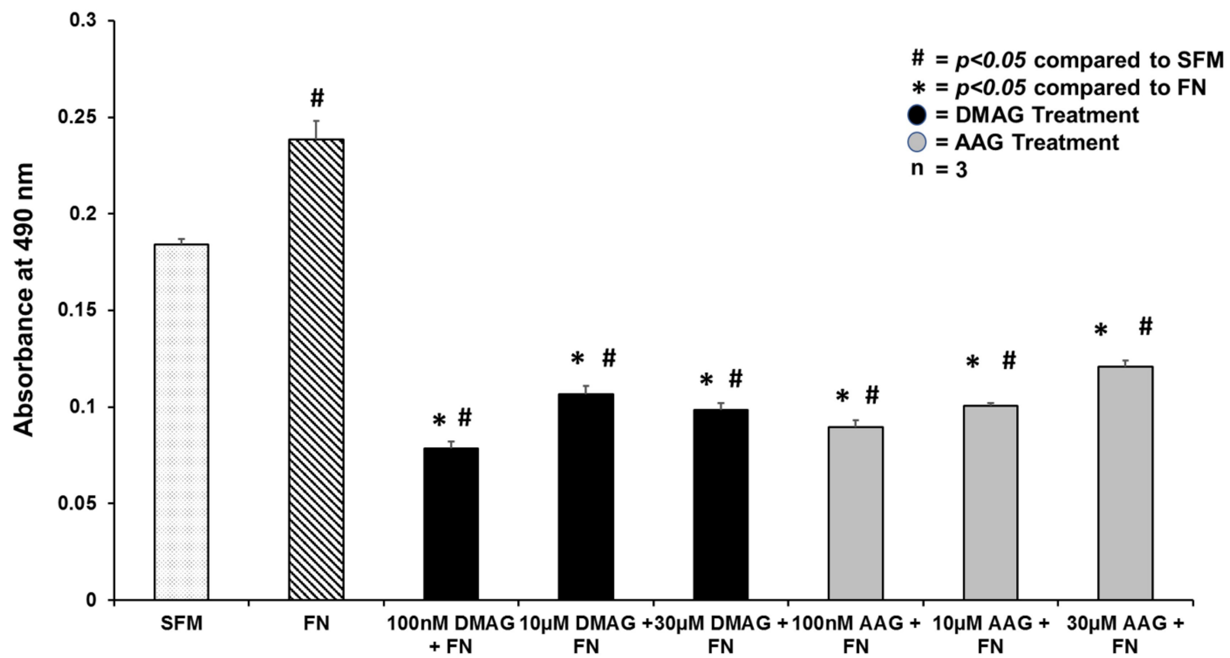

Figure 6.

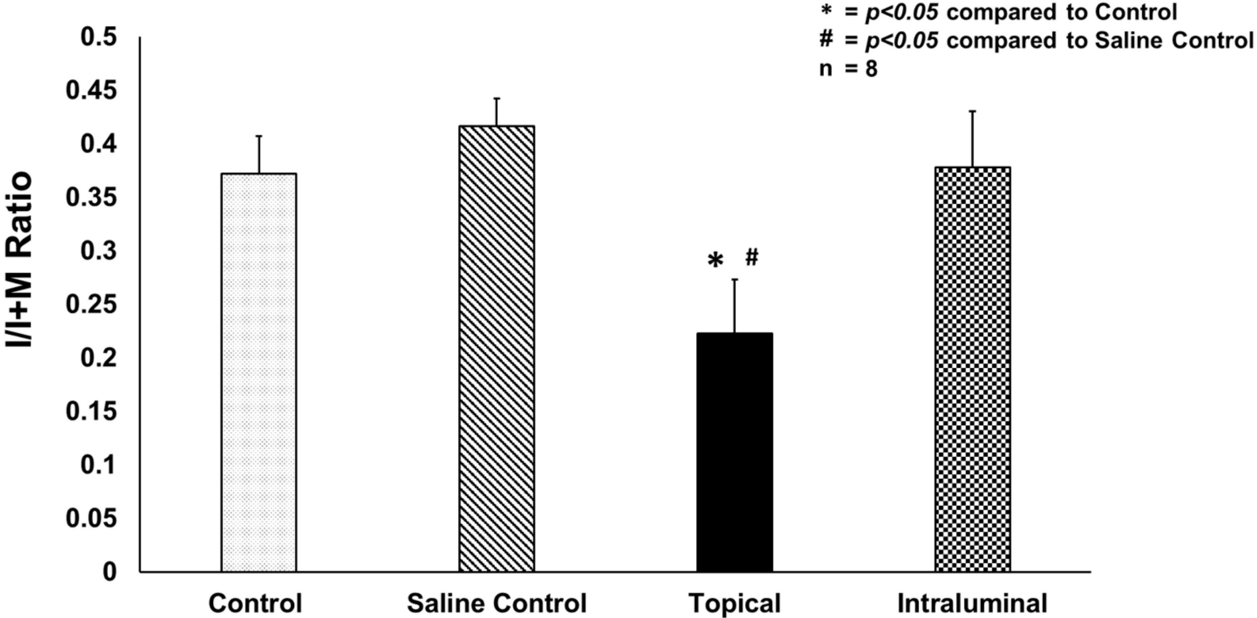

Figure 7.