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Targeting the 90 kDa Heat Shock Protein Improves Photodynamic Therapy

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Abstract

The geldanamycin derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), binds to the amino-terminal ATP binding pocket of the 90 kDa heat shock protein (Hsp-90) and inhibits this chaperone from stabilizing client proteins involved with the malignant phenotype. We examined the effects of a combined modality protocol involving photodynamic therapy (PDT) and 17-AAG in mouse mammary carcinoma cells and tumors. PDT increased the expression of the anti-apoptotic and pro-angiogenic proteins survivin, Akt, HIF-1 α , MMP-2 and VEGF in tumor tissue and this expression decreased significantly when 17-AAG was included in the treatment regimen. Tumor bearing mice treated with PDT and 17-AAG had improved long-term tumoricidal responses when compared with individual treatment protocols. We conclude that Hsp-90 plays an active role in modulating tumor responsiveness following PDT and targeting Hsp-90 with 17-AAG enhances the therapeutic effectiveness of PDT.

Keywords

photodynamic therapy; PDT; apoptosis; Hsp-90; 17-AAG; geldanamycin

1. Introduction

Photodynamic therapy (PDT) is used for treating malignant and non-oncologic disorders [1]. The treatment involves either the systemic injection of a photosensitizer or topical application of a prodrug followed by exposure of the target lesion to visible light [1,2]. The FDA approved photosensitizer, Photofrin (PH), as well as a variety of second generation photosensitizers are in PDT clinical trials [1–3]. Photochemical generation of cytotoxic reactive oxygen species within the treatment field leads to direct and indirect responses. Direct cell killing of the tumor occurs via necrosis, apoptosis and autophagy [4,5]. Indirect responses within the tumor microenvironment include vascular injury, hypoxia and inflammatory reactions [6,7]. Molecular actions following PDT involve increased expression of regulators of angiogenic and

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Conflict of Interest

There were no conflicts of interest associated with this work for either author.

apoptotic pathways including growth factors [6,8,9] matrix metalloproteinases [10] cytokines [7,11], prostanoids [11,12], signaling molecules [2,4,13] and members of the inhibitor of apoptosis (IAP) family [14]. Methods to improve PDT responsiveness are now examining combined modality approaches targeting these molecules and pathways.

The heat shock protein (Hsp) family functions as molecular chaperones and guide the folding, intracellular localization and proteolytic turnover of key regulators of cell growth, differentiation and survival. The 90 kDa Hsp (Hsp-90) comprises 1–2% of the total intracellular protein in normal eukaryotic cells, is constitutively over expressed in tumor cells, and is being evaluated as a target for cancer therapy [15,16]. Hsp-90 controls the balance between folding/maturation and proteasomal degradation of client proteins and is involved in promoting the growth and survival of malignant cells and tumors [15–18]. Geldanamycin and the clinically relevant derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), bind to the amino-terminal ATP binding pocket of Hsp-90 and block the ability of this chaperone to properly fold or stabilize client proteins [15,19]. 17-AAG displays higher affinity for Hsp-90 derived from tumor cells compared to Hsp-90 from normal cells [20]. Hsp-90 inhibitors synergize with chemotherapeutic agents and radiation and cause the destabilization and degradation of HIF-1 α [20,21]. We recently reported that 17-AAG attenuates PDT-induced expression of survivin and Akt and enhances apoptosis in a human breast cancer cell line [14]. The goal of the current study was to expand upon these findings by determining the effectiveness of 17-AAG in improving the *in vivo* tumoricidal action of PDT using a mouse mammary carcinoma model. We demonstrate for the first time that 17-AAG increases the efficacy of the PDT tumor treatment and suggest that a combined modality approach targeting multiple pathways maintained by Hsp-90 may be a beneficial adjuvant for PDT.

2. Materials and Methods

2.1. Drugs

The photosensitizer Photofrin porfimer sodium (PH) (a gift from Axcan Pharma, Mont-Saint-Hilaire, QC, Canada) was dissolved in 5% dextrose in water to make a 2.5 mg/ml stock solution and frozen at –20 C until used. The stock solution was diluted to 25 μ g/ml in culture medium prior to *in vitro* experiments or to a concentration of 0.5 mg/ml in saline prior to *in vivo* studies. 17-AAG (provided by the Cancer Treatment and Evaluation Program, National Cancer Institute, Bethesda, MD) was dissolved in dimethylsulfoxide (DMSO) to make a stock concentration of 1 mM for *in vitro* experiments and 100 mM for *in-vivo* studies. The 17-AAG stock solutions were kept at –20 C and diluted to 1 μ M with medium immediately before *in vitro* experiments and to a concentration of 10 mM in saline before *in-vivo* administration.

2.2. Cell Culture and Tumor Models

Mouse mammary carcinoma (BA) cells were grown as monolayer cultures in RPMI 1640 and supplemented with 10% FCS plus antibiotics. BA tumors were generated by subcutaneous trochar injection of 1-mm³ pieces of tumor to the right hind flank of 8- to 12-week-old female C3H/HeJ mice [10]. Treatments were started when tumors reached 6–7 mm in diameter. All *in vivo* experiments were approved by our institutional animal care and use committee.

2.3. In vitro and In vivo Treatment Protocols

BA cells were seeded onto plastic Petri dishes and incubated for 24 hours in complete growth medium to allow for attachment [10]. Prior to light exposure, cells were incubated in the dark with PH at a dose of 25 μ g/ml for 16 hours at 37 C. Cells were then incubated for an additional 30 minutes in fresh growth medium containing 5% FCS and rinsed in medium without serum prior to exposure to varying doses of broad spectrum (570–650 nm) red light generated by a parallel series of red Mylar-filtered 30 W fluorescent bulbs delivered at a dose rate of 0.35

mW/cm². For combination experiments, PDT treated cells were incubated for an additional 24 hours with growth medium in the presence or absence of 17-AAG. In-vivo PDT treatment of BA tumors included an i.v. injection of PH (5 mg/kg) followed 24 hours later with non-thermal laser irradiation of the tumor using a solid-state diode laser (Diomed, Andover, MA) emitting red light at 630 nm [12]. Light was delivered via quartz fiber microlens and outputs were measured with a power meter. A light dose rate of 75 mW/cm² and a total light dose of 200 J/cm² were used for all in-vivo PDT treatments. 17-AAG was administered via ip injection at a dose of 75 mg/kg thrice weekly (M,W,F) for up to two weeks. Mice were monitored three times per week for detection of tumor recurrence and regrowth measurements. A treatment cure was defined as being tumor free for 90 days after PDT.

2.4. Western Immunoblot Analysis

Protein expression profiles were obtained by Western immunoblot analysis as previously described [11,12]. Briefly, cells were collected at specified times after treatment and sonicated in 1X lysis buffer containing phenylmethanesulfonyl fluoride from Cell Signaling Technology (Danvers, MA). Tumor samples were collected either 2 or 24 hr following treatment, homogenized with a polytron in 1 X reporter lysis buffer from Promega (Madison, WI) and evaluated for protein expression. Protein samples were size-separated on discontinuous polyacrylamide gels (10–16%) and transferred overnight to nitrocellulose membranes. Filters were blocked for 2 hours with 5% nonfat milk and then incubated overnight with either rabbit polyclonal antibody to phospho-survivin (Thr34) from Novus Biologicals (Littleton, CO), rabbit polyclonal antibody to survivin from Chemicon International (Temecula, CA), rabbit polyclonal antibody to Akt1/2 or Raf-1 from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal antibody to HIF-1 α from Transduction Laboratory (Lexington, KY), rabbit polyclonal antibody to Ser473 phospho-Akt from Cell Signaling Technology (Danvers, MA), rabbit polyclonal Hsp-90 antibody and mouse monoclonal antibody to Hsp70/Hsc70 from Stressgen Bioreagents (Victoria, BC, Canada). Filters were then incubated with either an anti-mouse or an anti-rabbit peroxidase conjugate from Sigma (St. Louis, MO), and the resulting complexes were visualized by enhanced chemiluminescence autoradiography from Amersham Life Science (Chicago, IL). Protein loading was evaluated by incubating the same filters with a mouse monoclonal anti-actin antibody from MP Biochemicals (Aurora, OH). Autoradiographs were quantified by scanning densitometry.

2.5 ELISA Assay

A Quantikine M mouse ELISA kit from R&D Systems (Minneapolis, MN) was used according to the manufacturer's instructions to quantify VEGF levels in control and treated tumor lysates [9]. Results were normalized for total protein concentrations.

2.6 SDS-PAGE Zymography

Aliquots from tumor lysates were denatured, run on 10% gelatin gels from Invitrogen Life Technologies (Carlsbad, CA) and renatured as previously described [10]. Gels were stained with 0.5% Coomassie Blue, destained in a solution containing 10% methanol and 7.5% acetic acid, and dried [10]. Proteins having gelatinolytic activity were visualized as white bands on a blue gel.

2.7. Statistics

The log-rank test was used to compare differences between treatment groups for analysis of tumor cure rates. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. 17-AAG inhibits Hsp-90 function in BA mammary carcinoma cells and tumors

The biological activity of 17-AAG was first evaluated by examining the expression profiles of the biomarkers Raf-1 and Hsp-70 in BA cells and tumors exposed to the geldanamycin derivative. Raf-1 is a proto-oncogene with serine/threonine kinase activity. Raf-1 is also an Hsp-90 client protein and the expression of this molecule is blocked via a direct interaction of 17-AAG on Hsp-90 function. Conversely, expression of the Hsp-70 molecular chaperone increases following 17-AAG exposure. Levels of these two biomarkers are used to monitor the efficacy of Hsp-90 inhibitors [22]. Figure 1A and B show representative Western immunoblot results following exposure of BA cells and tumors to 17-AAG respectively. Raf-1 expression was blocked following exposure to 17-AAG while the level of Hsp-70 was increased. These results confirm the in vitro and in vivo efficiency of the 17-AAG doses used in our PDT studies to target Hsp-90 chaperone activity.

3.2. 17-AAG blocks cellular expression of PDT-induced pro-survival molecules

Photofrin mediated PDT, at doses producing between 20–40 percent clonogenic cytotoxicity [12], induces cellular expression and phosphorylation-mediated activation of the Hsp-90 client proteins survivin and Akt. Figure 2A provides representative Western immunoblots showing expression of Akt, Ser473 phospho-Akt, survivin and Thr34 phospho-survivin levels in cells exposed to PDT, 17-AAG or PDT plus 17-AAG. The PDT treatment resulted in the increased expression of survivin, phospho-survivin and phospho-Akt. Combining PDT with 17-AAG decreased the expression of these proteins but did not alter the expression profiles of Hsp-90 or actin. Densitometric averages for three separate experiments are shown in Fig 2 (B–F) for Akt, phospho-Akt, survivin, phospho-survivin and Hsp-90 respectively and confirmed the reproducibility of the inhibitory effects of 17-AAG on the expression of these Hsp-90 client proteins.

3.3. 17-AAG blocks the expression of angiogenic and pro-survival molecules in PDT treated tumors

We next examined the in vivo expression profiles of the Hsp-90 client proteins Akt, HIF-1 α and survivin in BA tumors following exposure to PDT and/or 17-AAG. Tumors received a 200 J/cm² PDT dose. One group of mice received a 75 mg/kg ip dose of 17-AAG delivered immediately following light exposure. Mice were sacrificed two hours following PDT and the tumors were removed for protein analysis. Figures 3A and B show Western immunoblots and corresponding protein densitometric measurements for tissue lysates from tumors exposed to PDT, 17-AAG or PDT plus 17-AAG. Administration of 17-AAG resulted in decreased expression of HIF-1 α , Akt and survivin within treated tumors. The reduced expression of Akt and survivin was accompanied by a concomitant decrease of their active phosphorylated forms. As expected, 17-AAG did not alter the expression of Hsp-90 or actin. Expression of survivin/phospho-survivin was also observed in tumor tissues assayed 24 hours post PDT and the combination protocol of PDT and 17-AAG also attenuated expression of this anti-apoptotic protein at this time period (data not shown).

3.4. 17-AAG attenuates PDT expression of VEGF and MMPs in tumor tissue

BA tumors were treated with either PDT, 17-AAG or PDT $\pm$ 17-AAG as described above and tissue samples were then collected 24 hours later and evaluated for MMP expression/activity via SDS-PAGE gelatinase zymography and for VEGF levels using a commercial ELISA kit. Figures 4 A and B show zymographic results and corresponding densitometric reading of MMP expression. PDT induced increased expression and activation of MMP-2 and MMP-9. The addition of 17-AAG to the treatment protocol inhibited the PDT-induced expression and

proteolytic activation of these MMPs. Figure 4 C shows that PDT induced expression of VEGF within treated tumor tissue was reduced to background values when 17-AAG was combined with PDT. These results demonstrate that 17-AAG effectively attenuates invasion and angiogenic molecules induced within PDT treated tissue.

3.5. 17-AAG enhances PDT treatment of BA mammary tumors

The therapeutic effectiveness of combining 17-AAG with a standard PDT protocol was evaluated with the same BA mammary tumor model described above to examine Hsp-90 client protein expression profiles. Tumors measuring 6–7 mm in diameter were treated with PDT using a light dose of 200 J/cm². Immediately after PDT, one group of mice was started on a two-week regimen of 17-AAG (75 mg/kg) administered thrice weekly (M,W,F). Tumor response was monitored for a 90-day period. Figure 5 shows the percentage of tumor-free mice as a function of days after treatment for mice receiving 17-AAG, PDT or PDT combined with 17-AAG. The 17-AAG alone protocol did not result in any long-term tumor cures but did result in a 2–3 day delay in tumor regrowth (data not shown). PDT alone resulted in a 25% cure rate while the combination protocol of PDT and 17-AAG resulted in 50% long-term cures. The differences between PDT and PDT plus 17-AAG were found to be statistically significant ($p < 0.5$).

4. Discussion

PDT is a first line therapy for cutaneous and bronchial malignancies and is an effective treatment option for multiple tumor types following recurrence after chemotherapy or radiation therapy [23,24]. However, similar to other treatment modalities, PDT mediated oxidative stress also induces the expression of prosurvival and angiogenic signaling molecules within the tumor microenvironment which can negatively impact treatment effectiveness [6,7,25–28]. Preclinical studies confirm that targeting individual signaling, anti-apoptotic or angiogenic molecules during PDT can improve therapeutic efficacy but may not maximize treatment responsiveness due to the overlapping pathways of many proteins that promote tumor survival and growth [8,9,10,26]. The molecular chaperone Hsp-90 has emerged as a target for cancer therapy because of its role in maintaining the conformation, stability and activity of multiple oncogenic client proteins including several induced by PDT [21]. Inhibiting Hsp-90 activity induces pleiotrophic effects including interference with sustained angiogenesis, avoidance of apoptosis, growth factor independence, unlimited replication, and blockage of tissue invasion and metastasis [21,22]. The goal of our study therefore was to determine if a combined modality approach involving PDT together with targeted inhibition of Hsp-90 could enhance tumoricidal activity in a murine mammary carcinoma.

We examined the tumoricidal effects of 17-AAG with PDT due to the inhibitor's clinical relevance and availability as well as to follow up on our previous observation that 17-AAG increases PDT-mediated apoptosis in a human breast cancer cell line [14,29–31]. 17-AAG interacts directly with the Hsp-90 amino-terminus ATP binding site and this leads to client protein destabilization and ubiquitin labeling followed by proteasome mediated degradation [20,21,29]. Phase I and II clinical trials suggest that 17-AAG is well tolerated and can inhibit Hsp-90 client proteins in a variety of malignancies including those relevant to PDT such as carcinomas of the breast and prostate [30,31]. However, exposure to 17-AAG increases expression of Hsp-70 and up-regulation of this stress protein can induce an anti-apoptotic phenotype within cancer cells and may be detrimental to the anticancer activity of geldanamycin derivatives [18]. A definitive role of Hsp-70 on PDT sensitivity has not been obtained [32–34] but increases in 17-AAG mediated Hsp-70 expression may negatively modify combined modality protocols. In this regard, we administered 17-AAG after PDT in order to take advantage of the rapid decrease in tumor mass following laser irradiation as well as to

minimize any effects of 17-AAG mediated Hsp-70 expression on PDT responsiveness. Currently there are approximately twenty Hsp-90 inhibitors in various stages of development and this includes studies to identify effective Hsp-90 inhibitors that do not increase Hsp-70 expression [20,21,29]. Shepherdin, a peptidomimetic inhibitor of the survivin-Hsp-90 complex acts upon a different portion of the ATP binding site than 17-AAG, eliminates expression of Hsp-90 client proteins, does not alter HSP-70 expression, and is being tested as an alternative to geldanamycin derivatives [35].

Our results clearly illustrate the potential for targeting multiple signaling pathways and oncogenic molecules via Hsp-90 inhibition during PDT. Western immunoblot analysis confirmed that 17-AAG administered after in vivo PDT effectively blocks client protein expression. Nevertheless, vascular damage and inflammation associated with PDT may alter the pharmacokinetics of 17-AAG localization within tumor tissue. Our 17-AAG administration procedure of thrice weekly injections of 75 mg/kg for two weeks is a less aggressive protocol than other in vivo studies and this may have played a role in why we observed only a doubling of the long-term tumoricidal response (from 25 to 50 percent long-term cures) when PDT was combined with 17-AAG [36]. It will be important to optimize this treatment regimen in order to more thoroughly understand the therapeutic options of this combination.

In summary, our data supports the premise that a functional Hsp-90 chaperone can negatively impact PDT-mediated tumoricidal responses by stabilizing client proteins with prosurvival and angiogenic properties. These observations provide a strong rationale for targeting Hsp-90 as an adjunctive therapy to PDT.

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Figure 1.

Biomarker confirmation of Hsp-90 inhibition; 17-AAG inhibits the expression of Raf-1 and induces the expression of Hsp-70 in BA cells and tumors. Cells were incubated for 24 hr in the presence or absence of 17-AAG (1 μ M). C3H mice with subcutaneous mammary tumors received an ip injection of 17-AAG (75 mg/kg) and tumor tissues were collected 24 hours later. Cell (A) and tumor (B) lysates were analyzed for Raf-1 and Hsp-70 protein expression by Western immunoblot using specific antibodies against each protein. Expression of actin, a non-Hsp-90 client protein, was used as a loading control.

Figure 2. 17-AAG decreases the expression of Hsp-90 client proteins associated with cell growth and proliferation in PDT-treated BA cells. (A) Western immunoblot analysis documenting expression of survivin, phospho-survivin (Thr34), Akt, phospho-Akt (Ser473), Hsp-90, and actin in controls and PDT-treated cells. Cells were incubated with PH (25 µg/ml) in the dark for 16 hr and exposed to red light (210 or 420 J/m²). Cells were then incubated in the dark for an additional 24 hr in the presence or absence of 17-AAG (1 µM). Lysates from control and treated cells were collected and analyzed for protein expression using commercially available antibodies. Actin expression was used to monitor protein loading. Densitometric measurements (averages ± SE) from immunoblots of three separate experiments are shown for Akt (B), phospho-Akt (Ser473) (C), survivin (D), phospho-survivin (Thr34) (E) and Hsp-90 (F).

Figure 3.

17-AAG decreases the expression of Hsp-90 client proteins associated with cell growth and angiogenesis in PDT-treated BA tumors. C3H/HeJ mice with subcutaneously transplanted BA tumors measuring 6–7 mm in diameter were injected iv with PH (5 mg/kg) and then 24 hr later tumors were exposed to a 200 J/cm² dose of 630 nm light. A group of mice received an ip injection of 17-AAG at a dose of 75 mg/kg either alone or immediately after PDT. Tumor lysates were collected 2 hr after treatment. (A) Western immunoblot analysis show expression profiles for HIF-1α, Akt, phospho-Akt (Ser473), survivin, phospho-survivin (Thr34), Hsp-90 and actin using commercially available antibodies. (B) densitometric readings (average ± SE) obtained from the immunoblots of 3 samples.

Figure 4.

17-AAG attenuates the expression of proteins associated with invasion and angiogenesis in PDT-treated BA tumors. C3H/HeJ mice with subcutaneously transplanted BA tumors measuring 6–7 mm in diameter were injected iv with PH (5 mg/kg) and then 24 hr later tumors were exposed to a 200 J/cm² dose of 630 nm light. A group of mice received an ip injection of 17-AAG at the dose of 75 mg/kg either alone or immediately after PDT. Tumor lysates were collected 2 hr after treatment for analysis. (A) SDS-polyacrylamide gelatin zymography documenting the presence of MMP-9 and MMP-2 in treated samples and control. (B) Corresponding densitometric readings of the proteolytic bands associated to MMP-9 and MMP-2 enzymatic activity. (C) Levels of VEGF determined by the Quantikine M mouse ELISA kit. Means $\pm$ SE are plotted. N=3 per group.

Figure 5.

17-AAG enhances the tumoricidal action of PDT. Mice with transplanted BA tumors were entered into treatment groups when lesions reached 6–7 mm in diameter. Test groups included controls, PDT alone, 17-AAG alone, and PDT plus 17-AAG. For PDT, mice received PH (5 mg/kg) by iv injection and 24 hr later tumors were exposed to a 200 J/cm² dose of 630 nm light delivered at 75 mW/cm². 17-AAG (75 mg/kg) was administered by ip injection thrice weekly (M,W,F) for two weeks and the 17-AAG administration was started immediately after the completion of the PDT treatment. Mice were monitored for recurrences three times a week for a 90-days period. Percent cures are plotted as a function of time after treatment. N=12 per group. A statistically significant difference ($p < 0.05$) was observed for PDT plus 17-AAG versus PDT alone.