A rat retinal damage model predicts for potential clinical visual disturbances induced by Hsp90 inhibitors

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A B S T R A C T
In human trials certain heat shock protein 90 (Hsp90) inhibitors, including 17-DMAG and NVP-AUY922, have caused visual disorders indicative of retinal dysfunction; others such as 17-AAG and ganetespib have not. To understand these safety profile differences we evaluated histopathological changes and exposure profiles of four Hsp90 inhibitors, with or without clinical reports of adverse ocular effects, using a rat retinal model. Retinal morphology, Hsp70 expression (a surrogate marker of Hsp90 inhibition), apoptotic induction and pharmacokinetic drug exposure analysis were examined in rats treated with the ansamycins 17-DMAG and 17-AAG, or with the second-generation compounds NVP-AUY922 and ganetespib. Both 17-DMAG and NVP-AUY922 induced strong yet restricted retinal Hsp70 up-regulation and promoted marked photoreceptor cell death 24 h after the final dose. In contrast, neither 17-AAG nor ganetespib elicited photoreceptor injury. When the relationship between drug distribution and photoreceptor degeneration was examined, 17-DMAG and NVP-AUY922 showed substantial retinal accumulation, with high retina/plasma [R/P] ratios and slow elimination rates, such that 51% of 17-DMAG and 65% of NVP-AUY922 present at 30 min post-injection were retained in the retina 6 h post-dose. For 17-AAG and ganetespib, retinal elimination was rapid (90% and 70% of drugs eliminated from the retina at 6 h, respectively) which correlated with lower R/P ratios. These findings indicate that prolonged inhibition of Hsp90 activity in the eye results in photoreceptor cell death. Moreover, the results suggest that the retina/plasma exposure ratio and retinal elimination rate profiles of Hsp90 inhibitors, irrespective of their chemical class, may predict for ocular toxicity potential.

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Introduction

Heat shock protein 90 (Hsp90) is a ubiquitously expressed molecular chaperone required for the post-translational stability of its target substrates, known as client proteins, many of which are critical for cell growth, differentiation and survival (Taipale et al., 2010). In addition to playing an indispensable role in the normal homeostatic maintenance of organs and tissues, it is now recognized that the chaperone functions of Hsp90 can become subverted during tumorigenesis (Whitesell and Lindquist, 2005). In this setting Hsp90 can serve as a biochemical buffer to promote the structural and functional stability of a number of oncogenic signaling proteins causally implicated in human cancers (Trepel et al., 2010; Whitesell and Lindquist, 2005). Note, pharmacologic inhibition of Hsp90 results in destabilization and targeted protosomal destruction of these clients and a unique feature of Hsp90 blockade is that it provides a means to simultaneously inhibit multiple oncogenic pathways. For these reasons, Hsp90 has emerged as an attractive molecular target for the development of novel cancer therapeutics (Banerji, 2009; Kim et al., 2009).

Ocular toxicities are a common side-effect of systemic chemotherapeutic drugs (al-Tweigeri et al., 1996) and have also emerged as an important clinical concern for newer molecularly-targeted agents entering standard oncology practice (Renouf et al., 2012). The first class of targeted Hsp90 compounds characterized was the benzoquinone ansamycins, which include the natural product geldanamycin and its derivatives 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) and 17-AAG (17-allylamino-17-demethoxygeldanamycin) (Taldone et al., 2008). In recent years, an increasing number of synthetic small molecule inhibitors of Hsp90 have also been developed that are based on a diverse variety of chemical scaffolds, including resorcinol, purine and benzamide structures (Biamonte et al., 2010; Taldone et al., 2008). In human clinical trials some Hsp90 inhibitors, including 17-DMAG, have been associated with visual disorders including blurred vision, flashes, delayed light/dark accommodation, night blindness and photophobia (Kummar et al., 2010; Pacey et al., 2011; Samuel et al., 2010; Shapiro et al., 2010). These ocular adverse effects are often reversible though dose-limiting; moreover, the clinical evaluation...
of the second-generation aminobenzamide-based inhibitor, PF-04929113 (SNX-5422), was recently discontinued by Pfizer based on significant drug-related ocular toxicity in both preclinical and clinical studies (Rajan et al., 2011). The molecular mechanisms underlying such visual disturbances remain undefined, although recent animal toxicology studies have suggested that retinal dysfunction linked to photoreceptor degeneration and cell death may be a contributing factor (Rajan et al., 2011).

Interestingly, a number of other Hsp90 inhibitors that have undergone clinical evaluation, including 17-AAG and ganetespib (a second generation resorcinol compound), have not manifested the same degree of visual abnormalities seen for these other compounds (Banerji et al., 2005; Cho et al., 2011; Demetri et al., 2011; Goldman et al., 2010; Ramalingam et al., 2008; Ramanathan et al., 2005, 2007; Solit et al., 2007; Wong et al., 2011). The reasons for the superior ocular safety profile exhibited by 17-AAG and ganetespib are presently unknown. Here, histopathological changes and retinal drug distribution profiles of four Hsp90 inhibitors, with or without reported clinical visual disturbances, were evaluated in a rodent model in order to understand the observed differences in ocular toxicity profile among agents in this class. A characteristic feature of targeted Hsp90 inhibition is the induction of heat shock protein 70 (Hsp70), a related molecular chaperone that plays a key role in the chaperone complex machinery (Mayer and Bukau, 2005). In this regard, Hsp70 upregulation is a commonly used surrogate biomarker for Hsp90 blockade (Whitesell et al., 2003) and was used as a biological readout for Hsp90 inhibition in the rat retina. Results presented in this report suggest that Hsp90 plays a critical role in normal retinal function and suggest that aberrant inhibition of Hsp90 activity in the eye results in structural damage of the retina and photoreceptor cell death. In addition, the data suggest that the retina/plasma exposure ratio and retinal elimination rate profiles of individual Hsp90 inhibitor compounds, irrespective of their chemical class, represent predictive factors for assessing compounds’ potential for ocular damage.

Materials and methods

Hsp90 inhibitors. 17-DMAG and 17-AAG were purchased from LC Laboratories (Woburn, MA) and NVP-AUY922 from Selleck Chemicals (Houston, TX). Ganetespib was synthesized by Synta Pharmaceuticals Corp.

Animals, tissue processing and immunohistochemistry. All in vivo procedures were approved by the Synta Pharmaceuticals Corp. Institutional Animal Care and Use Committee and carried out in strict accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Male Sprague Dawley (SD) and Long Evans rats (220 ± 60 g; Charles River Laboratories, Wilmington, MA) were maintained on a 12 h light–dark cycle. Rats were dosed with either 17–DMAG (20 mg/kg), 17–AAG (80 mg/kg), ganetespib (20 mg/kg), NVP-AUY922 (10 mg/kg) or vehicle (17-DMAG was formulated in 10% DMSO/14% Cremophor RH40/76%D5W) and the other three compounds in DRD (LI-COR, Lincoln, NE).

Histological assessment. Histology slides were reviewed in a blind fashion by two independent investigators followed by a board certified pathologist. TUNEL stained sections were scored semi-quantitatively as follows: negative (−), ≤20 cells/overall section; minimal (±), 21–50 positive cells/overall section; mild (±+), >50 positive cells in less than 30% of overall retinal section; moderate (+++), >50 positive cells in 30–60% of the overall retinal section; severe (++++), >50 positive cells in greater than 60% of the overall retinal section. Quantitative image analysis was performed on the Hsp70 immunostained sections. Three prominently stained retinal regions per rat were randomly selected from the peripheral area, proximal to the optical nerve, and within the intervening region. Images were captured under identical electronic exposure profiles and analyzed using Image-Pro Plus software (Media Cybernetics Inc., Rockville, MD) at 20× magnification, and measured as the percentage of Hsp70 positive and negative areas from the inner segment of the photoreceptor layer to the ganglion cell layer. The average % Hsp70-positive area/retinal cells were calculated and expressed as a fold change (median ± SD) compared to the vehicle group. Statistical significance was set at P < 0.05 using one-way ANOVA.

Retinal morphological evaluation. As an index of photoreceptor cell loss, a quantitative measurement of mean outer nuclear layer (ONL) thickness was performed according to previously published procedures (LaVail and Lawson, 1986; Williams and Howell, 1983). Rats were treated with vehicle or 17–DMAG at 20 mg/kg/day, every other day (q.o.d.) for 2 weeks. This dose schedule provided repeated exposure to the inhibitor, and was identified in pilot studies as the maximally tolerated dose. Twenty-four hours after the final dose, eyes were enucleated, fixed in Modified Davidson’s solution and embedded in paraffin. Retinal tissue was sectioned (4-μm thickness) along the vertical meridian through the optic nerve and stained with H&E. In each of the superior and inferior hemispheres, the ONL thickness was measured quantitatively using Image-Pro Plus software. The ONL thickness of each of the superior and inferior hemispheres was measured in nine sets of three measurements each (total of 27 measurements per hemisphere). Each set was centered on adjacent 500 ± 50 μm segments of the retina, with the first point of measurement taken approximately 170 ± 30 μm from the optic nerve head and subsequent sets located toward the periphery. Comparisons of ONL thickness were made between vehicle and 17–DMAG-treated animals using 6 rats per group.

Western blotting. Retinal tissue (including the retinal pigment epithelium layer) from both eyes was dissected using previously described methodology (Barres et al., 1988) and homogenized in a lysing matrix tube (MP Biosciences, Santa Ana, CA) containing lysis buffer (Cell Signaling Technology). Lysates were clarified by centrifugation and equal amounts of protein resolved by SDS–PAGE before transfer to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Membranes were blocked and then immunoblotted with antibodies directed against Hsp70/72 (C92F3A-5; Enzo Life Sciences, Farmingdale, NY) or GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the antibody–antigen complex was visualized and quantitated using the Odyssey system (LI-COR, Lincoln, NE).

Pharmacokinetic analysis. 17–DMAG (20 mg/kg), 17–AAG (80 mg/kg), ganetespib (20 mg/kg) and NVP-AUY922 (10 mg/kg) were i.v. administered to male SD rats (n = 3). For consistency with the experimental protocol, animals were given two consecutive daily doses of inhibitor prior to PK assessment. Following retro–orbital blood collection, plasma samples were protein precipitated and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Noncompartamental pharmacokinetic analysis was performed using Phoenix WinNonlin ver. 6.3 (Pharsight, Mountain View, CA) for individual animals and mean data reported for the group.

For the determination of plasma and retinal tissue drug concentrations, inhibitors were i.v. administered to rats (n = 3) as two consecutive daily doses and plasma and retina samples were collected at 0.5, 6 and 18 h after the final dose for bioanalysis. To do this, animals were euthanized and retinal samples from each time point were pooled and
processed with the Covaris Cryoprep system (Woburn, MA) followed by homogenization in phosphate buffered saline with an IKA homogenizer. Blood was collected from the vena cava for plasma preparation. Plasma and homogenized retinal samples were then extracted by protein precipitation and analyzed by LC–MS/MS. A Phenomenex Kinetex 2.6 μm C18 (30 × 2.1 mm) column was used with a run time of 3.5 min per sample.

Statistical analysis. Data were expressed as means ± SD. Mean or median values were analyzed using one-way ANOVA with Dunn’s Method for multiple comparisons versus the control groups. P values less than 0.05 were considered statistically significant.

Results

Chemical structure and clinical visual disturbance profile of Hsp90 inhibitors

Four small molecule Hsp90 inhibitors, all of which exhibit competitive binding for the ATP pocket at the N-terminus of Hsp90, were used in this study and their chemical structures are presented in Fig. 1. 17-DMAG and 17-AAG both belong to the ansamycin class. Despite their close structural similarity, visual disturbances have been associated with 17-DMAG, but not 17-AAG, in human clinical trials (Kummar et al., 2010; Pacey et al., 2011). Ganetespib is a novel second generation resorcinolic triazolone compound that is structurally distinct from the first-generation ansamycins and does not cause visual disturbances (Ying et al., 2012). NVP-AUY922 is another resorcinol-based second generation inhibitor of Hsp90 (Eccles et al., 2008) and, in contrast to ganetespib, ocular toxicities have recently emerged as an undesirable side-effect for this compound (Samuel et al., 2010).

17-DMAG induced retinal degeneration involves the photoreceptor outer nuclear layer in rats

Histologically, the retina is composed of multiple layers of neuronal cells. H&E staining of a normal retina from a vehicle-treated Sprague Dawley (SD) rat revealed an orderly structural organization (Fig. 2A), within which the ganglion cell layer (GCL), the photoreceptor inner nuclear layer (INL) and outer nuclear layer (ONL) (containing the rod and cone cells) could be readily delineated. First a repeat-dose experiment using SD rats treated with i.v. administration of 17-DMAG (20 mg/kg) or vehicle (D5W) q.o.d. for two weeks was performed. This dose was determined to be the maximally tolerated dose (MTD) for the drug, above which animal mortality was observed. When retinal morphologies were initially examined by H&E staining no discernible differences were seen in vehicle-treated animals; in contrast, 17-DMAG exposure resulted in marked attenuation of the thickness of the ONL and prominent cellular degenerative changes within this region, as evidenced by extensive nuclear condensation and pyknosis (data not shown). These changes suggested that photoreceptor degeneration resulted as a consequence of exposure to the Hsp90 inhibitor. Therefore the mean ONL thickness in vehicle- and 17-DMAG-treated SD rats was examined as a measure to evaluate the degree of photoreceptor cell loss. The ONL thickness was measured along the entire vertical meridian, and the mean thickness values (n = 6 rats) are plotted in Fig. 2B. Compared to the vehicle control rats, the mean ONL thickness in 17-DMAG treated animals was markedly reduced along the entire length of the retina; by 20–37% within the inferior hemisphere and by 20–62% within the superior hemisphere. Within the more sensitive superior hemisphere, the reductions in thickness were greatest toward the periphery compared to the more central regions (Fig. 2B).

Fig. 2C (upper panel) shows representative H&E images of retinas obtained from vehicle and 17-DMAG treated SD rats, corresponding to segment 9 of the superior hemisphere shown in Fig. 2B. The loss of ONL thickness was readily apparent (reduced from approximately 9–10 rows of nuclei to 3–4 following 17-DMAG treatment), as was a major disorganization and substantial loss of photoreceptor inner and outer segment membranes. TUNEL staining of adjacent tissue sections (Fig. 2C, lower panel) revealed the presence of apoptotic cells, confined to the ONL, consistent with a model of selective photoreceptor cell loss occurring in response to 17-DMAG exposure.

In subsequent experiments, rats were treated with vehicle or 20 mg/kg 17-DMAG for two consecutive days and retinas harvested 24 h following the last dosing. TUNEL staining confirmed that the cellular damage occurring within the ONL was due to apoptotic loss of photoreceptors (Fig. 2D, upper panel). Because SD rats lack a pigmented epithelial cell layer, we performed a comparative study using Long Evans rats to determine whether the presence of this layer conferred any protection from Hsp90 inhibitor-induced cytotoxicity. As shown in Fig. 2D (lower panel), 17-DMAG exposure also resulted in extensive apoptotic induction throughout, and restricted to, the ONL in these animals. The structural damage and widespread cell death observed within the retinal photoreceptor layer underscore the ocular toxicity seen in rats treated with 17-DMAG.

Up-regulation of the inducible heat shock protein 70 (Hsp70) following 17-DMAG exposure accompanies apoptotic induction

Hsp90 is ubiquitously observed throughout all layers of the retina (Dean and Tytell, 2001) and no significant changes in total protein levels were seen by Western blot following 17-DMAG treatment (Supplementary Fig. 1). Therefore levels of the stress protein Hsp70 were examined as a surrogate marker for Hsp90 inhibition, since Hsp70 expression is induced following treatment with Hsp90 inhibitors (Whitesell et al., 2003). As shown in Fig. 3A, Hsp70 was robustly up-regulated following 17-DMAG treatment, displaying strong cytoplasmic staining in the inner segment and synaptic regions of the photoreceptor...
cells. Hsp70 induction was restricted to the photoreceptor and ONL layers — a distribution consistent with targeted Hsp90 inhibition occurring selectively within the cells of this region. Next the kinetics of Hsp70 regulation were determined by Western blot (Fig. 3B). Hsp70 protein levels were maximally induced 24 h following the last 17-DMAG treatment (Day 1), remained elevated for at least 3 days, and then returned toward the baseline level at Day 5. This temporal induction of Hsp70 was confirmed by immunostaining (Fig. 3C, upper panel). Interestingly, when concomitant TUNEL staining was performed we observed almost identical kinetics of apoptotic induction (Fig. 3C, lower panel). The relative levels of Hsp70 protein expression are quantitated in Fig. 3D and apoptotic scores shown in Fig. 3E. Taken together, these data show that 17-DMAG treatment aberrantly inhibits Hsp90 activity in retinal photoreceptor cells and this exposure promotes subsequent cell death.

**Comparison of retinal degeneration in SD rats treated with the Hsp90 inhibitors 17-AAG, ganetespib and NVP-AUY922**

Retinal damage profiles of 17-AAG, ganetespib and NVP-AUY922 were examined. Rats were treated with 2-day consecutive dosing of vehicle (DRD), 17-AAG (80 mg/kg), ganetespib (20 mg/kg) or NVP-AUY922 (10 mg/kg) before retinal harvest 24 h later. The 17-AAG dose used was the maximally tolerated dose (MTD) for this drug in order to provide a direct comparison with 17-DMAG in the rat model system. Continuing development of these two ansamycin Hsp90 inhibitors as cancer therapeutics has largely been halted; however both of the second-generation compounds ganetespib and NVP-AUY922 are currently undergoing clinical evaluation in multiple trials and cancer types. Therefore the doses of ganetespib and NVP-AUY922 used in this study were selected based on equivalency to clinically-relevant human dosing levels. Similar to what was observed for 17-DMAG,
NVP-AUY922 treatment induced extensive photoreceptor cell death. In stark contrast, and consistent with an absence of clinically reported visual changes, neither 17-AAG nor ganetespib produced any detectable apoptotic photoreceptor injury (Fig. 4A, Table 1). As predicted by these findings, only NVP-AUY922 treatment elicited robust up-regulation of Hsp70 within the retinal tissue (Fig. 4B).

Comparative pharmacokinetics

The relationship between drug distribution profiles and photoreceptor degeneration using pharmacokinetic (PK) analyses was examined for each Hsp90 inhibitor. The plasma concentration–time profiles presented in Fig. 5A were obtained after the i.v. administration of each
compound at the above doses in SD rats and the main PK parameters are summarized in Table 2. Collectively the data show that inhibitor differences in standard PK parameters did not correlate with the capacity to induce retinal effects. Notably, the peak concentrations of 17-DMAG and NVP-AUY922, both of which were retinal damage inducers, were substantially lower than those for 17-AAG and ganetespib (Table 2). Indeed, ganetespib showed an almost 25-fold higher Cmax value than 17-DMAG when administered at the same 20 mg/kg dose (Fig. 5A) and, over time, was present at higher circulating concentrations than NVP-AUY922. Reflecting its dosing level (80 mg/kg), 17-AAG showed the highest initial plasma drug concentrations however the short half-life of the compound (T1/2, 2.8 h) promoted rapid clearance from the bloodstream (Fig. 5A). Because the extent of plasma protein binding can influence the activity of a drug we additionally determined the unbound fraction of each compound, listed in Table 2. Interestingly, despite their different potentials for retinal degeneration, the two resorcinolic compounds showed almost identical mean unbound plasma fraction values (2.4% and 2.5% for NVP-AUY922 and ganetespib, respectively) suggesting that the availability of these two potent inhibitory agents for on-target Hsp90 effects was comparable.

Retina/plasma exposure ratio and retinal elimination rate are linked to photoreceptor toxicity induced by Hsp90 inhibitors

A comprehensive evaluation of the SD rat tissue distribution profiles for each Hsp90 inhibitor measured at 0.5, 6 and 18 h post-dosing is

**Table 1**

Comparison of Hsp90 inhibitor ocular toxicity in SD rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosage (mg/kg/day × 2, i.v.)</th>
<th>IHC staining (apoptosis(^a), IHC staining (Hsp70 increase vs. control(^a))</th>
<th>Western blot (Hsp70 increase vs. control(^b))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>19/20 (−), 1/20 (±)</td>
<td>0 ± 9</td>
</tr>
<tr>
<td>17-AAG</td>
<td>80</td>
<td>0/7 (−)</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>Ganetespib</td>
<td>20</td>
<td>0/7 (−)</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>20</td>
<td>9/9 (++) (--)(+++++)</td>
<td>122 ± 24(^*)</td>
</tr>
<tr>
<td>NVP-AUY922</td>
<td>10</td>
<td>6/6 (±)(+++++)</td>
<td>117 ± 7(^*)</td>
</tr>
</tbody>
</table>

\(^a\) Evaluations performed 24 h following final dose.

\(^b\) Histological score for apoptosis (TUNEL staining): (−) negative, (±) minimal, (+) mild, (++) moderate, and (+++) severe.

\(^*\) P < 0.05. Multiple comparisons versus control group (Dunn's Method).

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Fig. 5. Comparative pharmacokinetic analyses. (A) Mean plasma concentration–time profiles after the i.v. administration of Hsp90 inhibitors to SD rats. 17-DMAG, 17-AAG, ganetespib and NVP-AUY922 were dosed at 20 mg/kg, 80 mg/kg, 20 mg/kg and 10 mg/kg, respectively. Data represent mean ± SD (n = 3). (B) Retinal concentration–time profiles after the i.v. administration of Hsp90 inhibitors to SD rats. Retinal samples were collected at 0.5, 6 and 18 h post-dose. Data are presented on a logarithmic base 2 scale. (C) Comparison of Hsp90 inhibitor retina/plasma (R/P) drug concentration ratios and (D) retinal elimination rates over time. Plasma and retinal samples were collected at 0.5 and 6 h post-dose and subject to pharmacokinetic analysis. Data are presented from pooled samples representing 3 animals/time point. The bar graph inset in panel (C) denotes the relative R/P ratios at the initial 0.5 h time point. NVP-AUY922 and 17-DMAG, which produced severe ocular effects, displayed the highest R/P ratios whereas the lower R/P ratios of ganetespib and 17-AAG were associated with less risk of retinal damage. Retinal elimination profiles correlated with the degree of observed toxicity. 17-AAG and ganetespib, both of which showed an absence of adverse ocular effects, were more rapidly eliminated from the retina compared to 17-DMAG and NVP-AUY922.
presented in Table 3. Drug concentrations following i.v. administration of each compound were found to be generally higher in the retina than in the plasma, confirming the ability of each compound to penetrate the blood–retina barrier, however the overall plasma concentration alone did not predict for retinal exposure. When retinal concentrations were plotted as a function of time (Fig. 5B) it was found that treatment with the two second generation compounds resulted in comparable levels of drug at the 0.5 h time point, yet the retinotoxic NVP-AUY922 displayed higher sustained concentrations up to 18 h compared to ganetespib. Similarly, while dosing the ansamycin inhibitors at their respective MTDs resulted in greater overall retinal values initially, 17-DMAG showed comparatively higher tissue retention than 17-AAG over time (Fig. 5B). In order to more directly compare the distribution profiles, we determined the retina/plasma (R/P) drug concentration ratio for each compound (Table 3, Fig. 5C), and this parameter was further used to more directly compare the distribution of each compound to penetrate the retina, accounting for the low R/P ratio. Ganetespib exhibited a similar profile, with 70% retinal elimination of the drug over the same time period. Both 17-DMAG and NVP-AUY922 showed substantially slower rates of elimination from the retina at 6 h (Fig. 5D, Table 3). Even at 18 h, NVP-AUY922 showed only 43% clearance compared to 87% for ganetespib. Taken together, these data suggest that both the degree and duration of Hsp90 inhibitor drug exposure in the retina are linked to ocular toxicity potential.

Table 3
Summary of plasma and retinal drug concentrations following Hsp90 inhibitor treatment.

<table>
<thead>
<tr>
<th>Hsp90 inhibitor</th>
<th>Dose (mg/kg)</th>
<th>Time (h)</th>
<th>Plasma (μM)</th>
<th>Retina (nmol/g)</th>
<th>R/P ratioa</th>
<th>Retinal elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>80</td>
<td>0.5</td>
<td>444</td>
<td>59.8</td>
<td>0.14</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.36</td>
<td>6.18</td>
<td>1.36</td>
<td>2.8</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.52</td>
<td>1.47</td>
<td>18</td>
<td>2.8</td>
<td>98%</td>
</tr>
<tr>
<td>Ganetespib</td>
<td>20</td>
<td>0.5</td>
<td>7.29</td>
<td>7.79</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.03</td>
<td>23.9</td>
<td>2.8</td>
<td>90%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.005</td>
<td>0.99</td>
<td>190</td>
<td>8%</td>
<td>–</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>20</td>
<td>0.5</td>
<td>4.71</td>
<td>32</td>
<td>6.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.32</td>
<td>16.2</td>
<td>51</td>
<td>49%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.29</td>
<td>8.64</td>
<td>30</td>
<td>73%</td>
<td>–</td>
</tr>
<tr>
<td>NVP-AUY922</td>
<td>10</td>
<td>0.5</td>
<td>1.33</td>
<td>5.91</td>
<td>4.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.013</td>
<td>3.85</td>
<td>288</td>
<td>35%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.004</td>
<td>3.36</td>
<td>752</td>
<td>43%</td>
<td>–</td>
</tr>
</tbody>
</table>

Discussion

The advent of molecularly targeted therapies has ushered in a new era for cancer treatment. While these agents are generally considered to be more tumor-selective and hence elicit fewer side effects than traditional chemotherapeutic drugs, toxicities in normal tissues and organs are still commonly observed. Because of their relatively small mass and rich vascular supply, the eyes are particularly susceptible to insult from systemically administered cytotoxic agents (Peponis et al., 2010). In addition, their high metabolic rate and inability to regenerate following damage are also causative factors. Exposure to toxic compounds can modify the physiology of retinal neurons and this in turn can lead to major, and in some cases irreversible, visual sensory loss (Renouf et al., 2012). To date, the pathogenic mechanisms of many retinotoxic drugs have not been well characterized although it is reasonable to suggest that on-target effects resulting from suppression of oncogenic targets and signaling pathways, which also exist within ocular tissues, play a significant role.

In this study, we initially evaluated histopathological changes induced within the retina by four Hsp90 inhibitors, two from the prototypical ansamycin class (17-DMAG and 17-AAG) and two second-generation resorcinolic compounds (ganetespib and NVP-AUY922). Although not included in the present report, ocular adverse events have not been noted in clinical trials of the purine scaffold-based synthetic Hsp90 inhibitor, BIBO21 (Dickson et al., 2013; Eflky et al., 2008). Consistent with their clinical visual symptom profiles, 17-DMAG and NVP-AUY922 each induced marked photoreceptor cell death, but similar effects were not produced by either 17-AAG or ganetespib treatment. Interestingly, the morphologic and structural changes observed in the rat retina (degeneration of the photoreceptor segments and thinning of the ONL) are similar to what has been seen following retinal photic injury (Shahinfar et al., 1991; Voussif et al., 2011), suggesting that pharmacological insult and light damage may manifest similar pathologic outcomes in this tissue. Moreover, the identical retinal damage produced by two structurally distinct Hsp90 inhibitors (17-DMAG and NVP-AUY922) provides further evidence that retinal toxicity induced by this group of agents is a drug target-dependent process.

In rat ocular tissue, Hsp90 is abundantly expressed within the retina (Dean and Tytell, 2001) suggesting that its chaperoning activities play essential roles in normal photoreceptor function. Our findings indicate that prolonged or aberrant inhibition of Hsp90 activity in the retina results in photoreceptor death. Hsp90 controls the folding of numerous key signaling molecules required to maintain normal cell function, including ubiquitously expressed apoptotic regulators such as AKT and its effector molecule glycogen synthase kinase 3 (Sato et al., 2000). Therefore, blockade of pro-survival signals could readily account for the rapid onset of apoptotic loss observed following sustained 17-DMAG or NVP-AUY922 exposure. In addition, the high degree of specialization exhibited by photoreceptor cells predicts for unique chaperone requirements (Kosmaoglou et al., 2008). In this regard, photoreceptor cell-type specific Hsp90 client proteins may represent critical targets whose function may be profoundly impacted by Hsp90 inhibition. For example, aryl hydrocarbon receptor interacting protein-like 1 (AIP1L1) is expressed by cone and rod photoreceptor cells and plays a critical role in cellular viability (Kirschman et al., 2010). Mutations in this gene underlie one of the most severe forms of inherited retinal dystrophy, Leber Congenital Amaurosis (LCA) (den Holland et al., 2008; Sohocki et al., 2000). Notably, AIP1L1 has been shown to interact with Hsp90 (Hidalgo-de-Quintana et al., 2008) and is thought to represent a retina-specific co-chaperone that is required for the assembly of essential photoreceptor enzymes including the phototransduction protein, phosphodiesterase (Kirschman et al., 2010; Kosmaoglou et al., 2008). Studies investigating the precise client proteins and signal cascades that are destabilized following inhibition of Hsp90 in this tissue are underway. It is also interesting to note that, despite multiple reports of Hsp90 inhibitors inducing cytotoxic effects in cultured retinal
pigment epithelium (RPE) cells in vitro (Kaarniranta et al., 2005; Wu et al., 2010; Yao et al., 2010), only low endogenous levels of Hsp90 protein are found in these cells in situ (Dean and Tytell, 2001) and we observed no apoptotic changes in the SD rat RPE following Hsp90 inhibitor treatment.

Importantly, this study provides the first evidence of a mechanistic basis for different ocular toxicity potentials exhibited by small molecule inhibitors of Hsp90. The similar toxicity profiles and biological responses elicited by 17-DMAG and NVP-AUY922 indicated that it was not simply chemical class that dictated the capacity to induce retinal degeneration. Indeed the lack of retinal damage seen with 17-AAG and ganetespib, also in accordance with their clinical safety profiles, supported this premise. Among the compounds studied, 17-DMAG and NVP-AUY922 contain salt-forming amine groups and are more hydrophilic than either 17-AAG or ganetespib, though it is unlikely that this pharmaceutical property alone is sufficient to account for the different toxicities seen within this group of therapeutic agents. Instead, our pharmacokinetic examination of the relationship between retinal drug distribution profiles and photoreceptor degeneration showed that the retention characteristics of each agent appeared to be a predictive factor for retinal damage. All four compounds showed distribution into the retinal tissue; however the potential for photoreceptor damage was related to the two interconnected parameters of retina/plasma ratio and retinal elimination rate. Indeed, the higher initial R/P ratios for 17-DMAG and NVP-AUY922 indicated that both compounds distributed more readily to the retinal compartment compared to the non-retinotoxic inhibitors and actual drug concentrations of each were sustained at higher levels over time. In addition, these characteristics were sufficient to account for the robust upregulation of Hsp70 seen 1 to 3 days following 17-DMAG exposure. Hsp70 induction, while a useful marker of Hsp90 inhibition, also reflects the activation of an evolutionarily conserved cellular stress response wherein this chaperone may exert cytoprotective activity and play a central role in the triage of damaged proteins following proteotoxic stress (Lanneau et al., 2010). Overall, while all inhibitors successfully penetrated the blood–retina barrier, the degree and duration of retinal exposure differed even among compounds of the same class, and it was these characteristics that determined the extent of photoreceptor injury.

Studies of ocular drug reactions are best performed in species for which the information can be most relevantly applied to the clinical setting. The SD rat is commonly used for regulatory toxicity testing and the retinotoxic responses to individual Hsp90 inhibitors observed in this strain reflected the safety profiles seen in patients. However since these albino animals lack melanin in the RPE or the uveal tract, an important consideration for this study was whether pigmentation of the eye could affect the outcome of Hsp90 inhibitor exposure, given that a large number of structurally and pharmacologically unrelated drugs show significant affinity for binding to melanin. In this regard, while drug binding to melanin itself is not predictive of retinal toxicity (Leblanc et al., 1998), the presence of this biological pigment may impact local drug concentrations by promoting accumulation within the retinal compartment. As shown in Fig. 2, the pigmented LE rat strain remained susceptible to 17-DMAG-induced retinal damage — which was similarly restricted to the photoreceptor layer. Moreover, additional tissue distribution studies performed in LE rats also demonstrated higher R/P ratios for the retinotoxic 17-DMAG and NVP-AUY922 inhibitors compared to ganetespib and 17-AAG (D. Zhou, unpublished observations). These data are consistent with the observations found in the SD rat and suggest that the presence of melanin in the pigment epithelium unlikely plays a major role in determining the relative retention kinetics of these four compounds within the rat eye.

Finally, our findings validate the use of a rodent model to assess risks of visual disturbances and retinal dysfunction induced by targeted Hsp90 inhibition. Evaluation of retinal pathology, heat shock protein modulation and profiles of retinal drug exposure in rats represented a relatively straightforward, sensitive and robust approach to detect retinal cell death within 24 h after drug administration. This methodology provides a practical advantage over more sophisticated experimental approaches, such as electrotetroretinography (ERG), which requires specialized equipment to assess clinical visual changes that take considerably longer times to manifest and monitor. Perhaps most significantly, our results correlate with the adverse event profile in humans for each of the agents examined, and suggest that this strategy may predict the potential for retinal damage when applied to pre-clinical screening of candidate Hsp90 inhibitor compounds. Despite some species-related differences (e.g. types and density of cone cells) rat and human eyes share the same basic structure and function and rodent models have traditionally proven useful for the investigation of ocular disease pathogenesis, response to therapies, and toxicology screening. It is important to note, however, that many of the visual disorders seen in patients are reversible — therefore the photoreceptor death observed in the rat retina likely does not account for all pathological processes occurring in the human eye as a consequence of aberrant Hsp90 inhibition.

In summary, Hsp90 client proteins play important roles in normal retinal function and prolonged Hsp90 inhibition can lead to vision disorders such as those that have been seen in the clinical setting. Our findings reveal that the drug retinal/plasma exposure ratio and elimination rate profiles play crucial roles in ocular toxicity and may be used as indicators of Hsp90 inhibitor-induced damage in rats. Importantly, the retinotoxicity potential of each of the Hsp90 inhibitors defined in the rodent model was entirely consistent with their observed clinical profile in humans. Based on these findings, it appears that adverse ocular effects may be successfully minimized by administration of Hsp90 inhibitors with favorable drug properties that include, although are not necessarily limited to, lower retinal/plasma exposure ratios and faster retinal elimination. In addition, improved understanding of pathophysiological mechanisms that result in retinal damage will have important implications for appropriate monitoring protocols designed to prevent visual impairment as a result of targeted Hsp90 therapies.

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Conflict of interest statement

All authors with the exception of Qin Huang are current or former employees of Synta Pharmaceuticals Corporation.

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