Evidence for Efficacy of New Hsp90 Inhibitors Revealed by Ex Vivo Culture of Human Prostate Tumors

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Abstract

Purpose: Targeting Hsp90 has significant potential as a treatment for prostate cancer, but prototypical agents such as 17-allylamino-17 demethoxygeldanamycin (17-AAG) have been ineffective in clinical trials. Recently, a phase I study aimed at defining a biologically active dose reported the first response to an Hsp90 inhibitor in a patient with prostate cancer, which supports the development of new generation compounds for this disease.

Experimental Design: The biological actions of two new synthetic Hsp90 inhibitors, NVP-AUY922 and NVP-HSP990, were evaluated in the prostate cancer cell lines PC-3, LNCaP, and VCaP and in an ex vivo culture model of human prostate cancer.

Results: In cell lines, both NVP-AUY922 and NVP-HSP990 showed greater potency than 17-AAG with regard to modulation of Hsp90 client proteins, inhibition of proliferation, and induction of apoptotic cell death. In prostate tumors obtained from radical prostatectomy that were cultured ex vivo, treatment with 500 nmol/L of NVP-AUY922, NVP-HSP990, or 17-AAG caused equivalent target modulation, determined by the pharmacodynamic marker Hsp70, but only NVP-AUY922 and NVP-HSP990 showed antiproliferative and proapoptotic activity.

Conclusions: This study provides some of the first evidence that new generation Hsp90 inhibitors are capable of achieving biologic responses in human prostate tumors, with both NVP-AUY922 and NVP-HSP990 showing potent on-target efficacy. Importantly, the ex vivo culture technique has provided information on Hsp90 inhibitor action not previously observed in cell lines or animal models. This approach, therefore, has the potential to enable more rational selection of therapeutic agents and biomarkers of response for clinical trials. Clin Cancer Res; 18(13): 3562–70. ©2012 AACR.

Introduction

The molecular chaperone Hsp90 is responsible for folding and stabilizing client proteins into their active conformation. Hsp90 has more than 200 clients, including many oncproteins (1), thus pharmacologic inhibition of Hsp90 in cancer cells has the potential to simultaneously target multiple signaling pathways implicated in cancer progression. Targeting Hsp90 in prostate cancer is a particularly attractive therapeutic strategy as Hsp90 is commonly overexpressed in prostate tumor cells compared with normal epithelium (2). Moreover, the androgen receptor, the key driver of prostate cancer progression (3), is an Hsp90 client protein.

Given the promising preclinical studies of Hsp90 inhibition in prostate cancer cells (4–6), the lack of efficacy observed for these agents in prostate cancer clinical trials has been disappointing (7–9). The ansamycin derivatives 17-allylamino-17 demethoxygeldanamycin (17-AAG) and 17-(dimethylaminoethyl-amino)-17-demethoxygeldanamycin (17-DMAG) are the most extensively characterized Hsp90 inhibitors. Their clinical failure has been attributed to poor solubility and pharmacokinetics, hepatotoxicity and susceptibility to MDR (10). However, the first response to an Hsp90 inhibitor in a patient with castration-resistant prostate cancer (CRPC) was recently reported in a phase I trial of 17-DMAG that aimed to define a biologically effective dose rather than maximum tolerated dose (MTD; ref. 11). This encouraging result supports the development
Efficacy of New Hsp90 Inhibitors in Prostate Tumors

Translational Relevance

The study uses a unique application of ex vivo human prostate cancer tissue culture to provide the first evidence that the new generation Hsp90 inhibitors NVP-AUY922 and NVP-HSP990 have significant potential as anticancer agents for men with prostate cancer. We also show that induction of Hsp70, the widely used pharmacodynamic marker for this class of drugs, is not associated with biologic response to Hsp90 inhibition. Clinically, the ex vivo culture model used in this study has the potential to improve preclinical development of novel agents by assessing drug actions in the context of the normal tumor microenvironment. This approach will facilitate more rational selection of bioactive agents and the identification of more robust markers of clinical responses.

of new generation inhibitors with improved pharmacokinetics but also suggests that evaluating these agents at the level of the tumor, rather than by MTD, may be essential for this class of agent (12).

The chaperone activity of Hsp90 is ATP dependent (13). Most Hsp90 inhibitors bind to the conserved ATP pocket of Hsp90, preventing maturation of client proteins and causing their proteasomal degradation. NVP-AUY922 (hereafter called AUY922) and NVP-HSP990 (HSP990) likewise bind to the ATP-binding pocket of Hsp90 and represent 2 of the most potent synthetic small molecule inhibitors reported to date (14, 15). AUY922 is a resorcylic isoxazole amide with antitumor activity against a range of cancer cell lines and animal models, including PC-3 prostate cancer cells and xenografts (14, 16–18), and in a phase I study in patients with advanced solid malignancies, disease stabilization (16%) was observed (19). HSP990 is an orally available aminopyrimidine with antiproliferative activity in a variety of cancer cell lines and xenografts (15, 20) and is currently in phase I clinical trials (www.clinicaltrials.gov). Unlike 17-AAG, AUY922 and HSP990 are not susceptible to MDR mechanisms such as P-glycoprotein–mediated efflux or polymorphic metabolism by the enzyme NQO1/DT-diaphorase (14, 15).

The objective of this study was to compare the potential of AUY922 and HSP990 with 17-AAG to modulate Hsp90 client proteins and influence proliferation and death of prostate cancer cells, in vitro, and in ex vivo cultures of human primary tumors.

Materials and Methods

Cells and reagents

LNCaP, VCaP, and PC-3 human prostate carcinoma cells were obtained from the American Type Culture Collection. All cell lines underwent verification by short-tandem repeat profiling in 2010 by Cell Bank Australia. LNCaP and PC-3 cells were maintained in RPMI-1640 containing 10% or 5% FBS, respectively. VCaP cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% FBS, 1% sodium pyruvate, 1% MEM non–essential amino acids, and 0.1 mmol/L 5α-dihydrotestosterone. AUY922 and HSP990 were obtained from Novartis and dissolved in dimethyl sulfoxide (DMSO). 17-AAG was obtained from the National Cancer Institute and dissolved in DMSO. Primary antibodies are detailed in Supplementary Table S1.

Cell viability

Trypan blue exclusion and 4', 6-Diamidino-2-phenylindole (DAPI) staining were carried out as described previously (21).

Cell cycle

Flow cytometric analysis of cell-cycle distribution was carried out on cells treated with vehicle (DMSO) or Hsp90 inhibitors, with or without the pan-caspase inhibitor zVAD-fmk (20 μmol/L; BD Biosciences), as described previously (21). Analysis was done on a FACSCalibur (BD Biosciences) running CellQuest software (BD Biosciences). DNA frequency histograms were obtained using FlowJo software, using the Dean-Jett-Fox model.

Ex vivo culture of human prostate tumors

Prostate cancer tissue was obtained with written informed consent through the Australian Prostate Cancer BioResource from men undergoing robotic radical prostatectomy at the Royal Adelaide Hospital. Histopathologic features of tumors used in this study are detailed in Table 1. An 8-mm core of tissue was dissected into 1-mm cubic pieces and cultured in duplicate on a precoated gelatin sponge (Johnson and Johnson) in 24-well plates containing 500 μL RPMI-1640 with 10% FBS, antibiotic/antimycotic solution, 0.01 mg/mL hydrocortisone and 0.01 mg/mL insulin (Sigma). Tissue were cultured at 37°C for 48 hours, then formalin-fixed and paraffin embedded or preserved in RNAlater (Invitrogen).

Protein preparation

Whole-cell lysates were prepared as described previously (21). Cultured prostate tumors stored in RNAlater were lysed in ice-cold radioimmunoprecipitation assay buffer (10 mmol/L Tris-Cl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100) containing protease inhibitor cocktail (Sigma), using the Pecellys24 tissue homogenizer (Bertin Technologies).

Immunoblotting

Proteins (20 μg) were analyzed by immunoblotting as described previously (21). Densitometry was carried out using the Fluorchem SP Digital Imaging System (Alpha Innotech Corporation).

Immunohistochemistry

Sections (2 μm) on SuperFrost-plus slides underwent immunohistochemical staining as detailed in Supplementary Table S2. Images were captured with a Nanozoomer scanner. The percentage of Ki67 and cleaved caspase-3–positive nuclei were determined by blind
manual counting of at least 200 malignant cells over 5 to 10 fields at x40 magnification. Due to variation in staining, areas of highest density staining were selected for counting. Samples were removed from analysis only if there were insufficient malignant cells present, or if the Ki67 positivity in the DMSO-treated sample was 2% or less. On the basis of these criteria, 6 of 19 patient samples were removed due to lack of malignancy and 3 of 19 due to Ki67 levels. All remaining tissues were included for statistical analysis.

**Statistical analysis**

Data are displayed as the mean ± SE. Differences were determined using one-way ANOVA with Tukey or Dunnett post hoc test as indicated, using GraphPad Prism Software. Repeated measures ANOVA were used where stated. A P value 0.05 or less was considered statistically significant.

**Results**

**Enhanced potency of AUY922 and HSP990 compared with 17-AAG in human prostate cancer cell lines**

The efficacy of the synthetic Hsp90 inhibitors, AUY922 and HSP990, was assessed in androgen receptor–negative (PC-3) and androgen receptor–positive (LNCaP, VCaP) prostate cancer cell lines and compared with that of 17-AAG. In LNCaP cells, AUY922 caused a significant, dose-dependent inhibition of cell proliferation (P < 0.001) compared with vehicle, with maximal inhibition achieved at approximately 20 nmol/L (Fig. 1A; Supplementary Fig. S1). HSP990 and 17-AAG had lower antiproliferative activity than AUY922 at equivalent concentrations. VCaP was the least sensitive cell line, and PC-3 cells were markedly more sensitive than LNCaP cells with respect to inhibition of proliferation by HSP990 and AUY922; 17-AAG displayed the least efficacy in all cell lines (Fig. 1A). Both AUY922 and HSP990 (10–40 nmol/L) induced a dose-dependent increase in death of all 3 cell lines (P < 0.001; Fig. 1B). 17-AAG did not induce death in any of the cell lines with the doses used (10–80 nmol/L; Fig. 1B).

**AUY922 and HSP990 induce G2–M cell-cycle arrest and apoptosis**

Culture of PC-3 and LNCaP cells with AUY922 or HSP990 (40 nmol/L) caused accumulation of cells in phase G2–M of the cell cycle (Fig. 2A and B). No cell-cycle changes were observed for cells cultured with 17-AAG (Supplementary Fig. S2). AUY922 and HSP990 induced a significant
increase in the sub-G₁ peak from 3% in control cells to 27% and 24%, respectively, in PC-3 cells treated for 72 hours (P < 0.001, Fig. 2A and C), indicative of induction of apoptosis by these agents. In LNCaP cells, significant accumulation of cells in sub-G₁ was evident after 96 hours of treatment with AUY922 (13%) or HSP990 (7%) compared with vehicle (3%, P < 0.05, Fig. 2A and C). Consistent with their antiproliferative efficacy, both inhibitors induced a greater magnitude of change in PC-3 cells compared with LNCaP cells. Coculture of PC-3 cells with the pan-caspase inhibitor zVAD-fmk completely prevented the observed increase in sub-G₁ fraction in AUY922- and HSP990-treated cells and caused a concomitant increase in cells in the G₂–M phase (P < 0.001, Fig. 2A and C). In LNCaP cells, zVAD-fmk prevented cells from entering sub-G₁ in cells treated with AUY922 (P < 0.001) but not HSP990 (Fig. 2B and C).

DAPI staining showed that PC-3 or LNCaP cells cultured for 24 hours with AUY922 or HSP990 (40 nmol/L) exhibited chromatin condensation (Fig. 2D), which is characteristic of apoptosis. As above, PC-3 cells showed the greatest response to treatment with AUY922 (32.6%) or HSP990 (23%) when compared with vehicle...
AUY922 and HSP990 modulate Hsp90 client proteins in prostate cancer cell lines

Induction of Hsp70 and degradation of Cdk4 and c-Raf are common clinical markers of Hsp90 inhibition (7, 22). Induction of Hsp70 and a concomitant reduction in c-Raf, Cdk4, Her2, and Akt were observed in PC-3 and LNCaP cells treated with 40 nmol/L AUY922 and HSP990 compared with control, but AUY922 was consistently more potent than HSP990 (Fig. 3A and B). Marked depletion of androgen receptor and prostate-specific antigen (PSA), an androgen receptor–regulated protein, was observed following treatment of LNCaP cells with both inhibitors (Fig. 3B). Modulation of client proteins was absent or modest in cells treated with 40 nmol/L 17-AAG, a concentration that was maximally effective for AUY922 and HSP990. No change in Hsp90 expression was observed with any treatment in either cell line (Fig. 3A and B).

AUY922, HSP990, and 17-AAG induce Hsp70 in human prostate tumors ex vivo

To assess the activity of the synthetic Hsp90 inhibitors in clinical prostate cancer, we used an ex vivo methodology to culture human prostate tumors (Fig. 4A). Initially we assessed whether the Hsp90 inhibitors could induce Hsp70 expression in the human prostate tumors. Matched tissues from the same patients were cultured in medium with increasing concentrations of an Hsp90 inhibitor. A dose-dependent increase in Hsp70 levels was observed in tumors cultured with 17-AAG, AUY922, or HSP990 (at 100, 500, or 1,000 nmol/L) compared with control (Fig. 4B).

On the basis of these initial studies, the ability of each Hsp90 inhibitor to induce Hsp70 and decrease levels of 2 Hsp90 client proteins with relevance to prostate cancer (androgen receptor and Akt) was directly compared at a single, effective dose (500 nmol/L) in 8 independent tumors. Compared with control, Hsp70 expression was significantly increased in tumors treated with each inhibitor ($P < 0.01$; Fig. 4C), and androgen receptor expression was significantly decreased ($P < 0.01$; Fig. 4C). A similar reduction in Akt levels was also observed in tumors cultured with the inhibitors (Fig. 4C).

As immunoblotting was carried out on whole tissue lysates, immunohistochemistry was used to validate the observed induction of Hsp70 in tumor cells. Significant Hsp70 induction was seen in the tumor cells of human prostate tissues treated with 17-AAG, AUY922, or HSP990 when compared with vehicle control ($P < 0.05$, Fig. 5A).

Antiproliferative and proapoptotic activity of AUY922 and HSP990, but not 17-AAG, in human prostate tumors

We next assessed whether modulation of Hsp70 in the human prostate tissues was associated with altered biologic endpoints such as proliferation or apoptosis. When we assessed Ki67 positivity in the same tumor tissues assessed above for Hsp70, only 1 of 7 tumors cultured with 17-AAG at a dose (500 nmol/L) that was effective in modulating levels of Hsp70 (Fig. 5B) elicited a decrease in proliferation compared with control tissues. Overall, there was no significant change in Ki67 positivity between 17-AAG-treated (32.3%) and control tissues (32.5%; Fig. 5B). In contrast, Ki67 positivity was markedly and consistently reduced to 1.8% and 0.5% in tissues cultured with 500 nmol/L AUY922 or HSP990, respectively ($P < 0.001$; Fig. 5B).
Tissues were then assessed for induction of cell death by immunohistochemical detection of cleaved caspase-3. Consistent with the proliferative data, we observed low levels of cleaved caspase-3 positivity in control (8.7%) and 17-AAG–cultured tissues (7.5%), whereas both AUY922 (22%) and HSP990 (23%) significantly increased the number of positive cells ($P < 0.01$; Fig. 5C), indicating that these agents induce apoptotic death of human prostate cancer cells.

Extended dose-ranging studies (100–1,000 nmol/L), carried out for each agent on a subset of tumors in which there was sufficient tissue available, further showed the enhanced potency of AUY922 and HSP990 compared with 17-AAG (Supplementary Fig. S4).

Discussion

In this study, we report that the new synthetic Hsp90 inhibitors AUY922 and HSP990 are potent anticancer agents using human prostate cancer cell lines and ex vivo cultured human prostate tumors. Our findings are consistent with recent preclinical studies showing superior antitumor activity of other novel Hsp90 inhibitors, such as the nonquinone PF-04928473 and the mitochondria-targeted gamitrinibs, compared with 17-AAG in cell lines and animal models of prostate cancer (23–25), and highlight the potential of new generation inhibitors. Importantly, our findings using the human ex vivo approach provides the first evidence that these new agents are capable of achieving significant biologic responses in human prostate tumors and indicate that this class of agent may well achieve their full potential as a new therapeutic for prostate cancer.

Although both AUY922 and HSP990 displayed greater potency than 17-AAG in vitro, it is clear from earlier studies of 17-AAG that preclinical efficacy does not necessarily equate to clinical response. This may reflect the limitations of the current prostate cancer models, which do not take into account the tumor microenvironment or heterogeneous nature of the disease. In addition, most cell lines and xenograft models typically represent only late-stage disease. Consistent with clinical trial results

Figure 4. Hsp90 inhibitors induce Hsp70 expression in ex vivo cultured human prostate tumors. A, ex vivo tissue culture protocol. Within 60 minutes of being removed by robotic radical prostatectomy, human prostate tumor samples were obtained and dissected into 1-mm$^3$ pieces that were cultured on gelatin sponges inside wells containing medium. B, tumors cultured with medium containing increasing doses of 17-AAG, AUY922, or HSP990 for 48 hours were analyzed by immunoblotting for levels of Hsp70. GAPDH was used as a loading control. Graph represents densitometry analysis of immunoblots in which levels of Hsp70 are normalized to GAPDH. C, densitometry analysis of immunoblots from tissues cultured with vehicle control (C), 500 nmol/L 17-AAG (17), AUY922 (A) or HSP990 (H) for 48 hours in which levels of Hsp70, androgen receptor, or Akt have been normalized to GAPDH. Representative immunoblots are shown below each graph. Repeated measures ANOVA with Dunnett post hoc test: $^* P < 0.05$ treatment versus control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
In addition, our results not only support the view that modulation of Hsp70 is not a clinically informative biomarker of biologic response in tumors (9, 27) but also indicates that despite the prosurvival actions of Hsp70 (28), its induction does not preclude an antiproliferative response in human tumors. Although induction of Hsp70 has been widely used to monitor efficacy of Hsp90 inhibitors in clinical trials (7, 22), mainly in peripheral blood mononuclear cells (PBMC), increasing evidence suggests that Hsp70 induction is not sufficient to predict clinical activity of these agents. For example, in clinical trials of 17-DMAG in patients with advanced malignancies, Hsp70 levels measured in PBMCs showed no correlation with clinical response (9, 27). Measuring Hsp70 in serum, rather than PBMCs (22), detection of the Her-2 extracellular domain or IGFBP-2 (29) or use of FDG-PET imaging (19), have been suggested as possible alternatives, but our findings strongly argue that evaluation of functional endpoints such as proliferative response in human tumor tissues may allow more accurate monitoring of drug efficacy and be a preferable discovery platform to identify new biomarkers of response.

The ultimate use of Hsp90 inhibitors as prostate cancer therapy will depend on identifying agents that have clinically acceptable pharmacologic and toxicologic profiles and are effective in human prostate tumors. This study shows that ex vivo culture techniques can provide important information on drug efficacy in human tissues that is not possible with cell lines or animal models. The incorporation of this method into future studies may enable more robust preclinical development of novel agents, by providing a better approach for the prediction of drug effects and drug targeting that allows for more rational selection of bioactive agents from a library of compounds, and the identification of more relevant and robust markers of drug response.

### Table 1. Pathologic characteristics of tumors used in this study

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Patient age (y)</th>
<th>PSA (ng/mL)</th>
<th>Gleason grade</th>
<th>Pathologic stage</th>
<th>AR status (%)</th>
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<tr>
<td>28</td>
<td>63</td>
<td>14</td>
<td>3+4</td>
<td>pT3a</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>31</td>
<td>51</td>
<td>3</td>
<td>3+4</td>
<td>pT3a</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>32</td>
<td>70</td>
<td>6</td>
<td>3+4</td>
<td>pT3c</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>34</td>
<td>48</td>
<td>10</td>
<td>3+4</td>
<td>pT3a</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>35</td>
<td>71</td>
<td>8.3</td>
<td>4+3</td>
<td>pT3a</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>36</td>
<td>49</td>
<td>6</td>
<td>3+4</td>
<td>pT2c</td>
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</tr>
<tr>
<td>39</td>
<td>50</td>
<td>6</td>
<td>3+4</td>
<td>pT3a</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>40</td>
<td>70</td>
<td>15</td>
<td>3+4</td>
<td>pT2c</td>
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</tr>
<tr>
<td>41</td>
<td>65</td>
<td>10</td>
<td>3+4</td>
<td>pT2c</td>
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<td>68</td>
<td>3.8</td>
<td>3+4</td>
<td>pT3a</td>
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NOTE: All tumors are adenocarcinoma of acinar type and pathologic stages are as per AJCC TNM 7th Edition.
Abbreviation: AR, androgen receptor.
Disclosure of Potential Conflicts of Interest

H.I. Scher is a paid consultant for Novartis. G.V. Raj is a recipient of honoraria from Speakers Bureau of Amgen and is a consultant/advisory board member of Johnson and Johnson. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Ms. Lauren Giorgio and Ms. Shelley Hedwards for assistance with immunohistochemistry, Ms. Raima Amin for carrying out video image analysis, Ms. Margaret McGee for assistance with statistical analysis, and Dr. Luke Selth for critical reading of the manuscript and also thank the study participants, the urologists, nurses, histopathologists, and the South Australian Coordinators of the APCC, Ms. Helen Hughes and Ms Pamela Saunders, who kindly assisted in the recruitment and collection of patient material and information.

Grant Support

This work was supported by grants from the Prostate Cancer Foundation of Australia/Cancer Australia (ID 627229 to L.M. Butler, W.D. Tilley, and H.I. Scher), the Royal Adelaide Hospital Research Committee (to L.M. Butler and M.M. Centenera), the National Health and Medical Research Council (to M.M. Centenera), and the Prostate Cancer Foundation of Australia (to L.M. Butler).
Research Council of Australia (ID 627185) to W.D. Tilley, L.M. Butler and H.I. Scher. The Adelaide Prostate Cancer Research Centre is supported by an establishment grant from the Prostate Cancer Foundation of Australia (ID 2011/0452). The Australian Prostate Cancer BioResource is supported by the National Health and Medical Research Council of Australia (enabling grants 290456 and 614296) and Prostate Cancer Foundation of Australia. L.M. Butler holds a senior research fellowship from the Cancer Council of South Australia.

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Received March 6, 2012; revised April 26, 2012; accepted May 1, 2012; published OnlineFirst May 9, 2012.

References

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