A Pivotal Role for Heat Shock Protein 90 in Ewing Sarcoma Resistance to Anti-Insulin-like Growth Factor 1 Receptor Treatment: In vitro and In vivo Study

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Abstract

Ewing Sarcoma (ES) shows several deregulated autocrine loops mediating cell survival and proliferation. Therefore, their blockade is a promising therapeutic approach. We previously reported the in vitro effect of insulin-like growth factor 1 receptor (IGF1R)/KIT pathway blockade on ES cell lines, and now we extend our observations to changes induced by this treatment in interacting proteins/networks. A proteomic analysis revealed that Heat Shock Protein (HSP90) was differentially expressed between ES cell lines sensitive and resistant to specific IGF1R/KIT inhibitors. We therefore inhibited HSP90 with 17-allylamino-17-demethoxygeldanamycin (17-AAG) and siRNA, and observed that ES cell line growth and survival were reduced, especially in the resistant cell lines. Conversely, HSP90 induced–expression conferred resistance to anti-IGF1R/KIT treatment in the sensitive cell lines. 17-AAG treatment induced HSP90 client protein degradation, including AKT, KIT, or IGF1R, by inhibiting their physical interaction with HSP90. Xenograft models developed with A673 ES cell line confirmed that HSP90 inhibition, alone or combined with IGF1R inhibition, significantly reduced tumor growth and expression of client proteins. Remarkably, using two independent clinical sample sets, we have found that nearly half of IGF1R-positive tumors also show HSP90 overexpression. This delineates a subset of patients that could benefit from combination of anti-HSP90 agents when considering IGF1R-targeting therapies. Importantly, sensitivity to drugs such as ADW/IMA depends not only on the levels of expression and basal activation of IGF1R/KIT, but also, and for the first time reported in ES, on the development of the stress response mechanism. Accordingly, HSP90 expression could be a predictive factor of response to IGF1R-targeting therapies.

Introduction

Ewing Sarcoma (ES) is a neoplasm affecting children and young adults, in which 5-year survival rates remain around 70% (1, 2). ES expresses several deregulated autocrine loops that contribute to its pathogenesis. Insulin-like growth factor 1 receptor (IGF1R) and KIT are transmembrane receptors that mediate two of these loops (3–13). In vitro studies showed promising results on inhibiting IGF1R, and, arguably, KIT, (5, 9, 12–14), suggesting possible uses in clinical therapy.

Drug sensitivity depends on several molecular mechanisms, including response to stress. Key molecules involved in response to stress are Heat Shock Proteins (HSP). These are chaperone proteins that help to maintain protein stability, renature unfolded proteins, or target their degradation (15–17). Several of their client proteins are involved in signal transduction pathways that lead to proliferation, apoptosis, or cell cycle progression in several cancers (18, 19), and therefore, HSP inhibition became a new therapeutic strategy to inhibit multiple receptor pathways. Stress-protective HSP are often overexpressed in neoplastic tissues and cancer cell lines (18–22). In addition to their protein refolding activity, they are involved in apoptosis regulation, cell growth regulation, and anticancer drug resistance, and a correlation between elevated HSP levels and cancer progression has been shown (19). Among HSP, HSP90 is one of the major chaperones whose expression is increased in stress situations, having AKT, HIF, p53, ErbB2, or Raf-1 as client proteins (20–22). Currently, several specific HSP90 inhibitors are available, such as Geldanamycin or its derivate the 17-allylamino-17-demethoxygeldanamycin (17-AAG), the last one already being used in clinical trials with good results (23–27).

Based on our previous results (14), we performed a proteomic analysis of ES cell lines under drug blockade of IGF1R and/or KIT pathways to further study the following: (a) sensitivity differences to IGF1R/KIT inhibition among ES cell lines, (b) affected networks at the proteome level, and (c) to find and validate new possible therapy targets. Our results showed that HSP90 was the protein showing consistently the highest expression changes, raising the possibility that targeting HSP90 might be of therapeutic value in ES, especially in cases of previous resistance to IGF1R/KIT pathway blockade.

Materials and Methods

Cell lines. A673, TC-71, 5K-ES-1, and A4573 cell lines were obtained and maintained as previously described (14). TTC466 cell line (kindly given by Dr. Jaime Mora, Sant Joan de Déu Hospital, Barcelona, Spain) was cultured in RPMI (Life Technologies, Invitrogen) and supplemented with 10% fetal bovine serum. All cells were free of Mycoplasma, as screened by VenorGeM kit (Minerva Biolabs). These cell lines have different genetic background with respect to translocation type. The A673 and TC-71 cell lines has a EWS-FLI type-1 fusion, SK-ES-1 has a EWS-FLI1 type 2 fusion, A4573 has a
EWS-FLI type 3 fusion, and TTC-66 has a EWS-ERG fusion, as previously reported (28–31).

**Clinical samples.** Two sets of clinical samples collected before treatment were used. The first one, including 23 samples from ES \((n = 10)\) and other sarcomas used as controls, belongs to the consultation files of one of the authors (EA). The second one, used to confirm data in an independent set of samples [sections from two tissue arrays (TMA) including samples from 44 ES patients] was provided by other author’s files (C. Poremba). Both sets corresponded to patients with similar clinicopathologic features, belonging to 23 men and 18 women, with an average age of 26 years old \((18 \text{ in the ES cases})\). Fifty percent of the ES cases were localized in the lower extremities \((20.8\% \text{ in the tibia and 16}\% \text{ in the femur})\), 12.5\% in the upper extremities, and 21\% in the pelvis. Metastasis in the lung, bones, or medulla at the moment of diagnosis was detected in 42\% of them. Institutional Review Board approval was obtained in both institutions. Clinical data were not available in 26 cases.

**Drugs.** ADW742(ADW), AEW541(AEW), and Imatinib (Gleevec-Glivec, IMA) were kindly provided by Novartis Pharma AG. 17-AAG was purchased from Alexis Biochemicals. All these compounds were resuspended in DMSO \((10 \text{ mmol/L})\) and aliquoted at the desired working concentrations.

**Two-dimensional electrophoresis.** Cell lines were treated with 0.1 \(\mu\text{mol/L}\) ADW742 and/or 0.1 \(\mu\text{mol/L}\) Imatinib, for 24 and 72 h. Afterwards, cell lysates were obtained by scraping treated cells on ice with 500 \(\mu\text{L}\) of lysis buffer \(1\% \text{ NP}40, 150 \text{ mmol/L NaCl, 50 mmol/L EDTA, 10\% glycerol, 20 mmol/L Tris-HCl (pH 7)}, \text{ protease inhibitor cocktail (Roche), 50 mmol/L Na2 and 2 mmol/L Na2VO4}\). Proteins were precipitated with trichloroacetic acid/acetone and solubilized in a buffer containing 7 \(\text{mol/L urea, 2 mol/L thiourea, 2\% CHAPS, and protease inhibitors.}\)

Total protein \([100 \text{ \mug estimated using the PlusOne Quant kit (GE Healthcare)}\] was loaded in 250 \(\mu\text{L of rehydration solution [8 mol/L urea, 0.5\% (w/v) CHAPS, 0.2\% (w/v) DTT, 0.5\% (v/v) IPG Buffer, and 0.002% bromophenol blue]}\) and then used to reswell a 13-cm IPG strip pH 3-11 (GE Healthcare) for at least 12 h. Isoelectrofocusing was performed in an Etten IPGphor apparatus (GE Healthcare) at 30,000 Vh at 20\°C. Reduction, and alkylation were accomplished following manufacturer’s instructions (Bio-Rad). Second-dimension SDS-PAGE gels \((10\% \text{ or 12}\%)}\) were stained with silver nitrate. Differentially expressed protein spots were trypsinized and processed for MALDI-ToF mass spectrometry analysis. Proteins were identified by matching their peptide-mass fingerprinting with those available in 26 cases.

**Immunohistochemical analysis.** Three- to four-layer 3-\(\mu\text{m thick sections were prepared and immunohistochemical analysis was performed with anti-HSP90 and anti-AKT antibodies. All of them have previously analyzed for antigen specificity in our laboratory, being all conditions optimized for specific antigen detection, with elimination of nonspecific reactivity.**

**Proliferation studies.** First, dose-response proliferation of the cell lines under 17-AAG treatment was analyzed to determine the IC50 of proliferation. Second, the percentage of proliferation inhibition was evaluated in cell lines treated with 17-AAG combined with ADW742 or Imaatinib. Four to six independent experiments were performed, being final data presented as the average of all replicates.

To determine the rate of proliferation, we used the \(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)} method, as previously described (14).

**Apoptosis.** Apoptosis was measured after treatment with 17-AAG alone or with ADW or IMA for 72 h. Apoptotic and necrotic index were assessed by flow cytometry, as previously described (14). Three to five independent experiments were performed, being the final data presented as the average of all replicates.

**siRNA.** HSP90 validated siRNA and Silencer-negative control siRNA were purchased from Ambion. Briefly, A673 and TTC466 cell lines were cultured as above described. Cells in log-phase growth were harvested by centrifugation at 1,500 \(g \text{ for 5 min; resuspended in 200 \muL}} of medium with 75 \text{ mmol/L HSP90siRNA, 5 \muL NaCl 1.5 mol/L, and 20 \muL of DNA carrier; and transferred to a Bio-Rad 0.4-cm electrophoresis cuvette. Electroporation was done with a Gene Pulser II (Bio-Rad) at 126 V/800 \muF. Cells were transferred to 10 mL of culture medium and allowed to grow 24 h before drug treatment. Cells were grown using the same conditions described above. Proliferation and apoptosis induction were analyzed by the MTT and flow cytometry methods, respectively (as described).

**HSP90 transfection.** HSP90 clone was obtained from Invitrogen Ultimate ORF clones resource, and efficiently transferred to a Gateway Destination Vector (Invitrogen PCDN3A.1(+), catalog n° V790-20), previously ligated with Rf cassetteA from the Invitrogen Gateway Vector Conversion System (catalog n° 11828-029), using the Gateway LR Clonase Enzyme mix (catalog n° 11791-019), according to manufacturer’s instructions. The Gateway Destination Vector containing the HSP90 clone or the Vector Alone (Mock; 15 \mug) were transfected in A4573, SK-ES-1, and TC71 cell lines, by electroporation, as above described. Proliferation and apoptosis induction were analyzed by the MTT and flow cytometry methods, respectively.

**In vivo studies.** Four- to 5-wk-old female NOD/scid mice (supplied by Charles River’s Lab) were used, following the Spanish and European Union guidelines \((RD 1201/05 and 86/609/CEE, respectively)\). A673 ES cells in mid-log growth phase were used to induce tumors because it was the most representative cell line of drug resistance to ADW/IMA treatment. Cell suspensions \((5 \times 10^6 \text{ cells in 0.2 mL 1:1 cellular mediumMatrigel Matrix (ref. N° 35624; BD Bioscience)}) were injected s.c. into the right flank mice. Mice were randomized into controls and treated groups 1 wk after tumor started to be measurable \((2–3 wk after injection; day 0 of treatment), and mice with tumor volume higher than 1 cm3 were excluded from analysis. Treatment was as follows: vehicle 1 (25 mmol/L tartaric acid, p.o., twice daily, 7 d/wk for 2 wk); vehicle 2 (PBS Tween 80 0.05% DMSO 10\%, i.p., twice daily, 5 d/wk, for 2 wk); 17-AAG \((\text{alone, i.p., twice daily, 80 mg/kg/d, on days 0–5 and 8–12)}\); AEW541 \((\text{alone, p.o., twice daily, 50 mg/kg, 7 d/wk for 2 wk), or 17-AAG (same conditions) plus AEW541 (same conditions)}\). The tumors were measured every 2 to 3 d with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula \(a^2 b/6\) where \(a\) is the smallest diameter and \(b\) is the biggest one. At the end of the experiment, mice were sacrificed by anesthesia overdosing and tumors were collected for histopathology analysis.

**Pathologic analysis.** In the case of mice models, tissues were sampled in \(<15\text{ min after mice sacrifice and representative areas were (a) formalin fixed (12 h) and paraffin embedded, and (b) snap frozen and stored at }-80^\circ\text{C. In the case of the clinical samples, formalin-fixed, paraffin-embedded sections}}

were used. All sections were stained with H&E and prepared for immunohistochemical (IHC) study.

We used two sets of clinical samples of ES collected before treatment for confirmation of the in vitro/in vivo results.

For IHC, 2 cell conditioning periods of 8 min at 95°C and 4 min at 100°C on hot plate using buffer TRIS-EDTA (pH 8.0) buffer [except for IGFIR, in which citrate buffer (pH 6.0) was used] were performed on previously dewaxed formalin-fixed paraffin-embedded sections. Sections were then incubated at 37°C for 1 h with antibody anti HSP90 (Alexis Biochemicals ALX 804; dilution 1/500), IGFIR (Santa Cruz Biotechnologies; dilution 1/50), anti AKT (Cell Signaling Technologies #9272; dilution 1/500), and anti-mTOR (Cell Signaling Technologies #2972; dilution 1/50), and c-kit (Dako Cytomation A4502; dilution 1/200). Staining was performed with the IHC 3,3′-diaminobenzidine MAP system (Ventana Medical Systems). Results were evaluated blinded to clinicopathologic and molecular data. The number and intensity of immunoreactive cells were evaluated in three randomly selected fields. Number was expressed as the percentage of reactive cells. Intensity of staining was scored + to +++ (+, faint staining intensity; ++, strong staining intensity). We considered immunoreactivity >50% of cells as diffuse staining.

**Statistical analysis.** One-way ANOVA for independent samples was performed, using the VassarStats web site for statistical computation.

![Figure 1](image-url) Two-dimensional electrophoresis results (A and B) and HSP90 expression validation (C). A673 and A4573 cell lines were treated with 0.1 μmol/L ADW742 and/or 10 μmol/L Imatinib, for 24 and 72 h, and proteins were extracted. A, two-dimensional gels of A673 cell line proteins extracted under control conditions (left) or after treatment with ADW742 (right). After performing the second dimension, selection of differentially expressed protein spots was performed manually, and afterwards, spots were excised from the gels for MALDI-ToF analysis. The proteins highlighted in the image were identified as follows: 1 and 3, not determined; 2, PRDX1; 4, NASP; 5, HSP90. The close-overexpression presented shows HSP90 protein spots in both cases. Further data are compiled in Table 1. B, function of the proteins whose expression is altered by the ADW and/or IMA treatment. The largest groups of proteins showing expression changes were those related to stress-induced responses mechanism (21%) and apoptosis induction (17%). C, densitometric analysis of the WB study of the effects of ADW742 with or IMA on HSP90 expression. All ET cell lines were treated with 0.1 μmol/L ADW742 and/or 10 μmol/L Imatinib, for 24 and 72 h, and protein content extracted afterwards. HSP90 expression was increased at 24 h of treatment in A673 and TTC466 cell lines and decreased in the others. At 72 h of treatment, all cell lines had HSP90 expression decreased (especially in A673 cell line). These results confirmed those presented in Supplementary Fig. S2D, with A673 and A4573 cell lines. *, P < 0.05, determined with one-way ANOVA for independent samples.
Results

ADW742 and/or Imatinib treatment induce changes at the proteome level in ES cell lines. Because we previously reported on the effects of IGF1R/KIT circuit inhibition on ES proliferation, survival, and pathway inhibition, and found synergistic effects between several drugs (14), we now planned to perform an overall study of ES cell proteome to have a general view of ADW742/Imatinib effects. With this study, we aimed to assess the expression changes of several relevant proteins that we could test as possible targets/surrogate markers in ES.

We first treated ES cell lines A673 and A4573 (cell lines that, in our experience, deeply differ on their sensitivity to treatment) with 0.1 mmol/L ADW742 and/or 10 μmol/L Imatinib, for 24 and 72 hours. Cell extracts were prepared and protein expression levels were scrutinized for differences by two-dimensional electrophoresis and mass spectrometry (see Fig. 1A). A panel of proteins with different levels of expression was compiled, as seen in Table 1. The proteins obtained were involved in relevant cellular processes such as the regulation of proliferation (EBP1, RhoA), apoptosis (Set, 14-3-3T), and stress-induced responses (GRP75, HSP90, and HSP70; Fig. 1B). Ingenuity Pathway Analysis was then performed to analyze the most relevant networks altered with treatment (Supplementary Fig. S1D), selecting a more reduced panel of relevant proteins [the ones that are marked with an asterisk (*) in Table 1], including HSP90, to confirm the results obtained at protein and mRNA levels.

qRT-PCR and Western blotting (WB) showed that almost all of the "selected proteins" had actually changed their mRNA and protein expression levels with treatment (Supplementary Fig. S2D) and confirmed that results are not just due to unspecific particular cellular background. There were significant differences between 24- and 72-hour treatments, mainly showing the initial triggering of cellular stress responses at 24 hours. At 72 hours, effects observed were cell line specific and drug specific. There were significant differences between the cell lines studied. We globally observed that drug treatment alone had similar effects (with ADW742 or Imatinib) and was less effective than the combination of both drugs. In general, the overexpressed proteins were related to apoptosis, and the ones that were underexpressed were related with proliferation and stress-induced responses (Supplementary Fig. S2D).

HSP90 expression levels dramatically change with ADW742/Imatinib treatment. Based on the above-mentioned results, on the availability of HSP90-specific inhibitors, and its well-characterized involvement in cancer, we decided to study HSP90 role in the response to stress of ES cell lines.

Initially, we confirmed HSP90 expression changes after ADW and/or IMA treatment, for 24 and 72 hours, in a panel of 5 ES cell lines with different EWS-ETS fusion types (28–31).

![Table 1. Two-dimensional proteomic analysis results](image)

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<th>Description</th>
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<th>Cell line</th>
<th>Expression</th>
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<td>P27348*</td>
<td>1433T; 14-3-3 protein Theta (Tau)</td>
<td>162</td>
<td>A4573</td>
<td>Over</td>
</tr>
<tr>
<td>Q25010+ P84184</td>
<td>ACT3A; Actin, cytoplasmatic A3a</td>
<td>171</td>
<td>A4573</td>
<td>Under</td>
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<tr>
<td></td>
<td>ACT3B; Actin, cytoplasmatic A3b</td>
<td>138</td>
<td>Under</td>
<td></td>
</tr>
<tr>
<td>P23793</td>
<td>ARCA; arginine deiminase</td>
<td>193</td>
<td>A4573</td>
<td>Over</td>
</tr>
<tr>
<td>Q29499</td>
<td>DDX1; ATP-depend. Helicase DDX1</td>
<td>80</td>
<td>A4573</td>
<td>Over</td>
</tr>
<tr>
<td>Q29630</td>
<td>DYRK2; Dual specif. Tyr-Phos regul K2</td>
<td>67</td>
<td>A4573</td>
<td>Over</td>
</tr>
<tr>
<td>P26641</td>
<td>EF1G; Elongation Factor 1-γ</td>
<td>143</td>
<td>A673</td>
<td>Under</td>
</tr>
<tr>
<td>P38646*</td>
<td>GRP75; stress-70 prot, mit.percu</td>
<td>368</td>
<td>A4573</td>
<td>Under</td>
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<tr>
<td>P08238* P07900*</td>
<td>HS90B; Heat shock prot.90 β</td>
<td>176 + 174</td>
<td>A673</td>
<td>Over</td>
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<tr>
<td></td>
<td>HS90A; Heat shock prot.90 α</td>
<td>120 + 109</td>
<td>A4573</td>
<td>Over</td>
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<td>P11142*</td>
<td>HSP7C; Heat Shock cognate71kDaprot (HSP70)</td>
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<td>A4573</td>
<td>Over</td>
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<td>NASP; splice isoform 2 and 1</td>
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<td>Over</td>
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<td>Q9Y4L1</td>
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<td>A673</td>
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<tr>
<td>Q9CQ80*</td>
<td>PAG54; Prolif-ass. prot. 2G4 (EFP1)</td>
<td>206</td>
<td>A673</td>
<td>Under</td>
</tr>
<tr>
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<td>PSA5; Proteas. Suba. α type 5</td>
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<td>RBSA; 40S ribosomal prot. SA (p40)</td>
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<tr>
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<td>SET; SET protein (1-2PP2A)</td>
<td>64</td>
<td>A4573</td>
<td>Under</td>
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<tr>
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<td>STALP; AMSH-like protease</td>
<td>75</td>
<td>A4573</td>
<td>Over</td>
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<tr>
<td>P13693*</td>
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<td>A4573</td>
<td>Over</td>
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<td>333</td>
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<td>Over</td>
</tr>
<tr>
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<td>Vinculin; splice isoform 1 and 2</td>
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<td>A673</td>
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NOTE: Each protein was identified by matching peptide-mass fingerprinting against Swiss-Prot nonredundant human database. Minimal Score change cut was put at 64. Proteins marked with * were studied at mRNA and protein level.
As shown in Fig. 1C, HSP90 expression changed with treatment in all cell lines and depended on each cell line. Globally, HSP90 expression increased at 24 hours of treatment in A673 and TTC466 cell lines and decreased in the others. HSP90 expression decreased at 72 hours of treatment in all cell lines, especially in A673 cell line.

**HSP90 blockade by 17-AAG inhibits ES cell proliferation and survival by reducing activation of key HSP90 client proteins.** After confirming the expression changes of HSP90 in ES, we decided to study its role on cell proliferation and survival by performing specific inhibition using the geldanamycin derivate, the 17-AAG, in the same panel of ES cell lines.

All cell lines showed a dose-dependent inhibition of growth (Supplementary Fig. S3D), at 24 and 72 hours of treatment, ranging the IC_{50} at 24 hours from 1.0 to 1.62 μmol/L and at 72 hours of treatment from 0.42 to 0.72 μmol/L (Fig. 2A).

The effects of 17-AAG treatment on apoptosis induction depended on the ES cell lines treated. As depicted in Fig. 2B and C and Supplementary Fig. S4D, induction ranged between 30% and 25% in TTC466 and A673 cell line, and 8% to 20% in the others, with a much higher effect in the late apoptosis/necrosis levels than in early apoptosis.

These results were explained, at least in part, by 17-AAG treatment effects on HSP90 client protein expression. We observed that 17-AAG per se (Fig. 3A) induced IGF1R, KIT, and AKT expression, which, in turn, reduced phosphorylation levels of AKT, mTOR, and MAPK/24. These effects were more evident in A673 cell line than in A4573, in which there was nonsignificant degradation of IGF1R or c-kit. The IP studies showed that IGF1R, KIT, and AKT had their physical interaction with HSP90 reduced by 17-AAG treatment. Once again, we observed that the interaction between HSP90 and IGF1R or KIT was much weaker in sensitive cell lines to ADW/IMA (A4573, SK-ES-1, and TC71) than in A673 and TTC466 cell lines (Supplementary Fig. S5D; Fig. 3B).

**HSP90 blockade by 17-AAG sensitizes ES cell lines resistant to ADW742 or Imatinib treatment.** Because 17-AAG treatment alone seemed to be actually effective in A673 and TTC466 cell lines (which are cell lines that in our experience were highly insensitive to ADW or IMA treatment; ref. 14) and this treatment reduces p-AKT, p-mTOR, and p-MAPK/24, we decided to assess the combination of this drug with IMA or ADW and study their effects on proliferation and apoptosis induction.

We observed that combinations of 17-AAG with ADW or IMA resulted in an additional decrease of 15% to 50% in the proliferative rate, depending on the sensitivity of each cell line (Supplementary Fig. S6D). Drug combination with ADW was synergistic in almost all cell lines [combination index (CI range, 0.35–0.9)] with the exception of A4573 cell line. In the case of 17-AAG combination with IMA, we observed synergistic effects in A673 and TTC466 cell lines (A673: CI range, 0.53–0.9; TTC466: CI range, 0.44–0.79). When we studied the effect of combining 17-AAG with ADW or IMA, we observed synergistic effects in A673 and TTC466 cell lines sensitive to ADW/IMA treatment.

**HSP90 levels are determinant of the response of ES cell lines to ADW/IMA treatment.** To confirm that the inhibition obtained with 17-AAG treatment was due to HSP90 blockade, we performed an additional study based on a siRNA approach, in which we reduced HSP90 expression in the cell lines resistant to ADW/IMA treatment. Conversely, to prove our hypothesis that HSP90 high expression could be conferring resistance to ADW and/or IMA treatment, we studied the effect of HSP90 overexpression in cell lines sensitive to ADW/IMA treatment.

As expected, HSP90siRNA (that reduced HSP90 expression in 35–65%, as depicted in Fig. 4A) improved ADW or IMA effects in inhibiting proliferation and inducing apoptosis; the results being very similar to those obtained with 17-AAG (Supplementary Fig. S7D; Fig. 4B). We observed a decrease of 20% to 30% in the proliferative rate and 15% to 40% in the apoptosis induction of cell lines treated with HSP90siRNA, when compared with the nontreatment or HSP90siRNA-negative control conditions. These results confirmed not only our hypothesis but also that the results obtained with 17-AAG treatment are specific to HSP90 inhibition and not due to alternative effects of this drug.

Our hypothesis was also confirmed with the HSP90 overexpression studies. We obtained HSP90 overexpression in control conditions, and expression levels increased during ADW/IMA drug treatment (Fig. 4C). As depicted in Fig. 4D and Supplementary Fig. S8D, HSP90 overexpression increased proliferation, conferring resistance to apoptosis induction by ADW/IMA treatment in all cell lines analyzed. Apoptosis induction was drastically blocked (no >20% of apoptosis induction in all treatment conditions, values almost equal to control) and proliferation was induced, having values above nontreatment control. All conditions had proliferation values above 75%, much more than the nontreated conditions. These results were not simple transfection effects because transfection with Mock did not affect neither HSP90 expression (data not shown), nor proliferation or apoptosis of the cell lines.

**HSP90 inhibition, alone or combined with IGF1R inhibition, reduces tumor growth in vivo.** After the *in vitro* studies, we tested 17-AAG treatment effects, alone or combined with AEW541 in a xenograft model using A673, an IMA/ADW-resistant cell line. This drug is an ADW742 analogue that has the same IGF1R inhibition mechanism but is much more specific for IGF1R showing less toxic effects, the latter being important for ethical reasons (32).

This *in vivo* study confirmed our *in vitro* results. Although the control group showed an increase in median tumor size of 340% at the end of the study and of 270% in AEW group, the increase was only of 74% in the 17-AAG group, and ~38% in the combination group (Fig. 5A). Animals treated with AEW alone showed a similar behavior to the control group, whereas animals treated with 17-AAG alone did not show a tumor growth reduction but a slowing down during the 2 weeks of treatment, compared with the control group. However, animals treated with drug combination (AEW+17-AGG) not only experimented a slowing down of growth but a marked reduction in the median tumor size, next to 40% (Fig. 5A). The effect of drug combination was even higher in those animals that had initially tumor volumes below average tumor size, in which tumor size reduction reached 66%.

Histopathology studies showed that tumors treated with drug combination were not only smaller, but also showed more extensive necrotic areas (as depicted in Fig. 5B). Interestingly, expression of total IGF1R and AKT diminished in the tumors.
Figure 2. Effects of 17-AAG on proliferation and apoptosis of ET cell lines. Subconfluent cells were treated with 17-AAG (0.01–25 μmol/L) alone or combined with ADW742 (0.1–0.5 μmol/L) or Imatinib (5–15 μmol/L), for 24 or 72 h at different combinations, and then proliferation was measured by the MTT assay and apoptosis analyzed by flow cytometry, by Annexin-V/PI detection. A, IC50 of proliferation of all cell lines after 17-AAG treatment for 24 and 72 h of incubation (μmol/L). All cell lines showed a similar behavior, with a dose-dependent inhibition of growth. IC50 of proliferation depended on the cell line. B, graphic representation of apoptosis obtained for several treatment conditions of A673 cells treated with 17-AAG, ADW, and/or IMA, for 72 h. Although drugs alone were almost unable to produce apoptosis, the combinations of 17-AAG with either ADW742 or Imatinib were able to increase it (see Fig. 3C and Supplementary Fig. 4D for details). C, bar graph representation of all conditions. The higher the concentration of both drugs, the higher the ratio between apoptotic/necrotic cells and vital cells, indicating an increase in the induction of cell death. The effect of combining 17AAG with ADW742 or Imatinib resulted in an additional increase of 10% to 50% in apoptosis depending on the cell line. We mainly observed additive effects, but in A673, we saw synergistic effects when combining 17AAG+ADW or IMA, as described in Results. A, ADW742; I, Imatinib; A/N, apoptotic/N: necrotic.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>17-AAG μmol/L</th>
<th>ADW742* μmol/L</th>
<th>Imatinib* μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h 72h</td>
<td>24h 72h</td>
<td>24h 72h</td>
</tr>
<tr>
<td>A673</td>
<td>1.33 0.57</td>
<td>5.2 1.41</td>
<td>18.36 18.77</td>
</tr>
<tr>
<td>TC-71</td>
<td>1.12 0.49</td>
<td>5.03 0.67</td>
<td>17.45 22.00</td>
</tr>
<tr>
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<td>5.11 0.55</td>
<td>14.32 18.90</td>
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<tr>
<td>A4573</td>
<td>1.24 0.45</td>
<td>4.30 0.64</td>
<td>15.00 20.12</td>
</tr>
<tr>
<td>TTC466</td>
<td>1.62 0.72</td>
<td>5.1 1.20</td>
<td>17.8 19.20</td>
</tr>
</tbody>
</table>

*Previously published 14
treated with 17-AAG, alone or in combination with AEW, with respect to their untreated controls, in agreement to their role as HSP90-client proteins. In contrast, expression of total mTOR did not change with 17-AAG treatment (Fig. 5D).

A subset of IGF1R-positive clinical samples also shows expression of HSP90. HSP90, IGF1R, and KIT expression was analyzed in both ES clinical sample sets. Prevalence of expression of these three molecules was similar between both series of samples. IGF1R expression was cytoplasmic and relatively uniform among tumor samples, in both sets of samples (5 of 10, 50% in EA samples; 33 of 44, 75% in the TMA). Intensity was moderate to intense. HSP90 expression was cytoplasmic, sometimes with a dot-like pattern, and uniform within the tumor (3 of 10, 30% in EA samples; 19 of 44, 43%, in the TMA). Intensity was weak to moderate. KIT expression was detected at the cellular membrane, with a heterogeneous signal among tumor samples (7 of 11, 64% in EA samples; 21 of 44, 48% in the TMA). Intensity was also moderate to intense.

Interestingly, when comparing IGF1R and HSP90 expression, we found that almost half of IGF1R+ samples were also HSP90+. Conversely, almost 80% of the HSP90+ samples had also IGF1R expression (see Table S1 in Supplementary Data). KIT expression showed a clear overlap with IGF1R expression.

Discussion

Search for new therapeutic approaches is an urgent need in sarcomas. IGF1R blockade (12, 13) is a promising approach in the therapy of ES (14, 32–38). Having this in mind, and considering our previous experience on the synergistic effects of in vitro combination of IGF1R/KIT pathway blockade with vincristine and doxorubicin on ES cell lines (14), we studied the global proteomic changes induced by treatment with these drugs and perform a molecular validation of possible drug target candidates.

We assume that stress response is important in ES because drug treatment provoked changes in several proteins involved in different levels of chaperoning complex formation. Drug treatment changed the expression levels of HSP70 and 90, which are involved in the formation of the early and intermediate stress response complex (22), respectively, but also those of p23, a protein that is involved in the mature stress response complex formation. Changes in the expression patterns largely depended on the cell line used, not correlating to the translocation type or additional abnormalities previously reported, such as p16/INK4 loss or p53 mutation (29–31, 39–41).

Based on the results obtained, we had four evidences that pointed to a possible HSP90 involvement in ES:

1. The major group of proteins with expression changes (Fig. 1B) was related with stress responses;
2. HSP90 appeared four times (Supplementary Fig. S1D) connecting several networks in the Ingenuity Pathway Analysis;
3. HSP90 was one of the proteins whose expression changed in both cell lines studied (A673 and A4573), both at the mRNA and protein levels, suggesting a common cellular response mechanism; and

![Figure 3. 17-AAG effects on HSP90 client protein expression and phosphorylation in ET cell lines. A, A673 and A4573 cell lines were treated with 0.25 \mu mol/L 17-AAG for 24 and 72 h, IGF1 was stimulated for 10 min, and proteins were extracted. Drug treatment resulted in degradation of IGF1R, c-kit, and AKT, and in decreased AKT, mTOR, and MAPK42/44 phosphorylation. The levels of degradation largely depended on the cell line used, A673 being much more sensitive to treatment than A4573, in which we only saw weak degradation of IGF1R and KIT. B, 17-AAG effects on physical interaction of HSP90 with some client proteins. A673 cell line was treated with 0.1 \mu mol/L 17-AAG for 72 h. IP against HSP90, c-kit, AKT, and IGF1R was performed, and proteins were extracted. All studied proteins are present in Total Extract (TE; first lane from left) and c-kit, AKT, and IGF1R are coimmunoprecipitated with HSP90 (eighth lane from left). 17-AAG treatment resulted in loss of interaction of IGF1R, c-kit, or AKT with HSP90 (ninth lane from left). Client proteins do not “crossimmunoprecipitate” with each other (in each blot, we do not observe any other client protein signal than the one specifically being immnoprecipitated, together with the HSP90 signal) because HSP90 only can be interacting with one client protein at a time, IP signal only being observed for the client protein to whom it is chaperoning.](cancerres.aacrjournals.org)
4. HSP90 was precisely one of the proteins overexpressed in three cell variants of TC-71 cell line resistant to ET-743, as previously described (42).  

Therefore, we decided to check HSP90 expression in a larger panel of ES cell lines, including five cell lines with different fusion types. HSP90 protein expression dramatically increased in A673 and TTC466 cell lines (both insensitive to ADW/IMA, as we previously reported; ref. 14) at 24 hours of treatment, whereas it was slightly reduced in the other cell lines. We hypothesized that this finding could explain, at least in part, the insensitivity of A673

In vivo studies with 17-AAG, alone or combined with AEW541, and its correlation to clinical samples. NOD-Scid mice were s.c injected with $5 \times 10^6$ A673 cells. They were randomized into groups treated with vehicle (25 mmol/L tartaric acid or PBS Tween 80 0.05% DMSO 10%), 17-AAG alone (80 mg/kg/d), AEW alone (50 mg/kg/d), or 17-AAG+AEW (same conditions), for 15 d. Clinical samples were obtained from E. de Alava and C. Poremba. A, inhibition of tumor growth in mice by 17-AAG alone or combined with AEW541 treatment. Tumors in control group showed an increase in median tumor size of 340% and of 270% in AEW group, whereas in the 17AAG group, the increase was only of 72% and of 38% in the combination group. Animals treated with 17AAG alone had a tumor growth slowing down, and animals treated with drug combination (AEW+17AAG) had a marked reduction in the median tumor size, next to 40% (*, $P < 0.05$). B, visual and morphologic evaluation of mice tumors. H&E sections of tumors treated with 17AAG+AEW showed a marked increase in necrosis (light pink areas) and a dramatic decrease in size with respect to their controls (top). Bars, 1 cm. C, IHC evaluation of mice tumors. Expression of total IGF1R, HSP90, and AKT was almost abolished in tumor samples from animals treated with 17AAG+AEW (right) with respect to their controls (left). In contrast, mTOR expression did not change with 17AAG+AEW treatment ($>400$). D, IHC evaluation of clinical samples. HSP90 and IGF1R expression was cytoplasmic and uniform within the tumor. Intensity was weak to moderate for HSP90 and moderate to intense for IGF1R. KIT expression was detected at the cellular membrane, with a heterogeneous signal among tumor samples and intensity was also moderate to intense. A general view ($>4$), intermediate view ($>200$), and closer view ($>1,000$) of positive cases are shown and a intermediate view is shown for negative cases.
and TTC466 to KIT/IGF1R inhibiting drugs. We suggest that these cell lines have a well-developed mechanism of stress-induced response that helps to counterbalance drug treatment effects, being triggered at the early phases of treatment (up to 24 hours), and making it more resistant than the other cell lines. We then studied the effects of HSP90-specific inhibition, using the geldanamycin derivative 17-AAG in the same panel of 5 ES cell lines. This drug inhibited ES cell line proliferation and survival in a dose-dependent manner. There was a strong induction of apoptosis in A673 and TTC466 cell lines, which we showed to be resistant to ADW/IMA treatment but not in the other cell lines. We suggest that this is due to the fact that A673 and TTC466 do have a well-developed HSP90 stress response mechanism. It could be argued that A673 cell line would be more dependent on HSP90 inhibition because it has a BRAF mutation (39); however, TTC466 cell line has a similar behavior but lacks mutations in that particular gene, and there is no evidence for other genetic changes [such as p53 mutation (40) or p16/INK4 loss (41)] that could explain these results, suggesting independence on genetic abnormalities of these cell lines. We consider that these results are also transcript independent because TTC466 has a different fusion type than A673 and has similar behavior, whereas TC-71, that also has an EWS-FLI1 fusion type1, has a different behavior than A673.

We have shown that 17-AAG treatment down-regulated IGF1R, KIT, and AKT expression by reducing their physical interaction with HSP90. Phosphorylation of AKT, mTOR, and MAPK42/44 was also diminished, and therefore, inhibition of proliferation and apoptosis induction took place, at least, through inhibition of these signaling pathways. Direct physical interaction of HSP90 with KIT and AKT has already been reported (22, 43); our results being expected because AKT has been extensively described as a HSP90 client, and it has been reported that 17-AAG down-regulates KIT protein level (44). Although receptor tyrosine kinase or hormone receptors (19, 21–23, 45, 46) are HSP90 client proteins, and several groups have shown IGF1R inhibition by treatment with HSP90 inhibitors (47–49), as far as we are aware, IGF1R has not been previously reported to be a client protein of HSP90. In this case, we showed physical interaction by IP, although in future, a detailed study of these signaling pathways could explain these results, suggesting independence on genetic abnormalities of these cell lines. We consider that these results are also transcript independent because TTC466 has a different fusion type than A673 and has similar behavior, whereas TC-71, that also has an EWS-FLI1 fusion type1, has a different behavior than A673.

In vitro

In summary, we have shown through in vitro and in vivo studies that HSP90 is a potential therapeutic target in ES. In vitro inhibition of HSP90 with 17-AAG or HSP90 siRNA targets several signaling pathways relevant to sarcomas, being especially useful in cell lines insensitive to Imatinib or ADW742 and in vivo HSP90 inhibition, alone or combined with IGF1R inhibition, reduces tumor growth. We therefore suggest new mechanisms of resistance to treatment not yet described in ES. HSP90 inhibition also showed to be synergistic with ADW/IMA treatment, and therefore could be of therapeutic value in ES. Lastly, in view of the recent launch of clinical trials using IGF1R inhibition in ES, HSP90 expression could be evaluated as a predictive factor of response to treatment.

Acknowledgments

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References


A Pivotal Role for Heat Shock Protein 90 in Ewing Sarcoma Resistance to Anti-Insulin-like Growth Factor 1 Receptor Treatment: In vitro and In vivo Study

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