Phenotypic and Functional Effects of Heat Shock Protein 90 Inhibition on Dendritic Cell

Jooeun Bae,*† Constantine Mitsiades,* Yu-Tzu Tai,* Robert Bertheau,*† Masood Shammas,*† Ramesh Babu Batchu,*† Cheng Li,* Lawrence Catley,* Rao Prabhala,*† Kenneth C. Anderson,* and Nikhil C. Munshi[1]*†

The 90-kDa heat shock protein (Hsp90) plays an important role in conformational regulation of cellular proteins and thereby cellular signaling and function. As Hsp90 is considered a key component of immune function and its inhibition has become an important target for cancer therapy, we here evaluated the role of Hsp90 in human dendritic cell (DC) phenotype and function. Hsp90 inhibition significantly decreased cell surface expression of costimulatory (CD40, CD80, CD86), maturation (CD83), and MHC (HLA-A, B, C and HLA-DP, DQ, DR) markers in immature DC and mature DC and was associated with down-regulation of both RNA and intracellular protein expression. Importantly, Hsp90 inhibition significantly inhibited DC function. It decreased Ag uptake, processing, and presentation by immature DC, leading to reduced T cell proliferation in response to tetanus toxoid as a recall Ag. It also decreased the ability of mature DC to present Ag to T cells and secrete IL-12 as well as induce IFN-γ secretion by allogeneic T cells. These data therefore demonstrate that Hsp90-mediated protein folding is required for DC function and, conversely, Hsp90 inhibition disrupts the DC function of significant relevance in the setting of clinical trials evaluating novel Hsp90 inhibitor therapy in cancer. *The Journal of Immunology,* **2007, 178: 7730–7737.**

Molecular chaperones are highly conserved proteins which either work passively, by preventing the aggregation of damaged proteins, or actively, via an ATP-driven conformational folding of target proteins (1). The 90-kDa heat shock protein (Hsp90)[2] is an important ubiquitously expressed molecular chaperone that participates in the folding, assembly, maturation, and stabilization of various cellular proteins, particularly those involved in signal transduction, cell-cycle control, transcriptional regulation, and survival (2). Hsp90 also influences the activity of the number of client proteins that function as key regulators of cellular growth, differentiation, and apoptosis (3). More than 100 known Hsp90 client proteins regulate multiple signal transduction pathways in human malignancies: transmembrane tyrosine kinases (HER-2/*neu*, epidermal growth factor receptor; c-Met and insulin-like growth factor-1 receptor); signaling proteins (Akt, Raf-1, and IκB kinase); mutated signaling proteins (p53, Kit, Flt3, and v-*src*); chimeric signaling proteins (NPM-ALK, Bcr-Abl); steroid receptors (androgen, estrogen, and progesterone receptors); and cell-cycle regulators (cdk4, cdk6) (4–16). It also assists in maintaining the conformation of the MHC complex, T and B cell receptors, and many other key proteins involved in the normal function of immune cells (17–20). Hsp90, regulated by co-chaperone proteins, participates in a series of dynamic multiprotein complexes linked to its ATPase activity (21, 22). Of note, Hsp90 extracted from malignant cells was shown to have significantly higher ATPase activity and ATP-binding affinity compared with Hsp90 extracted from various normal cells (23). As a number of Hsp90 client proteins promote cancer cell growth and survival, Hsp90 has emerged as an important target for cancer therapy.

The naturally occurring ansamycin antibiotic geldanamycin binds to the conserved N-terminal binding pocket in the ATP-binding domain of Hsp90 (24–26) and inhibits ATP-dependent Hsp90 chaperone activity (27–29), thereby leading to proteasomal degradation of the client proteins (30, 31). Geldanamycin and its less toxic derivative 17-allylaminogeldanamycin (17-AAG) induce degradation of Hsp90 client proteins (32–34) and exert a potent antitumor activity (15). This agent is currently being evaluated in clinical trial to treat patients with relapsed multiple myeloma as well as other cancers (35–37).

Although the role of Hsp90 inhibition has been extensively studied in malignant cells, its effects on human immune cell function, especially the ability of dendritic cells (DC) to induce Ag-specific immunity, have not been well-known. In this study, we examined the impact of Hsp90 inhibition on the phenotype and function of immature (immDC) and mature DC (mDC) to evaluate how immune responses are modulated by inhibition of Hsp90. We show in this study that Hsp90 inhibition in DC significantly decreases expression of critical molecules, at the levels of RNA, surface protein as well as intracellular protein. This down-regulation by Hsp90 inhibition interferes with exogenous Ag uptake and interruption of the ability of mDC to stimulate allogeneic or Ag-specific autologous T cell responses.

Materials and Methods

Reagents

Recombinant human GM-CSF was obtained from Immunex. Recombinant human IL-2, IL-4, IFN-α, IFN-γ, and TNF-α were purchased from R&D Systems. Anti-CD40, -CD80, -CD83, and -HLA-A, B, and C mouse anti-human mAbs conjugated with FITC or PE were purchased from Immunotech. Anti-CD86 and -HLA-DP, DQ, DR mouse anti-human mAb conjugated with FITC or PE were purchased from BD Biosciences/BD

*Dana-Farber Cancer Institute and †Veterans Affairs Boston Healthcare System, Harvard Medical School, Boston, MA 02115

Received for publication January 19, 2007. Accepted for publication March 9, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Nikhil C. Munshi, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. E-mail address: nikhil_munshi@dfci.harvard.edu

[2] Abbreviations used in this paper: Hsp90, 90-kDa heat shock protein; DC, dendritic cell; immDC, immature DC; mDC, mature DC; MFI, mean fluorescence intensity.

Pharmingen. Geldanamycin was used as Hsp90 inhibitor, and it was obtained from the National Cancer Institute (Bethesda, MD).

Generation of monocyte-derived DCs and treatment with the Hsp90 inhibitor

PBMC were isolated from leukopaks obtained from normal donors by standard density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences). To generate immDC, the monocytes obtained as the adherent cells from PBMC were cultured for 7 days in the presence of 1000 U/ml GM-CSF and 1000 U/ml IL-4 in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (BioWhittaker). Fresh medium plus GM-CSF and IL-4 was added to the cultures every other day. The mDC were obtained by adding 1000 U/ml IFN-α plus 10 ng/ml TNF-α along with fresh GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) in 10% FCS-RPMI 1640 on day 7 and then incubating for an additional 3 days. Alternatively, DC were matured with 1 ng/ml LPS (Sigma-Aldrich) for determining IL-12 secretion from the culture. The immDC and mDC were treated with 1 μM Hsp90 inhibitor for 24 or 48 h at 37°C and 5% CO_2, evaluated for phenotypic and functional changes, and compared with untreated immDC and mDC.

Isolation of $CD3^+$ T cells

T cells from normal donors were obtained from the nonadherent cell fraction using the Pan T Cell Isolation kit from Miltenyi Biotec. In brief, T cell enrichment was accomplished by depletion of B cells, NK cells, early erythroid cells, platelets, and basophils by labeling with a mixture of hapten-conjugated anti-CD11b, -CD16, -CD19, -CD36, and -CD56 Abs with MAC microbeads coupled to an anti-hapten mAb. The effluent (negative cell fraction) was collected from the column as enriched $CD3^+$ T cells. Purity (mean ± SD) of the enriched $CD3^+$ T cells by flow cytometry was 95 ± 2%. Purified $CD3^+$ T cells were used as responder cells to evaluate the functional activity of mDC.

Cell survival, apoptosis, and phenotypic analysis

Cell viability of untreated or Hsp90 inhibitor-treated DC was determined by trypan blue exclusion staining. Results are expressed as the percent of live cells ((viable treated DC/viable untreated control DC) × 100). Effect of Hsp90 inhibitor on induction of apoptosis was examined using the Annexin V/PI-Apoptosis kit (Molecular Probes) as per the manufacturer's instruction. The surface protein expression on immDC and mDC was evaluated using mouse anti-human mAb conjugated with FITC or PE, which are specific to CD40, CD80, CD83, CD86, HLA-A, B, C, or HLA-DP, DQ, DR Ags. Following staining, the cells were analyzed using a FACSCalibur flow cytometer and CellQuest version 2.1 software (BD Biosciences). To estimate the recovery status of DC surface protein, Hsp90 inhibitor treated or untreated DC were extensively washed, recultured in fresh medium containing GM-CSF (1000 U/ml) plus IL-4 (1000 U/ml) for immDC or IFN-α (1000 U/ml) plus TNF-α (10 ng/ml) along with GM-CSF and IL-4 for mDC. Then, their cell surface expression levels were re-evaluated after 24, 48, and 72 h of incubation. Results (mean ± SE, $n = 3$) are expressed as the mean fluorescence intensity (MFI) or percent-positive cells.

Oligonucleotide microarray analysis of gene expression

Total RNA was extracted from immDC or mDC treated with Hsp90 inhibitor (1 μM for 24 h) vs untreated control immDC or mDC using an RNeasy kit (Qiagen). RNA concentration was determined by absorbency at 260 nm and 15 μg of high-quality total RNA was used in a cDNA synthesis reaction using the Megascript T7 kit (Ambion). The resulting cDNA was subsequently used to generate biotin-labeled antisense cRNA for hybridization with Human Genome HG-U133A Affymetrix gene chips. Scanning of image output files and analysis of gene expression data sets were followed by filtering of up-regulated or down-regulated transcripts, based on conventional criteria for statistical significance as well as by hierarchical and functional clustering algorithms (38–40).

Western blotting

Approximately 100 μg of protein was suspended in Laemmli's sample buffer (0.1 M Tris-Cl buffer (pH 6.8), containing 1% SDS, 0.05% 2-ME, 10% glycerol, and 0.001% bromphenol blue), boiled for 2 min, and electrophoresed on 8–16% SDS-polyacrylamide gels (Invitrogen Life Technologies) for 2 h at 80 V. A 10-kDa protein ladder was used as a size marker (Invitrogen Life Technologies). Gels were electroblotted onto nitrocellulose paper (Trans-Blot, 0.2-μm transfer membrane; Bio-Rad) at 40 V for 2 h in Tris-glycine. Western blotting was performed following our earlier protocol with minor modifications (41). Briefly, transfer was confirmed by Ponceau S staining, and nonspecific binding on the blots were blocked with 3% nonfat dry milk in PBS containing 0.2% Tween 20 (PBST). Incubation with mouse anti-human CD40 or CD83 Abs (Santa Cruz Biotechnology) was performed for 1 h in PBST containing 1% BSA with constant rocking. Blots were washed three times with PBST, and then incubated in anti-mouse HRP conjugates for 1 h in PBST containing 3% nonfat dry milk. After washing, specific proteins were detected using ECL, according to the instructions provided by manufacturer (Amersham Life Sciences).

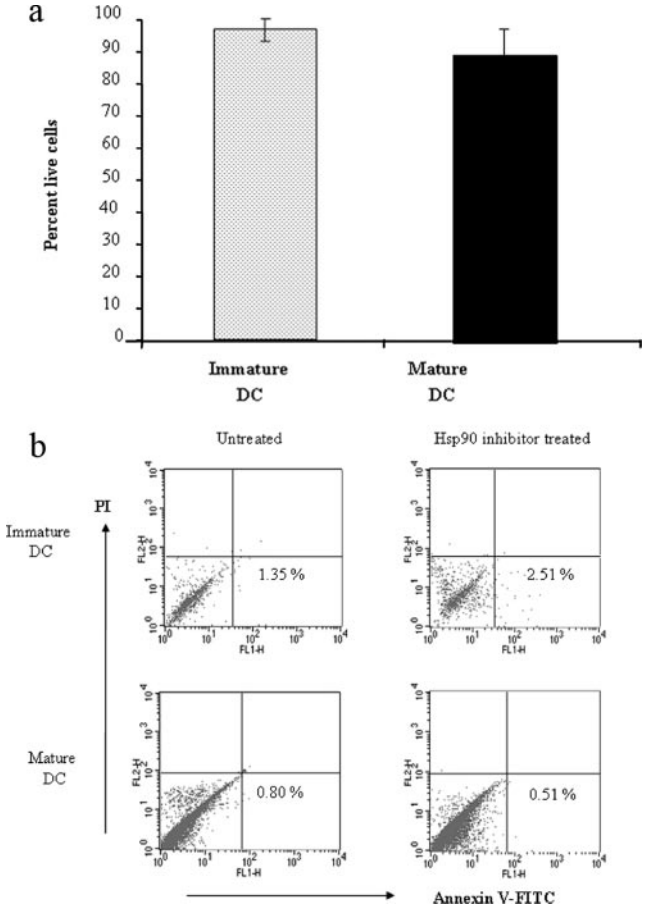

FIGURE 1. No apoptosis induction in immDC and mDC by Hsp90 inhibition. *a*, immDC and mDC were untreated or treated with Hsp90 inhibitor (1 μM for 24 h), washed, and stained with trypan blue to evaluate viable cell number (mean ± SE, $n = 3$). No significant difference was observed in Hsp90 inhibitor-treated immDC and mDC from the respective control DC. *b*, immDC and mDC were stained with Annexin V^{FITC} and propidium iodide (PI) following 24 h treatment with the Hsp90 inhibitor and analyzed for apoptotic cells by flow cytometry. Apoptotic cells (Annexin V^{FITC+}, PI^-) were not induced by Hsp90 inhibition of immDC and mDC. Data are representative of three independent experiments.

Induction of IL-12 secretion by mDC

IL-12 secretion by either Hsp90 inhibitor-treated or untreated mDC was measured using a human IL-12 ELISA kit (BD Biosciences). Briefly, supernatants from culture of DC matured with LPS (1 ng/ml), along with IL-12 as standards, were transferred into wells of a 96-well plate precoated with a monoclonal anti-human IL-12 capture Ab and incubated for 2 h at room temperature. After washing the plate with PBS/0.05% Tween 20, the buffer containing detection Ab and streptavidin-HRP conjugate was added to each well and incubated for 1 h at room temperature. The wells were washed and then developed by incubation with substrate solution for 30 min. Stop solution was added to each well and the absorbance was determined at 450 nm with a PerkinElmer Wallac Victor2 counter. The amount of IL-12 present in the mDC culture supernatant was calculated based on the IL-12 standard curve.

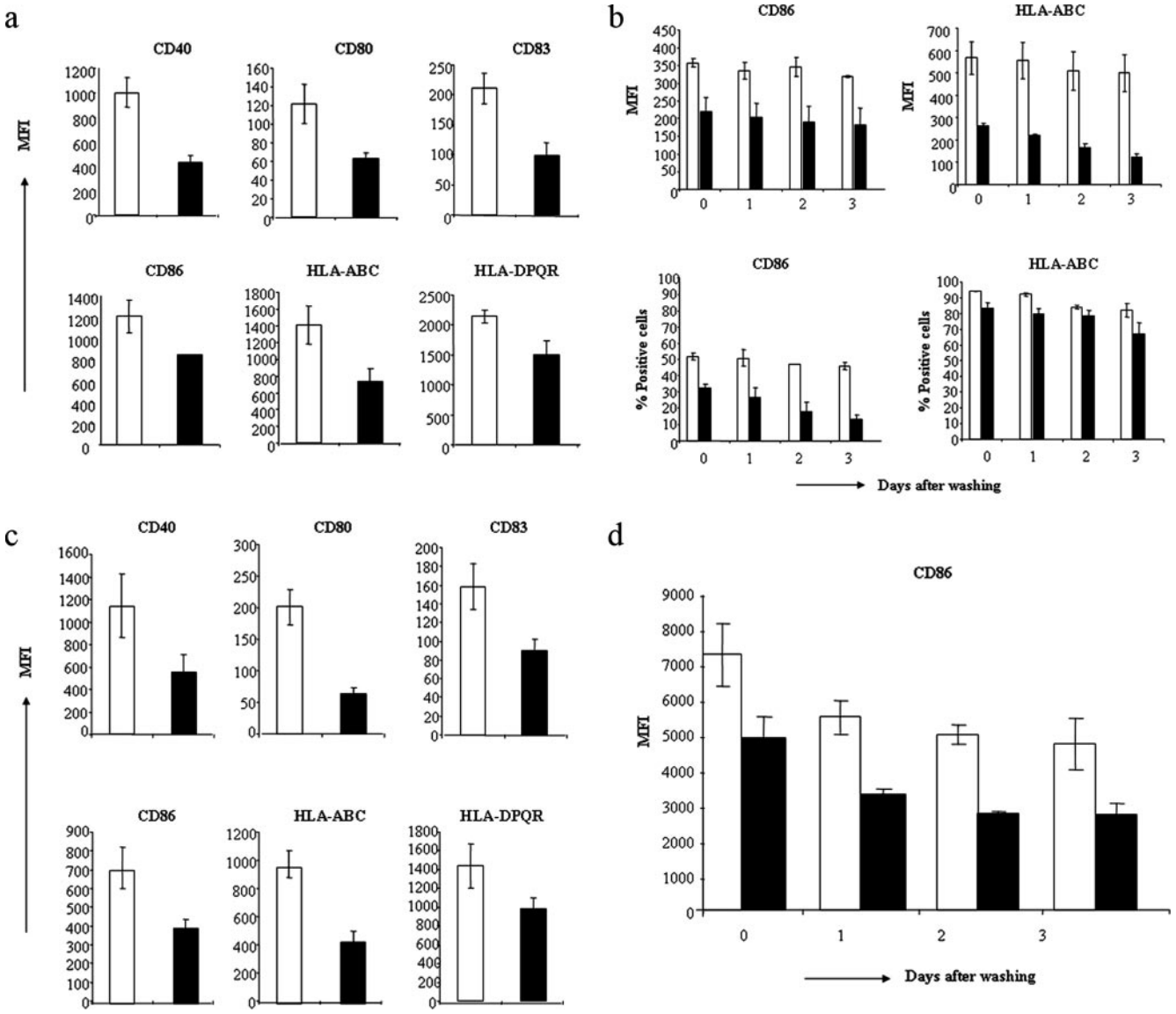

FIGURE 2. Irreversible down-regulation of key surface molecules on immDC and mDC by Hsp90 inhibition. *a*, immDC, untreated (□) or treated (■) with Hsp90 inhibitor for 24 h, were analyzed for expression of key cell surface molecules using specific Abs by flow cytometry; results are presented as MFI (mean ± SE, $n = 3$) of cell surface expression of each Ag (CD40, CD80, CD83, CD86, HLA-A, B, C, and HLA-DP, DQ, DR). Surface Ag expression was significantly decreased on the immDC after 24 h of Hsp90 inhibition. *b*, immDC were treated with the Hsp90 inhibitor for 24 h, extensively washed, and cultured for 3 days. Cells were analyzed by flow cytometry on days 1–3 for expression of costimulatory molecule (CD86) and MHC class I molecules (HLA-A, B, C) by MFI or the percentage of positive cells. No recovery of Ag expression was observed on the immDC on days 1, 2, or 3 following removal of the Hsp90 inhibitor. *c*, mDC, untreated (□) or treated (■) with Hsp90 inhibitor for 24 h, were analyzed for expression of key cell surface molecules (CD40, CD80, CD83, CD86, HLA-A, B, C, and HLA-DP, DQ, DR). Significant decrease was observed in MFI (mean ± SE, $n = 3$) of all Ags tested on mDC by Hsp90 inhibition. *d*, Expression of costimulatory molecule (CD86) was analyzed on mDC, treated with Hsp90 inhibitor for 24 h, extensively washed, and cultured for 3 days. As for immDC, mDC showed down-regulation of surface molecule expression, which did not recover up to 3 days after extensive washings of the Hsp90 inhibitor from the culture.

Ag uptake by immDC

Ability of immDC to uptake Ag was evaluated using a variety of sizes and forms of Ags including 40-kDa Dextran-FITC (1 mg/ml; Molecular Probes), 45-kDa protein A-Alexa Fluor 488 (200 μg/ml; Molecular Probes), or 20-kDa protein G-Alexa Fluor 488 (200 μg/ml; Molecular Probes). In brief, Hsp90 inhibitor-treated or untreated immDC (3×10^5) were pulsed with respective Ag, incubated at 37°C for 45 min, washed three times with cold RPMI 1640, resuspended in 1% paraformaldehyde, and analyzed by flow cytometry. To examine the modification of Ag uptake receptors on immDC, the expression level of mannose receptor, DEC-205 or FcγRIII, was analyzed by flow cytometry using specific Abs.

Ag processing and presentation by immDC

Hsp90 inhibitor-treated or untreated immDC were pulsed with tetanus toxoid (0.5 μg/ml; Calbiochem) overnight. Following washing, DC were matured with TNF-α (10 ng/ml) and IFN-α (1000 U/ml), and gamma-irradiated (10 Gy) using a cesium 137 (^{137}Cs) gamma irradiator source. The mDC were then cocultured with autologous T cells labeled with CFSE (Molecular Probes) to measure the proliferation status of the T cells. For CFSE labeling, T cells were washed, resuspended in 5% FCS-PBS at 2×10^6/ml, and stained with CFSE in DMSO at a final concentration of 5 μM for 10 min at 37°C. T cells were then washed three times and resuspended in AIM-V medium supplemented with 10% human AB serum (BioWhittaker) and 50 U/ml IL-2. After 6 days of coculture of CFSE-labeled T cells (1×10^6/well) with DC (1×10^5/well), proliferation status of the T cells was examined by flow cytometric analysis.

Ag presentation by mDC and induction of T cell proliferation

Allogeneic T cell stimulatory capacity of Hsp90-inhibited DC was assessed. DC were incubated with the Hsp90 inhibitor for 24 h during or after

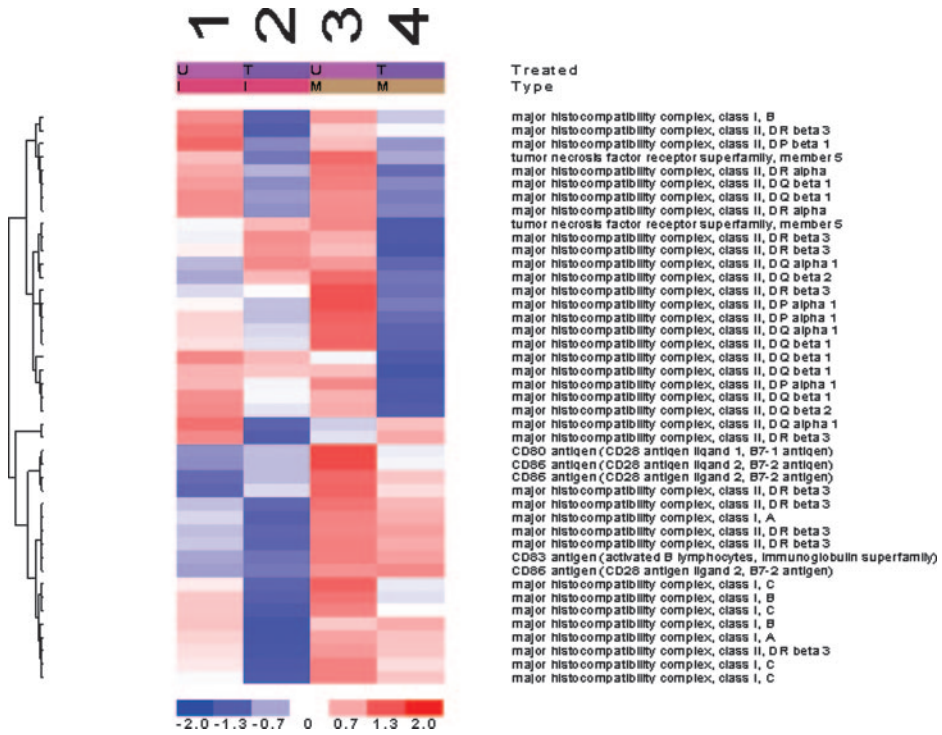

FIGURE 3. Change in transcript levels of key molecules in immDC and mDC by Hsp90 inhibition. Gene expression profile was analyzed in untreated or Hsp90 inhibitor-treated immDC and mDC using HG-U133A gene arrays (Affymetrix). Fold induction or reduction in expression is shown by intensity of red or blue colors, respectively. The color scale at the bottom of each figure represents fold change in the expression in Hsp90 inhibitor-treated cells relative to untreated cells. *Lane 1*, untreated immDC; *lane 2*, treated immDC; *lane 3*, untreated mDC; and *lane 4*, treated mDC. Expression of genes, including *CD40*, *CD80*, *CD83*, *CD86*, *HLA-A*, *B*, *C* and *HLA-DP*, *DQ*, *DR*, were decreased on immDC and mDC by Hsp90 inhibition.

DC maturation. Hsp90 inhibitor-treated or untreated mDC (1×10^4/well) were harvested, washed, irradiated (10 Gy), and cocultured with allogeneic $CD3^+$ T cells (1×10^5/well) in triplicate in 96-well U-bottom microtiter plates. Cultures established in the absence of responder or stimulator cells were prepared to monitor background proliferation. The cells were cultured in AIM-V medium supplemented with 10% human AB serum and 50 U/ml IL-2. After 5 days of culture, the cells were pulsed with 1 μCi of [^{3}H]thymidine overnight, harvested using the cell harvester, resuspended in liquid scintillation fluid, and evaluated for [^{3}H]cpm using a Beckman LS6500 scintillation counter. Results were demonstrated as mean cpm ± SD of triplicate wells.

Release of IFN-γ by allogeneic T cells stimulated with mDC

IFN-γ release by the allogeneic $CD3^+$ T lymphocytes following coculture with either Hsp90 inhibitor-treated or untreated mDC for 24 h was measured using a human IFN-γ ELISA kit (BD Biosciences). Briefly, purified IFN-γ as standard and T cell culture supernatants were transferred onto a 96-well plate precoated with a monoclonal anti-human IFN-γ capture Ab, incubated for 2 h at room temperature, and analyzed with ELISA as described above. Cytokine present in the CTL culture supernatant was quantitated based on an IFN-γ standard curve.

Results

Hsp90 inhibition decreases cell surface expression of key molecules on immDC and interferes with DC maturation

We first evaluated the effect of Hsp90 inhibition on survival of both immDC and mDC by examining viable cell number and induction of apoptosis, as measured by trypan blue staining and annexin V staining, respectively, following 24 h treatment with 1 μM Hsp90 inhibitor. Hsp90 inhibition did not affect cell survival (Fig. 1*a*) or induce apoptosis (Fig. 1*b*) in immDC and mDC.

The effect of Hsp90 inhibition on the expression of critical cell surface markers was next examined on immDC and mDC using flow cytometry. As seen in Fig. 2*a*, Hsp90 inhibition significantly ($p < 0.05$) down-regulated surface expression of costimulatory (CD40, CD80, CD86), maturation (CD83), and MHC (HLA-A, B, C and HLA-DP, DQ, DR) Ags on immDC at 24 h. To assess whether the immDC recover expression of the surface molecules following 24 h treatment with Hsp90 inhibitor, immDC were washed extensively to remove Hsp90 inhibitor and recultured in fresh medium containing GM-CSF and IL-4. The surface expression of the costimulatory, maturation, and MHC Ags was then analyzed for 3 days. As seen in Fig. 2*b*, the surface expression of Ags on immDC did not recover, as assessed by either MFI or percent-positive cells for 3 days, following removal of Hsp90 inhibitor, demonstrating that inhibition of Hsp90 irreversibly interrupts the expression of critical surface molecules on immDC.

Next, the effect of Hsp90 inhibition on DC maturation was evaluated by adding the inhibitor at the last 24 h of culture during DC maturation with IFN-α and TNF-α. As seen in Fig. 2*c*, Hsp90 inhibition decreased cell surface expression of costimulatory (CD40, CD80, CD86), maturation (CD83) and MHC (HLA-A, B, C and HLA-DP, DQ, DR) molecules on mDC. In addition, removal of the inhibitor by extensive washing followed by reculturing in fresh medium containing GM-CSF, IL-4, IFN-α, and TNF-α for next 3 days did not lead to recovery of surface expression of these molecules including CD86 (Fig. 2*d*), suggesting that inhibition of protein folding during DC maturation irreversibly interferes with the maturation process.

Hsp90 inhibition down-regulates gene as well as intracellular protein expression

To investigate the effect of Hsp90 inhibition on RNA expression of key molecules in both immDC and mDC, we evaluated the changes in the gene expression profiles of these molecules in immDC and mDC using HG-U133A gene arrays, with or without Hsp90 inhibitor treatment (1 μM for 24 h). As seen in Fig. 3, the expression of molecules critical for DC function, including CD40, CD80, CD83, CD86, HLA-A, B, C, and HLA-DP, DQ, DR, was down-regulated in immDC and mDC following Hsp90 inhibition.

To evaluate for corresponding changes in intracellular protein levels following Hsp90 inhibitor treatment, lysates of untreated and treated DC were analyzed by Western blots for the expression of CD40 and CD83. As can be seen in Fig. 4, Hsp90 inhibition decreased intracellular protein levels of CD40 and CD83 in both immDC (CD40: 2.50-fold, CD83: 3.33-fold) and mDC (CD40:

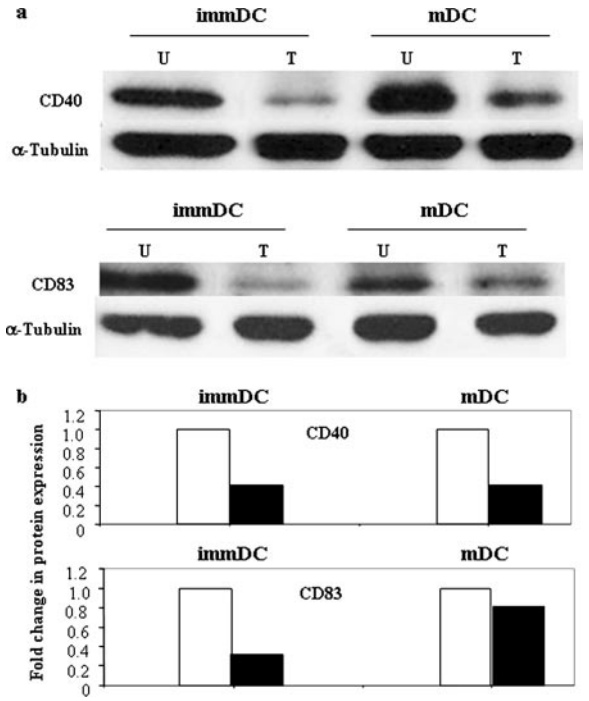

FIGURE 4. Decreased intracellular protein expression in immDC and mDC by Hsp90 inhibition. Equal amounts of protein were fractionated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were sequentially treated with primary Abs and HRP-conjugated secondary Abs; proteins were detected using ECL. Blots were then stripped and incubated with a mAb for α-tubulin as an internal control. Signal intensity of each band was quantitated and normalized to α-tubulin. *a*, Expression of CD40 and CD83 in immDC and mDC, either untreated (U) or treated (T) with the Hsp90 inhibitor at 1 μM for 24 h. *b*, Fold change in CD40 and CD83 expression normalized for α-tubulin in Hsp90-inhibited DC (■) with untreated DC (□). Intracellular protein expression was decreased on immDC and mDC by Hsp90 inhibition compared with control DC.

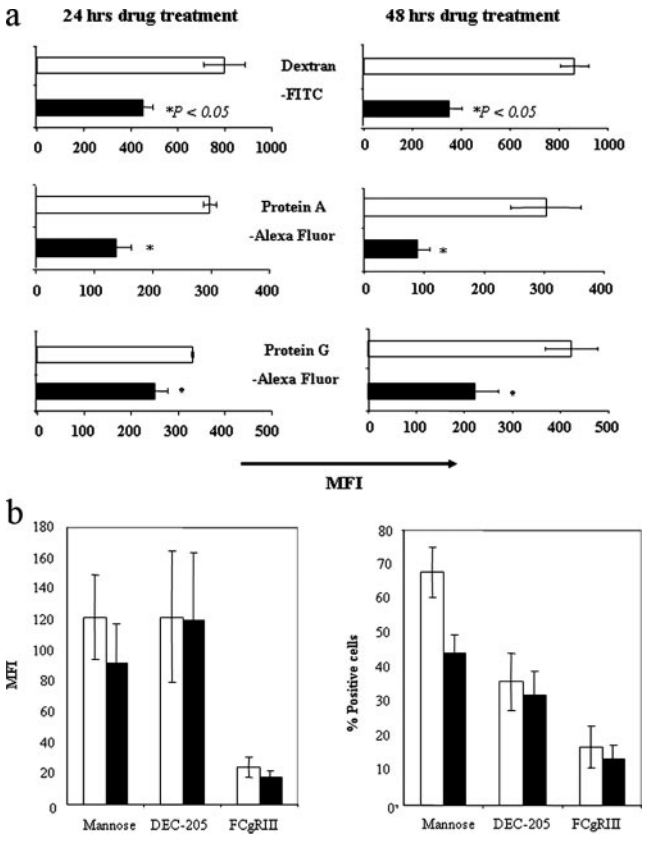

FIGURE 5. Reduction of Ag uptake by Hsp90 inhibition via down-regulation of mannose receptor on immDC. *a*, Hsp90 inhibitor-treated (1 μM, for 24 h or 48 h) or untreated immDC were pulsed with dextran-FITC (1 mg/ml), protein A-Alexa Fluor 488 (200 μg/ml), or protein G-Alexa Fluor 488 (200 μg/ml), incubated at 37°C for 45 min, and Ag uptake by the immDC was analyzed by flow cytometry. Results are presented as MFI (mean ± SE, $n = 3$) in untreated (□) or Hsp90 inhibitor-treated (■; 24 h or 48 h) immDC. The uptake of Ags including dextran, protein A and protein G was significantly decreased in Hsp90-inhibited immDC compared with control immDC, up to 48 h. *b*, Hsp90 inhibitor-treated or untreated immDC were analyzed by flow cytometry using specific mAb against mannose receptors, DEC-205, or FcγRIII. Data (mean ± SE, $n = 3$) are presented as MFI (*left panel*) and percent-positive cells (*right panel*); Hsp90 inhibited (■) compared with control (□) immDC.

2.50-fold, CD83: 1.40-fold) compared with control untreated DC. These results are consistent with the observed down-regulation of gene expression as well as cell surface protein expression following Hsp90 inhibition.

Hsp90 inhibition decreases Ag uptake, processing, and presentation by immDC

A key function of immDC is Ag uptake, before their processing and presentation to T cells. We therefore investigated the effect of Hsp90 inhibition on Ag uptake by immDC. Hsp90 inhibited (1 μM for 24 or 48 h) or control untreated immDC were pulsed with FITC-labeled Dextran, Alexa Fluor-labeled protein A or protein G at 37°C for 1 h. Uptake of these Ags by immDC was then measured by flow cytometry. As can be seen in Fig. 5*a*, Hsp90 inhibition led to a significantly reduced ($p < 0.05$) uptake of all three Ags with different molecular masses and Ag properties both at 24 and 48 h. Next, we investigated effect of Hsp90 inhibitor on cell surface expression of mannose receptor, DEC 205, and FcγRIII, the receptors involved in Ag uptake by DC. Hsp90 inhibition decreased expression of mannose receptor as demonstrated by MFI as well as percent-positive cells (Fig. 5*b*), which was associated with the observed reduction in Ag uptake. In contrast, neither DEC-205 nor FcγRIII expression was significantly changed by Hsp90 inhibition (Fig. 5*b*).

To assess the ability of Hsp90 inhibitor-treated DC to process and present Ag, we measured their ability to process and present tetanus toxoid as a recall Ag and to stimulate proliferation of autologous T lymphocytes. immDC, with and without Hsp90 inhibitor treatment (1 μM for 24 h), were pulsed with tetanus toxoid and used to stimulate CFSE-labeled T cells after the DC maturation. After 6 days incubation, T cell proliferation was measured as a shift in the proportion of cells labeled with CFSE by flow cytometry. In comparison with T cells alone (1%) (Fig. 6*a*) or T cells cultured with unpulsed DC (11%) (Fig. 6*b*), T cells stimulated with tetanus toxoid-pulsed DC (Fig. 6*c*) showed higher proliferation (73%), gated in M1. However, when Hsp90 inhibitor-treated DC pulsed with tetanus toxoid were used, T cell proliferation was significantly decreased (47%) (Fig. 6*d*). These results suggest that DC lose their Ag processing and presentation capability following Hsp90 inhibition.

Hsp90 inhibition reduces ability of mDC to induce allogeneic T cell response and to secrete IL-12

To further investigate the functional effects of Hsp90 inhibition on DC, we evaluated the ability of either untreated or Hsp90

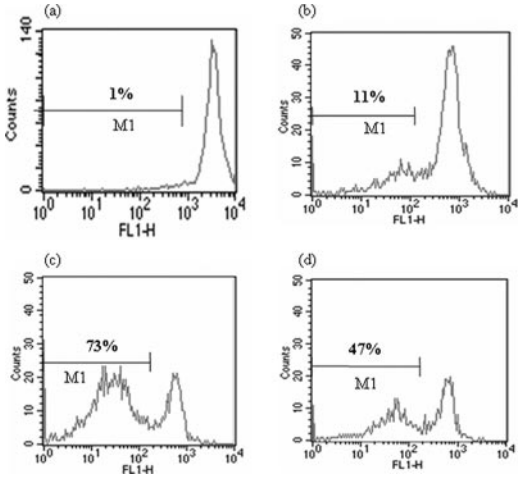

FIGURE 6. Decrease of Ag processing and presentation by Hsp90 inhibited immDC. CFSE-labeled T cells were cultured with Hsp90 inhibitor-treated or untreated autologous immDC pulsed with tetanus toxoid, and the T cell proliferation was measured after 6 days of coculture by flow cytometry. T cells without tetanus toxoid stimulation were used to determine background proliferation on day 0 or 6. The label inherited by daughter cells after cell division which shows as CFSE-low was gated in M1, and the proportion of proliferating cells is reported for each condition. T cell proliferation was examined by coculturing with (*a*) no DC; (*b*) unpulsed DC; (*c*) tetanus toxoid-pulsed untreated DC; or (*d*) tetanus toxoid-pulsed Hsp90 inhibitor-treated DC.

inhibitor-treated mDC to induce allogeneic T cell responses. Hsp90 inhibitor was added, either during or after DC maturation, for 24 h. DC were harvested, washed, irradiated (10 Gy), and cocultured with allogeneic $CD3^+$ T cells for 5 days and T cell proliferation was evaluated by measuring [^{3}H]thymidine incorporation. As seen in Fig. 7, untreated mDC induce allogeneic T cell proliferation through the presentation of mismatched MHC. However, Hsp90 inhibition, either during (Fig. 7*aI*) or after (Fig. 7*aII*) DC maturation, led to significant ($p < 0.05$) reduction in their ability to induce allogeneic T cell proliferation compared with untreated control DC. These results therefore demonstrate that DC lose their capacity to present Ag to allogeneic T cells following Hsp90 inhibition.

IL-12 plays a crucial role in polarizing Th1/Th2 responses, as well as cross-priming of CTL (42–45). We therefore next evaluated the effect of Hsp90 inhibition on the ability of mDC to secrete IL-12. The DC were matured with LPS (1 ng/ml) for 3 days and Hsp90 inhibitor was added to the DC culture for last 24 h of maturation. The secretion of IL-12 was examined from the culture of mDC obtained from three normal donors. As seen in Fig. 7*b*, Hsp90 inhibitor-treated mDC secreted significantly less IL-12 compared with untreated control mDC ($p < 0.05$).

Hsp90 inhibition leads to defective T cell response

The effects of Hsp90 inhibitor on mDC function were then further analyzed by measuring their ability to induce IFN-γ secretion by culturing with allogeneic T lymphocytes. The mDC generated from three normal donors were treated with Hsp90 inhibitor, washed, and cocultured for 24 h with allogeneic T lymphocytes obtained from two different normal donors. The high level of IFN-γ secretion due to allogeneic T cell immune responses was detected in T cell cultures (donor 4 or 5) mixed with untreated mDC (donor 1, 2 or 3). However, allogeneic T lymphocytes cultured with Hsp90 inhibitor-treated mDC from all donors secreted significantly lesser amounts of IFN-γ ($p < 0.05$) compared with untreated control mDC (Table I).

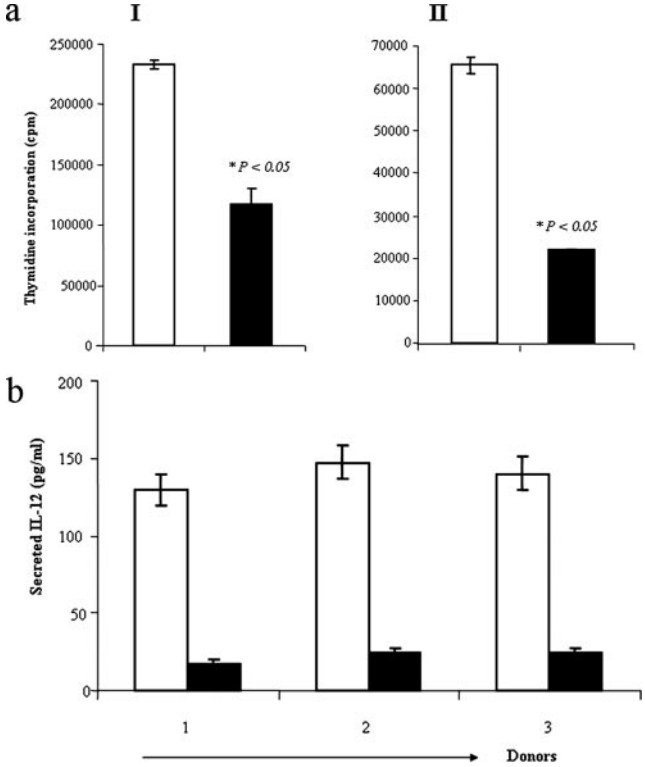

FIGURE 7. Lack of allogeneic T lymphocyte proliferation and IL-12 secretion by mDC treated with Hsp90 inhibitor. *a*, Proliferation of allogeneic T lymphocytes was measured by [^{3}H]thymidine incorporation on day 6 of culture in response to Hsp90-inhibited mDC, either during maturation (*I*) or after maturation (*II*). A significant (∗, $p < 0.05$) decrease in allogeneic T cell proliferation (mean ± SE, $n = 3$) was observed in response to Hsp90 inhibitor-treated mDC (■) compared with control untreated mDC (□). *b*, IL-12 secretion by either Hsp90 inhibitor-treated or untreated mDC was measured. Data are mean ± SE of three independent experiments using three different normal donors' cells. A significant (∗, $p < 0.05$) decrease in IL-12 secretion was observed with Hsp90 inhibitor-treated mDC (■) compared with control untreated mDC (□).

Discussion

The Hsp90 chaperone plays an important role in mediating immune functions. Hsp90 is involved in ATP-dependent assembly and function of the proteasomes, which produces the MHC class I ligands. The proteasome cap structure itself has chaperone activity, unfolding irreversibly damaged proteins and directing them to the active sites of the proteasomal core, where antigenic peptides are generated (46–50). In addition, like other cytoplasmic chaperones, Hsp90 participates in transporting, trimming, and presenting antigenic peptides to the MHC class I molecules to evoke T cell immune responses (51–53). For example, Yamano et al. (49) have

Table I. *Lower levels of IFN-γ secretion from allogeneic T cells stimulated with Hsp90 inhibited mDC*

		Allogenic T Cells	
Stimulator	Responder	Donor 4	Donor 5
Donor 1	Untreated	83.00	70.00
	Hsp90 inhibitor treated	8.50	8.70
Donor 2	Untreated	140	155.00
	Hsp90 inhibitor treated	8.50	8.50
Donor 3	Untreated	76.00	50.00
	Hsp90 inhibitor treated	7.00	8.00

shown that processing of Ags is regulated mainly through Hsp90 by demonstrating enhanced processing of OVA by Hsp90.

The objective of the current study was to examine how Hsp90 affects survival, phenotype, proliferation, and function of immature and mature human DC. In contrast to its effects on malignant cells such as multiple myeloma cells (54–56), Hsp90 inhibition did not affect survival of immDC or mDC. However, it significantly affected cell surface expression of key costimulatory, as well as maturation and MHC, molecules critical for DC function. This decreased cell surface expression of proteins induced by Hsp90 inhibitor can be mediated via various mechanisms: decreased mRNA expression, as observed with gene expression profile data; blocking degradation of proteins that require Hsp90 as chaperone for their conformational maturation (57, 58); and/or altering proper protein assembly as well as folding, thereby affecting the tertiary structure of these proteins, and cell surface anchoring. The mechanism of functional effect of Hsp90 inhibition on DC is a complex interplay of various molecular changes that may involve changes at expression and protein folding level as well as eventual proteasomal degradation.

In our study, significant reduction in proliferation of T cells was observed by their coculture with Hsp90-inhibited DC compared with untreated DC pulsed with tetanus toxoid. This could be due to decreased uptake and/or processing of tetanus toxoid by immDC following Hsp90 inhibition, thereby resulting in poor Ag presentation to T cells. Besides tetanus toxoid, we also observed significant decrease in DC uptake of various other types and sizes of Ags, including dextran, protein A, and protein G following Hsp90 inhibition. In addition, lower expression of costimulatory and MHC molecules on DC following Hsp90 inhibition could also contribute to reduced T cell proliferation to tetanus toxoid stimulation. Under normal condition, mDC, efficiently presents Ag and induces T cell stimulation compared with immDC by expressing higher levels of CD80, 86, MHC class I and II molecules (59, 60). Our study showed that cell proliferation was dramatically decreased when the allogeneic T lymphocytes were cultured with irradiated mDC treated with Hsp90 inhibitor, either during or after DC maturation. In addition, allogeneic T lymphocytes secreted significantly less IFN-γ when stimulated with Hsp90 inhibitor-treated mDC compared with untreated mDC. These results confirm that Hsp90 inhibition significantly affects normal immature and mDC function.

Molecular chaperones are required for stability and function of normal cellular proteins as well as mutated, chimeric, and overexpressed signaling proteins that promote cancer cell growth and/or survival (61–65). Hsp90 in tumor cells is largely contained in multiprotein complexes and its ATPase activity is markedly higher in transformed cells compared with untransformed cells, in which Hsp90 is mostly uncomplexed. The aberrant overexpression of Hsp90-dependent oncoproteins, such as kinases or transcription factors, has been proposed as the cause for this difference, which may at least in part, account for the sensitivity of tumor cells to Hsp90-targeting agents (66). The Hsp90 inhibitor, by interacting specifically with these molecular targets, causes destabilization and eventual degradation of Hsp90 client proteins, leading to apoptotic cell death. Hsp90 inhibitors have already shown promise as an antitumor agent in preclinical studies (67). For example, Hsp90 inhibitor induces apoptotic cell death in both primary myeloma cells and myeloma cell lines in vitro and in vivo in murine models of human myeloma (54). These preclinical data have led to an ongoing phase I clinical evaluation of the geldanamycin analog, 17 AAG, in multiple myeloma (56). Other highly potent Hsp90 inhibitors, such as radicicol (68) and purine scaffold inhibitors (69), are undergoing preclinical evaluation.

Because Hsp90-mediated protein processing and folding is critical for normal DC function, its inhibition could lead to significant modulation of DC-mediated immune responses with potential clinical effects. Our results suggest the need to closely monitor immune function in patients being treated with the Hsp90 inhibitor, in particular to monitor for immunosuppression and opportunistic infections. Also, its inhibitory effects on allogeneic MLR suggest that the Hsp90 inhibitor may be useful to treat or prevent graft-vs-host disease, as well as certain autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

References

1. Bukau, B., and A. L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* 92: 351–366.
2. Sun, J., and J. K. Liao. 2004. Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* 24: 2238–2244.
3. Sreedhar, A. S., C. Soti, and P. Csermely. 2004. Inhibition of Hsp90: a new strategy for inhibiting protein kinases. *Biochim. Biophys. Acta* 1697: 233–242.
4. Isaacs, J. S., W. Xu, and L. Neckers. 2003. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 3: 213–217.
5. Pratt, W. B., and D. O. Toft. 2003. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.* 2: 111–133.
6. Schulte, T. W., and L. M. Neckers. 1998. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother. Pharmacol.* 42: 273–279.
7. Xu, W., E. Mimnaugh, M. F. Rosser, C. Nicchitta, M. Marcu, Y. Yarden, and L. Neckers. 2001. Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J. Biol. Chem.* 2: 3702–3708.
8. Basso, A. D., D. B. Solit, P. N. Munster, and N. Rosen. 2002. Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* 21: 1159–1166.
9. Schulte, T. W., M. V. Blagosklonny, C. Ingui, and L. Neckers. 1995. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J. Biol. Chem.* 270: 24585–24588.
10. An, W. G., T. W. Schulte, and L. M. Neckers. 2000. The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210 bcr-abl and v-*src* proteins before their degradation by the proteasome. *Cell Growth Differ.* 11: 355–360.
11. Blagosklonny, M. V., J. Toretsky, and L. Neckers. 1995. Geldanamycin selectively destabilizes and conformationally alters mutated p53. *Oncogene* 11: 933–939.
12. Bonvini, P., T. Gastaldi, B. Falini, and A. Rosolen. 2002. Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), a novel Hsp90-client tyrosine kinase: down-regulation of NPM-ALK expression and tyrosine phosphorylation in ALK^+ $CD30^+$ lymphoma cells by the Hsp90 antagonist 17-allylamino,17-demethoxygeldanamycin. *Cancer Res.* 62: 1559–1566.
13. Yao, Q., R. Nishiuchi, Q. Li, A. R. Kumar, W. A. Hudson, and J. H. Kersey. 2003. FLT3 expressing leukemias are selectively sensitive to inhibitors of the molecular chaperone heat shock protein 90 through destabilization of signal transduction-associated kinases. *Clin. Cancer Res.* 9: 4483–4493.
14. Fumo, G., C. Akin, D. D. Metcalfe, and L. Neckers. 2004. 17-allylamino-17-demethoxygeldanamycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. *Blood* 103: 1078–1084.
15. Solit, D. B., F. F. Zheng, M. Drobnjak, P. N. Munster, B. Higgins, D. Verbel, G. Heller, W. Tong, C. Cordon-Cardo, D. B. Agus, et al. 2002. 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin. Cancer Res.* 8: 986–993.
16. Whitesell, L., and P. Cook. 1996. Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. *Mol. Endocrinol.* 10: 705–712.
17. Li, Z., A. Menoret, and P. Srivastava. 2002. Roles of heat-shock proteins in antigen presentation and cross-presentation. *Curr. Opin. Immunol.* 14: 45–51.
18. Schnaider, T., J. Somogyi, P. Csermely, and M. Szamel. 1998. The Hsp90-specific inhibitor, geldanamycin, blocks CD28-mediated activation of human T lymphocytes. *Life Sci.* 63: 949–954.
19. Schnaider, T., J. Somogyi, P. Csermely, and M. Szamel. 2000. The Hsp90-specific inhibitor geldanamycin selectively disrupts kinase-mediated signaling events of T-lymphocyte activation. *Cell Stress Chaperones* 5: 52–61.
20. Yorgin, P. D., S. D. Hartson, A. M. Fellah, B. T. Scroggins, W. Huang, E. Katsanis, J. M. Couchman, R. L. Matts, and L. Whitesell. 2000. Effects of geldanamycin, a heat-shock protein 90-binding agent, on T cell function and T cell nonreceptor protein tyrosine kinases. *J. Immunol.* 164: 2915–2923.
21. Smith, D. F., D. B. Schowalter, S. L. Kost, and D. O. Toft. 1990. Reconstitution of progesterone receptor with heat shock proteins. *Mol. Endocrinol.* 4: 1704–1711.

22. Kosano, H., B. Stensgard, M. C. Charlesworth, N. McMahon, and D. O. Toft. 1998. The assembly of progesterone receptor-hsp90 complexes using purified proteins. *J. Biol. Chem.* 273: 32973–32979.
23. Workman, P. 2004. Altered states: selectively drugging the Hsp90 cancer chaperone. *Trends Mol. Med.* 10: 47–51.
24. Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers. 1994. Inhibition of heat shock protein HSP90-pp60 v-*src* heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. USA* 91: 8324–8328.
25. Prodromou, C., S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90: 65–75.
26. Stebbins, C. E., A. A. Russo, C. Schneider, N. Rosen, F. U. Hartl, and N. P. Pavletich. 1997. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89: 239–250.
27. Obermann, W. M., H. Sondermann, A. A. Russo, N. P. Pavletich, and F. U. Hartl. 1998. In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J. Cell Biol.* 143: 901–910.
28. Panaretou, B., C. Prodromou, S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 1998. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J.* 17: 4829–4836.
29. Grenert, J. P., B. D. Johnson, and D. O. Toft. 1999. The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J. Biol. Chem.* 274: 17525–17533.
30. Miller, P., C. DiOrio, M. Moyer, R. C. Schnur, A. Bruskin, W. Cullen, and J. D. Moyer. 1994. Depletion of the *erbB-2* gene product p185 by benzoquinoid ansamycins. *Cancer Res.* 54: 2724–2730.
31. Mimnaugh, E. G., C. Chavany, and L. Neckers. 1996. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J. Biol. Chem.* 271: 22796–22801.
32. Schulte, T. W., and L. M. Neckers. 1998. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother. Pharmacol.* 42: 273–279.
33. Solit, D. B., H. I. Scher, and N. Rosen. 2003. Hsp90 as a therapeutic target in prostate cancer. *Semin. Oncol.* 30: 709–716.
34. Banerji, U., I. Judson, and P. Workman. 2003. The clinical applications of heat shock protein inhibitors in cancer—present and future. *Curr. Cancer Drug Targets* 3: 385–390.
35. Neckers, L. 2002. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol. Med.* 8(4 Suppl): S55–S61.
36. Sausville, E. A., J. E. Tomaszewski, and P. Ivy. 2003. Clinical development of 17-allylamino, 17-demethoxygeldanamycin. *Curr. Cancer Drug Targets* 3: 377–383.
37. Isaacs, J. S., W. Xu, and L. Neckers. 2003. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 3: 213–217.
38. Wright, J. M., P. L. Zeitlin, L. Cebotaru, S. E. Guggino, and W. B. Guggino. 2004. Gene expression profile analysis of 4-phenylbutyrate treatment of IB3-1 bronchial epithelial cell line demonstrates a major influence on heat-shock proteins. *Physiol. Genomics* 16: 204–211.
39. de Ruijter, A. J., R. J. Meinsma, P. Bosma, S. Kemp, H. N. Caron, and A. B. van Kuilenburg. 2005. Gene expression profiling in response to the histone deacetylase inhibitor BL1521 in neuroblastoma. *Exp. Cell Res.* 309: 451–467.
40. Kohlmann, A., C. Schoch, S. Schnittger, M. Dugas, W. Hiddemann, W. Kern, and T. Haferlach. 2003. Molecular characterization of acute leukemias by use of microarray technology. *Genes Chromosomes Cancer* 37: 396–405.
41. Batchu, R. B., M. A. Shammas, J. Y. Wang, J. Freeman, N. Rosen, and N. C. Munshi. 2002. Adeno-associated virus protects the retinoblastoma family of proteins from adenoviral-induced functional inactivation. *Cancer Res.* 62: 2982–2985.
42. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184: 747–752.
43. Clerici, M., M. L. Fusi, S. Ruzzante, S. Piconi, M. Biasin, D. Arienti, D. Trabattoni, and M. L. Villa. 1997. Type 1 and type 2 cytokines in HIV infection—a possible role in apoptosis and disease progression. *Ann. Med.* 29: 185–188.
44. Ahlers, J. D., N. Dunlop, D. W. Alling, P. L. Nara, and J. A. Berzofsky. 1997. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs: granulocyte-macrophage colony-stimulating factor and TNF-α synergize with IL-12 to enhance induction of cytotoxic T lymphocytes. *J. Immunol.* 158: 3947–3958.
45. Kostense, S., W. H. Sun, R. Cottey, S. F. Taylor, S. Harmeling, D. Zander, P. A. Small, Jr., and B. S. Bender. 1998. Interleukin 12 administration enhances Th1 activity but delays recovery from influenza A virus infection in mice. *Antiviral Res.* 38: 117–130.
46. Sreedhar, A. S., G. Nardai, and P. Csermely. 2004. Enhancement of complement-induced cell lysis: a novel mechanism for the anticancer effects of Hsp90 inhibitors. *Immunol. Lett.* 92: 157–161.
47. Imai, J., M. Maruya, H. Yashiroda, I. Yahara, and K. Tanaka. 2003. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J.* 22: 3557–3567.
48. Minami, Y., H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka, and I. Yahara. 2000. A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. *J. Biol. Chem.* 275: 9055–9061.
49. Yamano, T., S. Murata, N. Shimbara, N. Tanaka, T. Chiba, K. Tanaka, K. Yui, and H. Udono. 2002. Two distinct pathways mediated by PA28 and hsp90 in major histocompatibility complex class I antigen processing. *J. Exp. Med.* 196: 185–196.
50. Navon, A., and A. L. Goldberg. 2001. Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. *Mol. Cell* 8: 1339–1349.
51. Menoret, A., Z. Li, M. L. Niswonger, A. Altmeyer, and P. K. Srivastava. 2001. An endoplasmic reticulum protein implicated in chaperoning peptides to major histocompatibility of class I is an aminopeptidase. *J. Biol. Chem.* 276: 33313–33318.
52. Binder, R. J., N. E. Blachere, and P. K. Srivastava. 2001. Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. *J. Biol. Chem.* 276: 17163–17171.
53. Chen, D., and M. J. Androlewicz. 2001. Heat shock protein 70 moderately enhances peptide binding and transport by the transporter associated with antigen processing. *Immunol. Lett.* 75: 143–148.
54. McMullan, C. J., V. Poulaki, A. L. Kung, F. E. Davies, G. Morgan, M. Akiyama, R. Shringarpure, N. C. Munshi, P. G. Richardson, T. Hideshima, et al. 2006. Antimyeloma activity of heat shock protein-90 inhibition. *Blood* 107: 1092–1100.
55. Zaarur, N., V. L. Gabai, J. A. Porco, Jr., S. Calderwood, and M. Y. Sherman. 2006. Targeting heat shock response to sensitize cancer cells to proteasome and Hsp90 inhibitors. *Cancer Res.* 66: 1783–1791.
56. Richardson, P. G., C. S. Mitsiades, T. Hideshima, and K. C. Anderson. 2005. Novel biological therapies for the treatment of multiple myeloma. *Best Pract. Res. Clin. Haematol.* 18: 619–634.
57. Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers. 1994. Inhibition of heat shock protein HSP90-pp60 v-*src* heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. USA* 91: 8324–8328.
58. Stebbins, C. E., A. A. Russo, C. Schneider, N. Rosen, F. U. Hartl, and N. P. Pavletich. 1997. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89: 239–250.
59. Vidal, V., J. Dewulf, and G. M. Bahr. 2001. Enhanced maturation and functional capacity of monocyte-derived immature dendritic cells by the synthetic immunomodulator Murabutide. *Immunology* 103: 479–487.
60. Luft, T., P. Luetjens, H. Hochrein, T. Toy, K. A. Masterman, M. Rizkalla, C. Maliszewski, K. Shortman, J. Cebon, and E. Maraskovsky. 2002. IFN-α enhances CD40 ligand-mediated activation of immature monocyte-derived dendritic cells. *Int. Immunol.* 14: 367–380.
61. Bagatell, R., and L. Whitesell. 2004. Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol. Cancer Ther.* 3: 1021–1030.
62. Beliakoff, J., and L. Whitesell. 2004. Hsp90: an emerging target for breast cancer therapy. *Anticancer Drugs* 15: 651–662.
63. Goetz, M. P., D. O. Toft, M. M. Ames, and C. Erlichman. 2003. The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann. Oncol.* 14: 1169–1176.
64. Picard, D. 2002. Heat-shock protein 90, a chaperone for folding and regulation. *Cell. Mol. Life Sci.* 59: 1640–1648.
65. Aligue, R., H. Akhavan-Niak, and P. Russell. 1994. A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *EMBO J.* 13: 6099–6106.
66. Young, J. C., V. R. Agashe, K. Siegers, and F. U. Hartl. 2004. Pathways of chaperone-mediated protein folding in the cytosol. *Nat. Rev. Mol. Cell Biol.* 5: 781–791.
67. Neckers, L. 2003. Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. *Curr. Med. Chem.* 10: 733–739.
68. Soga, S., T. Kozawa, H. Narumi, S. Akinaga, K. Irie, K. Matsumoto, S. V. Sharma, H. Nakano, T. Mizukami, and M. Hara. 1998. Radicicol leads to selective depletion of Raf kinase and disrupts K-Ras-activated aberrant signaling pathway. *J. Biol. Chem.* 273: 822–828.
69. Chiosis, G., B. Lucas, A. Shtil, H. Huezo, and N. Rosen. 2002. Development of a purine-scaffold novel class of Hsp90 binders that inhibit the proliferation of cancer cells and induce the degradation of Her2 tyrosine kinase. *Bioorg. Med. Chem.* 10: 3555–3564.