Heat Shock Protein 90 Inhibitors Prolong Survival, Attenuate Inflammation, and Reduce Lung Injury in Murine Sepsis

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Rationale: Severe sepsis is the leading cause of death for patients in intensive care units. Patients with severe sepsis develop multiple organ failure, including acute lung injury (ALI), resulting from a deregulated inflammatory response. Inhibitors of the ubiquitous chaperone, heat shock protein 90 (Hsp90), block the activity of certain proinflammatory mediators *in vitro*. We hypothesized that Hsp90 inhibitors may ameliorate the inflammation and ALI associated with severe sepsis.

Objectives: To test the hypothesis that Hsp90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in a murine model of sepsis.

Methods: Male C57BL/6 mice received either one of two Hsp90 inhibitors, radicicol or 17-allylaminodemethoxygeldanamycin (17-AAG), 24, 12, 6, and 0 hours before receiving a lethal dose of endotoxin (6.75×10^4 endotoxin units/g body weight). Outcomes included survival and parameters of systemic inflammation (plasma neutrophil, cytokine, chemokine, and nitrite/nitrate levels), pulmonary inflammation (lung nuclear factor- κ B and myeloperoxidase activities, inducible nitric oxide synthase expression, inducible nitric oxide synthase-Hsp90 complex formation, and leukocyte infiltration), and lung injury (pulmonary capillary leak and lung function). **Measurements and Main Results:** Mice pretreated with vehicle and receiving endotoxin exhibited 100% 24-hour lethality, a dramatic increase in all parameters of systemic and pulmonary inflammation, increased capillary leak, and reduced lung function. Compared with them, mice receiving either radicicol or 17-AAG before endotoxin exhibited prolonged survival, reduced or abolished increases in systemic and pulmonary inflammatory parameters, attenuated capillary leak, and restored, normal lung function.

Conclusions: Hsp90 inhibitors may offer a new pharmacological tool in the management of severe sepsis and severe sepsis-induced ALI.

Keywords: Hsp90 inhibitors; sepsis; acute lung injury

Severe sepsis, defined as sepsis associated with acute organ dysfunction, results from a generalized inflammatory and procoagulant response to infection (1). Mortality from severe sepsis ranges from 30 to 50% despite advances in critical care (2). The incidence of sepsis has increased by 8.7% during a 20-year interval from 1979 to 2000 (3) and annual costs for treatment of sepsis are estimated at \$17 billion (4). Severe sepsis is associated with multiple organ failure, and a large fraction of these patients develop acute lung injury (ALI) or its severe form, the acute respiratory distress syndrome (ARDS) (3). Gram-negative sepsis is the leading cause of ALI/ARDS (5). ALI is character-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Patients with severe sepsis develop multiple organ failure, including acute lung injury, resulting from a deregulated inflammatory response. Inhibitors of heat shock protein 90 (Hsp90) block the activity of certain proinflammatory mediators *in vitro*.

What This Study Adds to the Field

Hsp90 inhibitors prolong survival, reduce or abolish systemic and pulmonary inflammation, and restore normal lung function in a murine model of sepsis.

ized by increased pulmonary neutrophil sequestration and disruption of pulmonary capillary integrity, resulting in pulmonary capillary leak and edema, reduced lung compliance, and impaired lung function.

Sepsis is associated with activation of proinflammatory mediators, including nuclear factor (NF)- κ B, an important proinflammatory transcription factor that mediates up-regulated expression of several proinflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)- α , IL-6, IL-8, and IL-1 β , critical for amplifying the inflammatory insult. Although these mediators are important for host defense against invading bacteria, their uncontrolled and excessive production ultimately contributes to multiple organ injury. Studies on animal models of severe sepsis have demonstrated a critical role of inflammation in the pathophysiology of sepsis. Inhibition of NF- κ B activation significantly improves survival in both lipopolysaccharide (6) and cecal ligation and puncture models of severe sepsis (7). The lung is an important and frequent target of inflammatory mediators in severe sepsis (8) and increased pulmonary NF- κ B activation is associated with adverse outcomes in sepsis-induced ALI (9). Activation of NF- κ B requires phosphorylation of, and subsequent dissociation from, its associated inhibitory protein I κ B (which masks its nuclear localization signal sequence) by I κ B kinase (IKK) (10). IKK exists in complexes with Hsp90, required for IKK stabilization and function (11). Consequently, Hsp90 inhibitors inhibit NF- κ B activation in various cell lines *in vitro* (12, 13).

Heat shock protein 90 (Hsp90), a homodimeric molecular chaperone constituting more than 3% of total cellular protein, is involved in folding and conformational regulation of several client proteins (14). ATP hydrolysis and ADP/ATP nucleotide exchange in the N-terminal domain of Hsp90 drive its chaperone function. Compounds referred to as "Hsp90 inhibitors," such as radicicol (RA) and geldanamycin and its derivatives, replace the nucleotide binding to Hsp90 with an affinity greater

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than that of ATP or ADP, resulting in client protein deactivation, destabilization, and degradation (15).

In this study, we tested the hypothesis that Hsp90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in a murine model of sepsis. Some of these data have been previously presented in abstract form (16, 17).

METHODS

Reagents and Animals

17-Allylaminodemethoxygeldanamycin (17-AAG) was obtained from the National Cancer Institute (Bethesda, MD). Anti-inducible nitric oxide synthase (iNOS) mouse monoclonal antibodies were from BD Biosciences Transduction Laboratories (Lexington, KY) for immunoprecipitation (cat. no. 610432) and from Upstate Cell Signaling Solutions/Millipore (Lake Placid, NY) for immunoblotting (cat. no. 06-573) experiments. Anti-Hsp90 mouse monoclonal antibodies were from BD Biosciences Transduction Laboratories (cat. no. 610419). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Male C57BL/6 mice (7–8 weeks age; Harlan, Indianapolis, IN) were used in all experiments. Mouse colonies were maintained under pathogen-free conditions with a 12:12 hour light:dark cycle. All animal care and experimental procedures were approved by the Animal Care Committee of the Medical College of Georgia (Augusta, GA).

Experimental Protocol

Stock solutions of LPS from *Escherichia coli* 0111:B4 were prepared in saline. Mice received vehicle (10% dimethyl sulfoxide [DMSO] in saline) or Hsp90 inhibitor (RA or 17-AAG, dissolved in 10% DMSO) intraperitoneally 24, 12, 6, and 0 hours before LPS administration (6.75×10^4 endotoxin units/g body weight). Mice were killed 0–18 hours after LPS by cervical dislocation and blood, collected by cardiac puncture, was immediately dissolved in 0.26 M ethylenediaminetetraacetic acid (EDTA) (5 μ l of EDTA:100 μ l of blood). Plasma was separated by centrifugation and stored at -80°C . The lungs were then flushed with 1 ml of ice-cold phosphate-buffered saline (PBS) (5 mM EDTA), excised, dipped in saline, and blotted dry. A portion of the lung was quickly snap-frozen in liquid nitrogen, crushed to powder in a pre-chilled mortar, and stored at -80°C . The remaining lung tissue was stored at -80°C .

Measurement of Plasma Cytokine and Chemokine Levels

Interleukin-12, TNF- α , IFN- γ , and monocyte chemoattractant protein (MCP)-1 levels were measured with cytometric bead arrays (cat. no. 552364; BD Biosciences Pharmingen, San Diego, CA). This is a sensitive flow cytometry-based immunoassay that uses a mixture of different beads, each having a distinct fluorescence intensity, and a capture surface for a specific protein that allows the simultaneous detection of all four proteins from a single plasma sample. Assay sensitivity is <11 pg/ml for all except MCP-1, for which the sensitivity is 52.7 pg/ml.

Measurement of Plasma Nitrite/Nitrate Levels

Plasma was diluted in PBS and deproteinized in Amicon YM-10 tubes (10-kD cutoff; Millipore, Bedford, MA) and stored at -80°C . Twenty-five microliters of diluted plasma or of known concentrations of sodium nitrate (serving as standards) was added to 25 μ l of nitrate reductase (1 U/1.5 ml) and 25 μ l of NADPH (0.134 mg/ml), both dissolved in 40 mM Tris, pH 7.6, and incubated at room temperature for 3 hours. One hundred microliters of Griess reagent (1:1 mix of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine) was then added and incubated for 10 minutes at room temperature, and the absorbance of the samples was recorded at 540 nm (reference, 650 nm). The concentration of nitrite/nitrate was determined by comparison with a standard curve generated with sodium nitrate.

Measurement of Circulating Blood Leukocytes and Neutrophils

Mice were anesthetized with pentobarbital (90 mg/kg, intraperitoneal) 12 hours after vehicle or LPS administration and blood was removed by cardiac puncture. An aliquot of whole blood was diluted 1:50 in 0.1 N

HCl (to lyse red blood cells) and placed on a hemocytometer, and the number of leukocytes was determined by manual counting. Neutrophil numbers were also determined manually (as the number of polymorphonuclear cells [PMNs] in 500 leukocytes counted and then corrected to the total number of circulating leukocytes in that animal) in a blood smear stained with Wright's stain.

Assay of Lung Myeloperoxidase Activity

Frozen lung samples were homogenized in 20 mM K_2HPO_4 buffer (30 μ l/mg tissue; pH 7.4) and centrifuged at $20,000 \times g$ for 30 minutes. The pellet was resuspended in 50 mM K_2HPO_4 (pH 7.4) containing hexadecyltrimethylammonium bromide (5 mg/ml; Fluka, Buchs, Switzerland), sonicated for 90 seconds, incubated for 2 hours at 60°C , and centrifuged at $14,000 \times g$ for 10 minutes. The supernatants were assayed for MPO activity, using kinetic readings over 3 minutes (25- μ l sample with 725 μ l of reaction buffer containing 50 mM K_2HPO_4 , *o*-dianisidine [0.167 mg/ml], and 0.15 mM H_2O_2). MPO activity was expressed as rate of change in absorbance (dA) per minute per 100 mg of tissue.

Immunohistochemical Detection of Lung Myeloperoxidase

Mice were killed by cervical dislocation and lungs were excised and immersed briefly in saline, blotted dry, and immersed into vials containing 10% neutral buffered formalin. Sections (4 μ m) were cut from paraffin blocks and mounted on treated slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Slides were air dried overnight, placed in a 60°C oven for 30 minutes, deparaffinized in xylene, and run through graded alcohol to distilled water. Endogenous peroxidase was quenched with 0.3% H_2O_2 for 5 minutes, followed by two rinses with distilled water. Slides were pretreated with target retrieval solution, citrate pH 6 (Dako Corporation, Carpinteria, CA), rinsed in distilled water, incubated in Power Block (Biogenex Laboratories, Inc., San Ramon, CA), rinsed in distilled water, placed in PBS for 5 minutes, incubated with primary antibody (anti-myeloperoxidase [anti-MPO], diluted 1:2,000; MP Bio-medicals, Irvine, CA) for 30 minutes at room temperature followed by two rinses in PBS, incubated with secondary peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (EnVision+; Dako) for 30 minutes, and rinsed in PBS. Bound antibody was detected with diaminobenzidine (DAB+ substrate kit; Dako). Hematoxylin was used for counterstaining.

Assay of Lung NF- κ B Activity

NF- κ B activity was measured as the nuclear translocation and DNA binding of the p65 subunit in 2.5- μ g nuclear extracts from lung tissues, using a commercially available ELISA (TransAM NF κ B p65, cat. no. 40096; Active Motif, Carlsbad, CA).

Assessment of Lung Capillary Leak

Mice were anesthetized intraperitoneally 12 hours after LPS administration, with ketamine (80 mg/kg) and xylazine-HCl (8 mg/kg). Evans blue dye (EB) dissolved in saline was injected (100 mg/kg) through the left jugular vein, using a 30-gauge needle inserted to PE-10 tubing. After 30 minutes, blood was withdrawn via cardiac puncture and stored at 4°C . The lungs were flushed with 1 ml of EDTA-PBS (pH 7.4, 4°C), excised, rinsed in saline, blotted dry, snap-frozen in liquid nitrogen, and stored at -80°C . Frozen lungs were homogenized in ice-cold PBS (1 ml/100 mg tissue), incubated with 2 volumes of formamide (60°C , 18 h), and centrifuged ($5,000 \times g$ for 30 min), and supernatant absorbance at 620 nm (A_{620}) and 740 nm (A_{740}) was recorded. Tissue EB content was calculated by correcting the A_{620} optical density for the presence of heme pigments: $A_{620}(\text{corrected}) = A_{620} - (1.426 \times A_{740} + 0.030)$ and by then comparing this value with a standard curve of EB in formamide-PBS. Total EB leak was expressed as lunge_{EB} content divided by serum_{EB} content.

Lung iNOS and Hsp90 Expression and Complex Analysis

Portions of stored lung samples were homogenized (Dounce; Fisher Scientific) in 1 ml of ice-cold modified radioimmunoprecipitation buffer with protease inhibitors, sodium orthovanadate, and sodium molybdate. After end-over-end rotation at 4°C , they were centrifuged at $21,000 \times g$ for 15 minutes and the supernatants were stored in -80°C . For immunoprecipitation experiments, 500 μ g of protein was precleared with 20 μ l of 50% A/G agarose beads (cat. no. sc-2003; Santa Cruz

Biotechnology, Santa Cruz, CA) for 1 hour at 4°C, beads were pelleted ($1000 \times g$ for 5 min), and the supernatant was incubated overnight at 4°C with 2.5 µg of anti-iNOS monoclonal antibody or IgG as negative control. Forty microliters of 50% A/G agarose beads was added and incubated for 1 hour at 4°C, centrifuged ($1,000 \times g$ for 5 min), washed three times, suspended in $2 \times$ sodium dodecyl sulfate sample buffer, immersed for 5 minutes in boiling water, and centrifuged, and the supernatant was loaded on a 7.5% polyacrylamide gel for electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences, Piscataway, NJ), reacted with primary antibodies of interest, incubated with appropriate horseradish peroxidase-conjugated secondary antibodies, and detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences).

Lung Function Measurements

Mice were anesthetized intraperitoneally with pentobarbital (90 mg/kg), tracheostomized with a metal 1.2-mm (internal diameter) cannula, and connected to a flexiVent (SCIREQ, Inc., Montreal, PQ, Canada) ventilator. Ventilation was initiated at a tidal volume of 10 ml/kg and a rate of 150/min. A TLC maneuver was performed, followed 15 seconds later by a sinusoidal 1-Hz oscillation. A single-compartment model was fit to these data to calculate dynamic compliance of the respiratory system. Subsequently, an 8-second forced oscillatory signal (0.5–19.6 Hz) was applied, the mechanical input impedance of the respiratory system was calculated, and a model containing a constant-phase tissue compartment was fit to input impedance in order to evaluate tissue elastance. Dynamic pressure–volume maneuvers were then performed by stepwise increasing airway pressure to 30 cm H₂O and then reversing the process.

Bronchoalveolar Lavage

Lungs were lavaged with three instillations of 1 ml of PBS, pH 7.2, containing 0.6 mM EDTA. Cells were pelleted and resuspended in Tris-buffered ammonium chloride, pH 7.2, to lyse the red blood cells, centrifuged, resuspended, fixed in 4% neutral buffered formalin, affixed onto slides, and stained with modified Wright's stain to determine the total number of white blood cells.

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical analyses between groups were performed using one-way analysis of variance or Student *t* tests between two groups, as appropriate. $P < 0.05$ was considered significant.

Figure 1. Heat shock protein 90 (Hsp90) inhibitors improve survival after lipopolysaccharide administration (post-LPS). C57BL/6 mice received four intraperitoneal injections of vehicle (LPS + dimethyl sulfoxide [DMSO]; $n = 8$), radicicol (LPS + RA; $n = 8$), or 17-allylaminodemethoxygeldanamycin (LPS + 17-AAG; $n = 7$) 24, 12, 6, and 0 hours before a single intraperitoneal injection of LPS. LPS + DMSO versus LPS + RA: $P < 0.0001$, log-rank analysis. LPS + DMSO versus LPS + 17-AAG: $P < 0.03$, log-rank analysis.

RESULTS

Hsp90 Inhibitors Prolong Survival after a Lethal Dose of LPS

Vehicle (DMSO)-pretreated mice receiving a bolus LPS injection exhibited 100% mortality by 22 hours post-LPS. Conversely, mice pretreated with either RA or 17-AAG before the same lethal dose of LPS was administered exhibited a significant prolongation in survival (Figure 1). 17-AAG pretreatment increased survival to 50% by 59 hours post-LPS, whereas 50% of RA-pretreated mice remained alive by 110 hours post-LPS, and 37.5% of them survived for at least 28 days post-LPS, at which time the study was terminated and the animals were killed. Pretreatment with 17-AAG or RA did not produce any observable behavioral effects, before LPS administration; however, mice exhibited normal grooming behavior, less diarrhea, and reduced ocular inflammation post-LPS, compared with vehicle-pretreated and LPS-injected mice.

Figure 2. Heat shock protein 90 (Hsp90) inhibitors attenuate the increase in plasma chemokines and cytokines in severe sepsis. (A–D) Plasma chemokine (A) and cytokine (B–D) levels in LPS-treated mice, pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1, and killed at the indicated times post-LPS. * $P < 0.05$ compared with 0-hour measurement; # $P < 0.05$ compared with corresponding time value of the LPS + dimethyl sulfoxide (DMSO) group ($n \geq 5$ at all time points and groups). Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; MCP-1 = monocyte chemoattractant protein-1; RA = radicicol; TNF-α = tumor necrosis factor-α.

Figure 3. Heat shock protein 90 (Hsp90) inhibitors prevent plasma nitrite and nitrate (NO_x) production and neutropenia in severe sepsis. (A) Time course of increase in plasma NO_x levels after a single intraperitoneal injection of LPS to mice ($*P < 0.01$ compared with 0-h value; $n \geq 6$ at all time points). (B) Levels of plasma NO_x 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1. $*P < 0.01$ compared with vehicle; $\#P < 0.01$ compared with vehicle + LPS ($n \geq 6$ in all groups). Error bars represent the SEM. (C) Blood neutrophil (PMN) numbers in LPS-treated mice, pretreated with vehicle (VEH) or Hsp90 inhibitors, as described in Figure 1, and killed 12 hours post-LPS. $*P < 0.05$ compared with vehicle; $\#P < 0.05$ compared with vehicle + LPS ($n \geq 5$ in all groups); $\dagger P < 0.05$ from LPS+RA. Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; RA = radicicol.

Hsp90 Inhibitors Prevent the Systemic Inflammatory Response to LPS

In vehicle-pretreated mice, LPS caused an immediate and dramatic increase in plasma MCP-1 and TNF- α levels. Whereas MCP-1 levels remained elevated for the subsequent 12 hours, TNF- α levels exhibited a time-dependent decline, but were still

Figure 4. Heat shock protein 90 (Hsp90) inhibitors attenuate lung nuclear factor (NF)- κB activation in severe sepsis. Shown is the amount of NF- κB subunit p65 in LPS-treated mice, pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1, and killed 2 hours post-LPS. $*P < 0.05$ compared with vehicle; $\#P < 0.05$ compared with vehicle + LPS ($n \geq 5$ in all groups). Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; O.D. = optical density; RA = radicicol.

significantly elevated at 12 hours post-LPS. Both Hsp90 inhibitors significantly prevented the rise in plasma levels of both MCP-1 and TNF- α to approximately one-fifth to one-eighth of the peak concentrations (Figures 2A and 2B). The increase in plasma levels of IFN- γ and IL-12 in vehicle-pretreated, LPS-

Figure 5. Heat shock protein 90 (Hsp90) inhibitors attenuate the increase in lung myeloperoxidase (MPO) activity in severe sepsis. (A) Time course of lung MPO activity (indicative of neutrophil sequestration in the lung) post-LPS ($*P < 0.05$ from vehicle; $n \geq 6$ at each time point). (B) Lung MPO activity 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1. $*P < 0.05$ from vehicle; $\#P < 0.05$ from vehicle + LPS ($n = 6$). Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; dA = rate of change in absorbance; RA = radicicol.

Figure 6. Heat shock protein 90 (Hsp90) inhibitors attenuate leukocyte infiltration in lung parenchyma and bronchoalveolar lavage (BAL) fluid in severe sepsis. (A–E) Myeloperoxidase (MPO) immunostaining of lung sections of mice pretreated with vehicle or Hsp90 inhibitors, 12 hours after administration of vehicle or LPS, as described in Figure 1. (F) Total white blood cell content of BAL, obtained 12 hours after vehicle or LPS was administered to mice pretreated with vehicle or Hsp90 inhibitors ($n = 8$). * $P < 0.05$ from vehicle; # $P < 0.05$ from vehicle + LPS group. (G–I) Hematoxylin-and-eosin staining of lung sections from mice pretreated with vehicle (VEH) or radicicol (RA), 12 hours after receiving vehicle or LPS. 17-AAG = 17-allylaminodemethoxygeldanamycin. Scale bar, 50 μm .

treated mice exhibited different dynamics: IFN- γ levels peaked at 12 hours post-LPS, whereas IL-12 levels reached a maximum at 6 hours and then declined to control levels by 12 hours post-LPS. Both RA and 17-AAG completely prevented the increase in IFN- γ (Figure 2C) and reduced plasma IL-12 levels to significantly below pre-LPS levels (Figure 2D).

Vehicle-pretreated, LPS-injected mice exhibited a time-dependent massive increase in plasma nitrite and nitrate (NO $_x$), which peaked at 12 hours post-LPS (Figure 3A). Both RA and 17-AAG essentially abolished the increase in NO $_x$, as shown at the 12-hour post-LPS measurement in Figure 3B.

Vehicle-pretreated, LPS-injected mice exhibited a profound decrease in circulating leukocytes ($1,997 \pm 110$ vs. $4,213 \pm 479$ for vehicle; $P < 0.01$) that was not prevented by pretreatment with Hsp90 inhibitors ($2,016 \pm 74$; $P < 0.01$ compared with vehicle). By themselves, Hsp90 inhibitors did not affect circulating white blood cell numbers ($4,852 \pm 378$; not significantly different from vehicle). Vehicle-pretreated, LPS-injected mice also exhibited a profound decrease in circulating PMNs; however, both RA and 17-AAG prevented the decrease in circu-

lating neutrophils (Figure 3C). Radicicol, in fact, increased circulating PMN levels significantly above levels observed in vehicle- and 17-AAG-treated mice.

Hsp90 Inhibitors Attenuate the Pulmonary Inflammatory Response to LPS

We tested the hypothesis that the observed protective effects of Hsp90-binding inhibitors in severe sepsis were associated with reduced activation of NF- κB *in vivo*. Preliminary time course analysis of activated p65 (one of the two NF- κB subunits) translocation into nuclear fractions of lung tissue revealed an early increase 2 hours post-LPS, which was reduced by 50% at 12 hours post-LPS (data not shown). We therefore selected the 2-hour post-LPS time point to investigate the effects of RA and 17-AAG. Vehicle-pretreated, LPS-injected mice exhibited a 3-fold increase in the amount of translocated, active p65 levels in lung nuclear extracts, compared with mice not given LPS (Figure 4). Pretreatment of mice with either of the two Hsp90 inhibitors significantly attenuated the LPS-induced increase in NF- κB activation and translocation (Figure 4).

Figure 7. Heat shock protein 90 (Hsp90) inhibitors prevent increased lung inducible nitric oxide synthase (iNOS) expression and Hsp90-iNOS complex formation in severe sepsis. (A) Lung Hsp90 and iNOS expression 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1 ($n \geq 6$). (B) Densitometric analysis of data in (A); * $P < 0.01$ compared with vehicle; # $P < 0.01$ compared with vehicle + LPS. (C) Hsp90-iNOS coimmunoprecipitation experiments 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1 ($n = 4$). (D) Densitometric analysis of data in (C); * $P < 0.01$ compared with vehicle; # $P < 0.01$ compared with vehicle + LPS. Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; IB = immunoblot; IP = immunoprecipitation; OD = optical density; RA = radicicol.

We investigated lung neutrophil infiltration by measuring the activity of lung MPO, a neutrophil-specific enzyme. In vehicle-pretreated mice, there was an immediate and sustained increase in lung MPO activity after LPS injection (Figure 5A); both RA and 17-AAG significantly prevented the increase in lung MPO activity, as shown in Figure 5B, 12 hours post-LPS. This observation was further investigated by anti-MPO immunocytochemistry of lung tissue 12 hours post-vehicle or LPS (Figure 6). Lung tissues from vehicle-pretreated, LPS-injected mice exhibited increased MPO staining (Figure 6B compared with Figure 6A) and frequent plugging of capillaries by MPO-expressing cells (*see insets*). MPO staining appeared reduced in tissues from animals pretreated with either Hsp90 inhibitor (Figures 6D and 6E) and capillary plugging by stained cells appeared absent (*see insets*). Similarly, there was a 50% increase in the number of leukocytes (mostly macrophages) in bronchoalveolar lavage fluid of vehicle-pretreated, LPS-injected mice, which was completely prevented by RA or 17-AAG pretreatment (Figure 6F). Histologic evaluation of lung tissue (hematoxylin-and-eosin staining) suggested a strong inflammatory response to LPS (Figure 6G), which appeared to be ameliorated by pretreatment with RA or 17-AAG (Figures 6H and 6I).

We hypothesized that reduction in iNOS expression and activity might contribute to the beneficial effects of Hsp90 inhibitors in LPS-induced sepsis. Vehicle-pretreated, LPS-injected mice exhibited a 14-fold increase in the expression of lung iNOS (Figures 7A and 7B). Even though no increase in Hsp90 expression was observed, there was a concomitant threefold increase in Hsp90-iNOS association (Figures 7C and 7D). Both Hsp90 inhibitors completely abolished the increase in iNOS expression and iNOS-Hsp90 association (Figure 7).

Hsp90 Inhibitors Prevent Lung Injury and Maintain Lung Function in LPS-induced Sepsis

Capillary endothelial barrier function (as reflected by the EB extravasation method) after LPS administration was compro-

mised in a time-dependent manner, reaching a twofold peak increase relative to control at 12 hours post-LPS (Figure 8A). Mice pretreated with RA or 17-AAG exhibited a significant and nearly 50% reduction in capillary barrier dysfunction 12 hours post-LPS (Figure 8B).

We investigated whether Hsp90 inhibitors would prevent the respiratory dysfunction associated with severe sepsis. At 12 hours post-LPS, vehicle-pretreated mice exhibited decreased respiratory system compliance (Figure 9A), increased lung tissue elastance (Figure 9B), and a rightward shift in the airway pressure-volume curve (Figure 9C), reflecting a significant deterioration of lung function. Both Hsp90 inhibitors completely restored lung function, as reflected in normal airway pressure volume curves, respiratory system compliance, and tissue elastance (Figure 9).

DISCUSSION

Hsp90 inhibitors have long been used experimentally, even before their true mechanism of action was appreciated. Thus, geldanamycin has been used as a tyrosine kinase inhibitor before it became evident that tyrosine kinases are Hsp90 client proteins and that geldanamycin attenuates their activity by inhibiting Hsp90 (18). More recently, two geldanamycin analogs, 17-AAG and DMAG, have been tested as new adjuncts to chemotherapy and have undergone successful phase I and II clinical trials (19). In these trials, the dose of 17-AAG (in mg/m² body surface area) was severalfold higher than that used in the present experiments (19). After intraperitoneal administration to mice, 17-AAG exhibits excellent bioavailability (99%), an elimination rate constant k_e of 0.03 minute⁻¹, and distribution to most tissues and major organs, including the lungs (20).

In the presence of ATP, the active Hsp90 promotes folding, stability, maturation, and/or activation of numerous client proteins regulating growth, survival, and inflammation (11, 14).

Figure 8. Heat shock protein 90 (Hsp90) inhibitors attenuate the increase in pulmonary capillary leak in severe sepsis. (A) Time course of lung capillary leak (inferred from Evans blue dye extravasation in the lung and expressed as the ratio of lung to serum Evans blue optical density [O.D.]) post-LPS in vehicle-pretreated mice (* $P < 0.05$ from vehicle; $n \geq 9$ at each time point). (B) Lung capillary leak at 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1 (* $P < 0.05$ compared with vehicle; # $P < 0.05$ compared with vehicle + LPS ($n = 9$). Error bars represent the SEM. 17-AAG = 17-allylaminodemethoxygeldanamycin; RA = radicicol.

Conversely, in the absence of ATP or in the presence of the Hsp90 inhibitors, recruitment of specific cochaperones (most prominently Hsp70) occurs, promoting client protein deactivation, ubiquitination, and proteasomal degradation (15, 21). There is accumulating evidence of a proinflammatory role of the active, Hsp90 dimer in various models of inflammation (13, 22, 23).

Gram-negative sepsis remains the leading cause of ALI/ARDS (24). Injection of bacterial LPS precipitates a systemic inflammatory response, which in certain—but not all—ways resembles the clinical profile of sepsis, including microvascular lung injury and ARDS (25). Using this model, we show that either RA or 17-AAG pretreatment significantly prolongs survival and reduces inflammation after an otherwise lethal dose of LPS. Mice that received LPS alone exhibited significant injury to the lung, which may have contributed to lethality. It is difficult to determine the exact cause of death in this paradigm, because severe sepsis is a complex syndrome characterized by systemic inflammation, multiple organ injury, coagulation, and impaired fibrinolysis. Patients with severe sepsis may also present with cardiovascular, pulmonary, renal, hepatic, and/or neurological abnormalities and death may result from failure of one or more of these critical organs. Because mice receiving Hsp90 inhibitors showed a dramatic abrogation of lung injury, it is possible that the improved respiratory function might have contributed to their survival. However, it is also likely that RA or

Figure 9. Heat shock protein 90 (Hsp90) inhibitors prevent lung dysfunction in severe sepsis. (A) Respiratory system compliance; (B) lung tissue elastance; (C) dynamic pressure-volume (P-V) loops. All measurements were performed 12 hours after vehicle or LPS administration in mice pretreated with vehicle or Hsp90 inhibitors, as described in Figure 1. Error bars represent the SEM. Where not shown, SEM bars are within the symbol. * $P < 0.05$ compared with vehicle; # $P < 0.05$ compared with vehicle + LPS-treated group ($n = 8$ in every group and for every pressure point). 17-AAG = 17-allylaminodemethoxygeldanamycin; RA = radicicol.

17-AAG might have reduced mortality by exerting protective effects on other major organs, a topic of current investigation. TNF- α is an initiator of the coagulation cascade in sepsis; other inflammatory mediators can elevate levels of plasminogen activator inhibitor, thus suppressing the fibrinolytic system. Moreover, TNF- α is an important mediator of acute renal failure in severe sepsis (26). Because TNF- α and other inflammatory mediators were maintained at near- or below-normal levels in mice receiving RA or 17-AAG, we speculate that these mice had reduced coagulation problems and improved cardiovascular and renal function, which also contributed to their prolonged survival. In addition, IFN- γ is a primary mediator of macrophage

activation in mice (27) and, therefore, the dramatic abrogation of IFN- γ plasma levels observed in RA and 17-AAG-pretreated mice likely contributed to reduced macrophage activation. Both Hsp90 inhibitors prevented LPS-induced neutropenia; it is unclear why radicicol-treated mice exhibited blood PMN levels higher than 17-AAG- or even vehicle-treated mice, but perhaps radicicol—in our experience, the most effective Hsp90 inhibitor—blocks neutrophil migration.

Gram-negative sepsis is associated with upregulated expression and activity of iNOS (NOS2) in numerous tissues including macrophages, neutrophils, and lung parenchymal tissue, which is thought to play a detrimental role in ALI/ARDS pathophysiology and to contribute to mortality (28). Furthermore, interaction of large (NOS2-derived) amounts of NO with reactive oxygen species ($O_2^{\cdot-}$) results in the formation of toxic intermediates, such as ONOO $^-$, which nitrate or S-nitrosylate proteins critical for maintaining normal homeostasis and for protecting the lung and other organs from oxidative injury (29). iNOS associates with Hsp90 to dose-dependently increase its activity and NO output; NO production is significantly impaired by Hsp90 inhibitors *in vitro* (30). We observed a rise in pulmonary immunoreactive iNOS. It is unclear whether the increased Hsp90–iNOS association was a result of increased iNOS expression, more efficient complex formation, or both. Regardless the source, increased iNOS–Hsp90 association likely contributed to significantly higher NO output, as confirmed by dramatically increased plasma levels of NO $_x$. Both Hsp90 inhibitors decreased iNOS expression and iNOS–Hsp90 association. iNOS is a major cause of inflammation, ALI, and mortality in several models of severe sepsis and selective inhibition of iNOS attenuates sepsis-induced ALI (31, 32). It is thus possible that, at least in part, Hsp90 inhibitors exert their protective actions in sepsis by reducing iNOS expression, iNOS–Hsp90 association, and iNOS-derived NO production. Furthermore, NO $_x$ levels produced by freshly isolated murine PMNs activated with LPS *in vitro* were also significantly reduced after pretreatment with Hsp90 inhibitors (data not shown). Hsp90 also associates with NOS3/eNOS (endothelial nitric oxide synthase), which, like iNOS, results in enhanced eNOS activation (33). LPS reduces mRNA and protein expression of eNOS in bovine endothelial cells (34). We observed a similar decrease in eNOS protein expression 12 hours post-LPS in lung homogenates (data not shown). Considering reports on the proinflammatory role of eNOS (35, 36), it is possible that reduced eNOS–Hsp90 complex formation and eNOS activation could have contributed to the protective effects of Hsp90 inhibitors.

Lung nuclear extracts of LPS-injected and vehicle-pretreated mice exhibited a dramatic increase in DNA-bound, activated p65 (NF- κ B subunit), which was significantly attenuated in Hsp90 inhibitor-treated mice, suggesting that NF- κ B may be one of the targets through which RA and 17-AAG might exert their protective effect in severe sepsis. In addition, Hsp90 associates with Toll-like receptor-4 (TLR4)–MD2 cluster in response to LPS (37) and may be involved in the innate recognition of LPS (38). Other Hsp90 client proteins include Akt, as well as STAT3 (signal transducer and activator of transcription-3) (39) and Src kinases (40), which also play important roles in augmenting the proinflammatory response in ALI (41, 42) and may thus be additional targets for the protective effects of Hsp90 inhibitors.

Increased levels of cytokines and chemokines have been consistently shown to correlate with mortality and adverse clinical outcome in patients with sepsis and ARDS (43). Numerous randomized clinical trials have been performed to test the hypothesis that manipulation of 1 of the more than 200 putative mediators of inflammation can improve survival in critically ill patients with sepsis. To date, only the use of activated recombi-

nant protein C has shown an improvement in mortality (44). Other selective strategies for improving survival have failed (45, 46), suggesting that targeting single molecules may not be a wise strategy. The advantage of Hsp90 inhibitors may lie in their pleiotropic activities.

Prior induction of stress proteins by “heat shock” protects against LPS-induced vascular leakage (47) and ischemia–reperfusion and ventilator-induced lung injury (48, 49). It was hypothesized that induction of Hsp70 by heat shock is the principal mediator of the observed cytoprotective effect. These data agree with our current findings, because Hsp90 inhibitors have been shown repeatedly to up-regulate Hsp70 expression. Hsp70 is an important component of the open-conformation Hsp90 dimer complex, necessary for the recruitment of ligases and subsequent ubiquitination and proteasomal degradation of many proinflammatory client proteins of Hsp90. Taking all these findings together, it is likely that the protective effects of Hsp70 are mediated through the degradation of one or (more likely) multiple proinflammatory Hsp90 client proteins.

In summary, we present data demonstrating significant improvement in mortality, lung function, and local and systemic inflammation in a mouse model of severe sepsis-induced ALI. Further investigation is warranted to dissect whether this new antiinflammatory pathway might be beneficial not only as prevention, but also as therapy, subsequent to insult, and not only in sepsis-induced ALI but in other inflammatory diseases as well.

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