Antileukemic Activity of Shepherdin and Molecular Diversity of Hsp90 Inhibitors

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Background: Heat shock protein 90 (Hsp90) is a molecular chaperone that is involved in signaling pathways for cell proliferation, survival, and cellular adaptation. Inhibitors of Hsp90 are being examined as cancer therapeutic agents, but the molecular mechanism of their anticancer activity is still unclear. We investigated Hsp90 as a therapeutic target for acute myeloid leukemia (AML) by use of the Hsp90 inhibitor shepherdin (a novel peptidyl antagonist of the interaction between Hsp90 and survivin, which is a regulator of cell proliferation and cell viability in cancer).

Methods: We studied protein interactions by molecular dynamics simulations and conducted competition experiments by use of enzyme-linked immunosorbent assay (ELISA). Shepherdin[79–83], a novel variant carrying the survivin sequence from Lys-79 through Gly-83, or its scrambled peptide was made permeable to cells by adding the antennapedia helix III carrier sequence. Apoptosis, Hsp90 client protein expression, and mitochondrial dysfunction were evaluated in AML types (myeloblastic, monocytic, and chronic myelogenous leukemia in blast crisis), patient-derived blasts, and normal mononuclear cells. Effects of shepherdin on tumor growth were evaluated in AML xenograft tumors in mice (n = 6). Organ tissues were examined histologically. Results: Shepherdin[79–83] bound to Hsp90, inhibited formation of the survivin–Hsp90 complex, and competed with ATP binding to Hsp90. Cell-permeable shepherdin[79–83] induced rapid (within 30 minutes) and complete (with concentrations inducing 50% cell death = .008) without evidence interval = 505.8 to 2426; M) killing of AML types and blasts, but it did not affect normal mononuclear cells. Shepherdin[79–83] made contact with unique residues in the ATP pocket of Hsp90 (Ile-96, Asp-102, and Phe-138), did not increase Hsp70 levels in AML cells, disrupted mitochondrial function within 2 minutes of treatment, and eliminated the expression of Hsp90 client proteins. Shepherdin[79–83] abolished growth of AML xenograft tumors (mean of control group = 1698 mm

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reflect deregulated cytokine signaling (24), especially that of granulocyte–macrophage colony-stimulating factor modulation of signal transduction and activation of transcription 3 (25), and has been linked to unfavorable outcomes in patients with adult (26) or childhood (27) AML.

We have investigated shepherdin, a novel peptidomimetic inhibitor of the survivin–Hsp90 complex and of the function of Hsp90 (28), as an anticancer agent in AML.

**Materials and Methods**

**Cells and Cell Cultures**

The following cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): the human myeloblastic leukemia cell line HL-60, monocytic leukemia cell line THP-1, monoblast leukemia cell line U937, and chronic myelogenous leukemia in blast crisis cell line K562. The epithelial tumor cell lines, including the cervical carcinoma line HeLa and the breast adenocarcinoma line MCF-7, were also obtained from the ATCC. Cells were maintained in culture at 37 °C and in an atmosphere of 5% CO₂ and 95% air in RPMI 1640 medium containing 10 μM HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum.

Peripheral blood samples were obtained by venipuncture from normal healthy volunteers after written informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated from these samples by Histopaque (Sigma-Aldrich, St. Louis, MO) gradient density centrifugation and maintained isolated from these samples by Histopaque (Sigma-Aldrich, St. Louis, MO) gradient density centrifugation and maintained in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics, as described above. For mitogenic stimulation, PBMCs were plated in a 96-well plate with each well containing 2 × 10⁵ cells in 200 μL of medium and incubated with phosphate-buffered saline (PBS) or phytohemagglutinin (10 μg/mL); Sigma-Aldrich) at 37 °C in an atmosphere of 5% CO₂ and 95% air for up to 1 week. A blood sample was obtained, after written informed consent was provided and approval from an Institutional Review Board was given, from an 85-year-old patient diagnosed with secondary AML without maturation (M1 according to French–American–British classification) with 82,000 white blood cells/mm³ and 97% blasts. Patient-derived peripheral blasts were isolated as described above.

**Peptide Synthesis**

Peptides were synthesized by the W. M. Keck Biotechnology Research Center at Yale University School of Medicine or by Peptron, Inc. (Daejeon, South Korea). All peptides were synthesized by use of solid-phase chemistry, purified to homogeneity (i.e., >98% pure) by reverse-phase high-pressure liquid chromatography, and assessed by mass spectrometry. The shepherdin peptide, named for its binding to the "shepherding" chaperone Hsp90, containing the survivin sequence between Lys-79 and Leu-87 or the corresponding scrambled peptide as a control were synthesized as described (28). A novel shepherdin variant containing the 5-amino acid survivin sequence between Lys-79 and Gly-83 (KHSSG, termed shepherdin[79–83]) was made cell permeable by adding the helix III from the cell-penetrating antennapedia homeodomain sequence (underlined) to its aminoterminal end: RQIKIWFQNRMMKWKHSSG-CONH₂. The sequence of the cell-permeable control scrambled peptide was RQIKIWFQONRRMKWKSSGKHS-CONH₂. In some experiments, peptides were synthesized with an amino-terminal biotin group for detection in cell culture and in vivo studies. All peptides were dissolved in water buffered with PBS at pH 7.4 immediately before use; working dilutions of the peptides were not stored.

**Molecular Dynamics Simulations**

For the simulation studies, the carboxyl and amino termini of shepherdin[79–83] were considered to have no charge to avoid electrostatic artifacts from the attractions between free opposite charges at these termini. The side chain of Lys-79 was considered to be protonated, with a net charge of +1. The peptide was solvated with water in a periodic truncated octahedron that was large enough to contain the peptide and 0.9 nm of solvent on all sides. All solvent molecules within 0.15 nm of any peptide atom were removed. One Cl⁻ counterion was added to ensure electroneutrality of the solution. The starting structure was totally extended to avoid biases in the conformational search. The simulation (production run) was 200 nanoseconds long. In all simulations, the temperature was maintained close to the intended value of 300 K by weak coupling to an external temperature bath with a coupling constant of 0.1 picosecond (29). The peptide and the rest of the system were coupled separately to the temperature bath (29). The GROMOS96 force field (30), the simple point-charge water model (31), the LINCS (linear constraint solver) algorithm (32), and the Settle (an analytical version of the SHAKE and RATTLE algorithms) algorithm (33) were used. The density of the system was adjusted as described (29). All molecular dynamics simulation analyses of trajectories were performed with the GROMACS software package (34). Conformational cluster analysis of the 200-nanosecond trajectory for shepherdin[79–83] was performed as described (35).

The representative structure of the most populated cluster of shepherdin[79–83] without the antennapedia cell-penetrating sequence, corresponding to the most visited structures in the molecular dynamics simulation, was used for docking experiments on the amino-terminal domain of Hsp90. We used the Autodock program (36) for these experiments. The crystal structure of Hsp90 was taken from the protein data bank (code 1YET pdb). The original x-ray structure contains the ligand geldanamycin, which was removed from the active site to yield the apo-open form of Hsp90 (37). The docking procedure was carried out as described (28). The results of the clustering procedure and subsequent molecular dynamics refinements were classified by a two-step procedure. First, the docked conformations of the ligand peptides were listed in increasing energy order. The structure of the complex that corresponded to the global minimum energy was used as the starting point of the first refinement molecular dynamics run. Second, the ligand conformation with the lowest energy was used as a reference, and all conformations with a center of mass to center of mass distance of less than 3 Å from the reference were taken to belong to the first class. After a ligand was assigned to a class, it was not used again for other classes. The process was then repeated for all hitherto unclassified conformations until all conformations were put in a class. The representative structure of the most populated class was then used for the second molecular dynamics refinement run.
The two molecular dynamics runs of the complexes obtained after the Autodock runs were each 70 nanoseconds long.

**Peptide Binding and Competition Experiments**

Binding of shepherdin variants to Hsp90 was assessed by use of the enzyme-linked immunosorbent assay (ELISA), as described (28). Briefly, plastic microtiter wells were coated with increasing concentrations (0.09 – 200 μg/mL) of shepherdin peptides or their scrambled peptides, and unbound sites were blocked with 3% gelatin. The recombinant Hsp90 N-domain region (Hsp90 residues 1 – 272, 1 μg/mL), which contains the binding site for shepherdin, was produced in BL-21 *Escherichia coli* as a glutathione S-transferase fusion protein, further isolated from glutathione S-transferase by thrombin cleavage (28), and added to peptide-coated plates. After a 2-hour incubation at 22 °C, protein binding under the various conditions tested was detected with an antibody against Hsp90 and visualized by a peroxidase-conjugated secondary species-specific antibody. The reaction was quantified by absorbance at 405 nm. Alternatively, the recombinant Hsp90 N-domain protein (10 μg/mL) was immobilized on plastic microtiter wells and incubated with increasing concentrations of shepherdin variants or their scrambled peptides (0.09 – 200 μg/mL) for 2 hours at 22 °C. After addition of recombinant survivin (1 μg/mL), binding of survivin to shepherdin peptide-treated Hsp90 was determined by ELISA, as described above (28).

To assess the ability of shepherdin to displace ATP from the Hsp90–ATP complexes (38), 3 μg of recombinant Hsp90 N-domain was incubated with 10 mM ATP, 150 μM shepherdin[79 – 83], or 150 μM scrambled peptide in 200 μL of molydate buffer (10 mM Tris-HCl at pH 7.5, 5 mM MgCl₂, 10 mM NaMoO₄, and 0.2% Tween 20) for 2 hours at 4 °C under constant agitation. Samples were further incubated with 50 μL of γ-phosphate linked ATP-Sepharose (Boca Scientific) for 2 hours at 4 °C, and after washes in molydate buffer, the Sepharose-bound material was eluted in 5% sodium dodecyl sulfate (SDS) and examined by western blot analysis (28).

**Fluorescence Microscopy**

HL-60 cells or PBMCs were plated onto poly-L-lysine-coated coverslips at 1 × 10⁶ cells per mL of medium and incubated with cell-permeable biotinylated shepherdin peptides or their scrambled sequences at a final concentration of 50 μM. After a 1-hour incubation, cells were washed in PBS (pH 7.4), fixed in methanol overnight at –20 °C, and stained with a 1:1000 dilution of streptavidin–Texas red (Amersham Biosciences, Buckinghamshire, UK); to bind to the biotin attached to the cell-permeable shepherdin peptides) in 300 μL of PBS (pH 7.4) containing 0.1% Triton X-100. Intracellular penetration of the various peptides was visualized by fluorescence microscopy.

**Western Blot Analysis**

We incubated 1 × 10⁶ cells with cell-permeable shepherdin peptides, their scrambled sequences, or 17-AAG (Alexis Biochemicals) as indicated. Cells were collected by centrifugation (2005g for 10 minutes at 4 °C) and washed in PBS (pH 7.4). Cell extracts were prepared by incubating cells in 20 mM Tris-HCl (pH 7.2), 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA for 1–2 hours at 0 °C. Supernatants were collected by centrifugation (15700g for 30 minutes at 4 °C), and protein-normalized supernatants were separated by SDS-polyacrylamide gel electrophoresis on 12% gels. After transfer of the protein bands to polyvinylidene difluoride membranes (Immobilon P Transfer Membrane; Millipore Corp., Billerica, MA), nonspecific binding sites on the blots were blocked in 5% skim milk, and the blots were incubated with various primary antibodies against survivin, cytochrome c, Akt, cyclin-dependent kinase 6 (CDK-6), Hsp70, Hsp90, or β-actin for 16 hours. Protein bands with bound antibodies were detected with horseradish peroxidase–conjugated species-specific secondary antibodies and visualized by chemiluminescence (Amersham Biosciences).

**Cell Viability, Apoptosis, and Analysis of Mitochondrial Membrane Potential**

HL-60, K562, THP-1, or U937 AML cell lines, patient-derived blasts, or unstimulated or phytohemagglutinin-stimulated PBMCs (each at 1 × 10⁶ cells per mL) were incubated in 96-well plates with cell-permeable shepherdin peptides or their corresponding scrambled peptides for 30 minutes at 37 °C. At the end of the incubation, cultures were analyzed in triplicate for cell viability with a 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay at an absorbance of 405 nm, as described (28). Alternatively, 1 × 10⁶ AML cells per 1 mL were treated with shepherdin peptides or their corresponding scrambled peptides for 30 minutes at 37 °C. Cells were harvested and simultaneously analyzed for caspase activity by measuring the level of DEVDase activity (Asp-Glu-Val-Asp cleavage activity; green channel) and plasma membrane integrity by measuring the level of propidium iodide (red channel) (CaspaTag; Intergen, Burlington, MA) by multiparametric flow cytometry. In other experiments, HL-60 cells were labeled for 10 minutes in the dark with the mitochondrial membrane potential–sensitive fluorescent dye JC-1 (10 μg/mL of PBS; Molecular Probes). After three washes in PBS (pH 7.2), samples were incubated with shepherdin[79 – 83] or control scrambled peptide from 2 to 30 minutes and analyzed by flow cytometry to monitor changes in the red/green (FL-2/FL-1) fluorescence ratio to obtain the index of mitochondrial membrane depolarization. JC-1–stained cultures treated with carboxyl cyanide 3-chlorophenyl hydrazone were used as positive control for these experiments.

**Xenograft Tumor Model**

All experiments involving animals were approved by an institutional animal care and use committee. We injected 1 × 10⁷ HL-60 cells suspended in 200 μL of sterile PBS into both flanks of 6- to 8-week-old CB17 SCID/beige mice (Taconic Farms, Germantown, NY) and allowed the injected cells to grow as palpable tumors for 10 days. When tumors reached a volume of 100–150 mm³, animals were randomly assigned to one of the two groups (two tumors per mouse and three mice per group) that received saline or shepherdin[79 – 83] at 50 mg/kg for 11 consecutive days by intraperitoneal injection (200 μL per injection). Tumor measurements were taken daily with a caliper, and tumor volume was calculated with the formula [(length in millimeters) × (width in millimeters)^2]/2. At the end of the...
experiment, the various AML tumors, liver, kidneys, and lungs were harvested from both groups of animals. Tissue samples were fixed in formalin, embedded in paraffin, and sectioned. Sections were placed on slides and stained with hematoxylin–eosin or with antibodies against survivin (NOVUS Biologicals, Littleton, CO) or Akt (Cell Signaling Technology, Beverly, MA). Antibody binding was detected by immunohistochemistry and peroxidase-conjugated species-specific secondary antibodies and visualized with 3,3-diaminobenzidine as a chromophore. Sections were reviewed by the University of Massachusetts Memorial Cancer Center Veterinary Pathology Core. Images were captured through an Olympus microscope with an on-line charge-coupled device camera.

**Statistical Analysis**

All data were analyzed with two-sided unpaired t tests in the GraphPad software package for Windows (Prism version 4.0). A P value of .05 was considered as statistically significant. Values are expressed as means of triplicate or duplicate experiments. For analysis of xenograft tumor growth, data were corrected to account for within-animal correlations by calculating the within-animal volumes and calculating a best estimate of the tumor volume for each mouse. The mean and SEM were then calculated with mice as the data units. All statistical tests were two-sided.

**RESULTS**

**Identification of an Optimized Variant of Shepherdin**

Because the shepherdin residues between Lys-79 and Gly-83 had previously been identified as being essential for Hsp90 binding (28), we synthesized a new five-residue peptide containing the survivin sequence between residues 79 and 83, KHSSG (named shepherdin[79–83]), and investigated whether it could interact with Hsp90. Both shepherdin[79–87] (sequence, KHSSGAFL) and shepherdin[79–83] bound recombinant Hsp90 in the same saturable and dose-dependent fashion, but the scrambled control peptide corresponding to either peptide did not bind to Hsp90 (Fig. 1, A). Shepherdin[79–87] or shepherdin[79–83], but not their corresponding scrambled peptides, also inhibited the binding of recombinant survivin to Hsp90 with similar affinities and in a concentration-dependent manner (Fig. 1, B).

We next studied the predicted structure of the shepherdin[79–83]–Hsp90 complex by molecular dynamics simulations. In these simulations, shepherdin[79–83] did not have a preferred ordered secondary structure, and so the hydrophilic side chains and the backbone carboxyl and amino groups tended to maximize their interactions with the surrounding water solvent. Cluster analysis of the 200-nanosecond simulations determined that the main conformational family of shepherdin[79–83] was characterized by a slight bend geometry involving residues His-80, Ser-81, and Ser-82. Docking experiments that used shepherdin with this geometry predicted that shepherdin[79–83] bound to the ATP pocket of Hsp90 (Fig. 1, C and D). Two different orientations of shepherdin[79–83] were observed: one that corresponded to the global free-energy minimum structure of the shepherdin–Hsp90 complex and one that represented the most frequently obtained structure after statistical clustering of all the structures studied during the Autodock simulations. The sites of contact between Hsp90 and shepherdin[79–83] in either configuration overlapped (Fig. 1, C and D). In the global free-energy minimum configuration (Fig. 1, C), the side chain of His-80 in shepherdin[79–83] made hydrophobic contacts with Ile-96 and hydrogen bonded with Gly-97 in Hsp90, the side chain of Ser-81 in shepherdin[79–83]...
hydrogen bonded with the side chains of Asp-102 and Asn-106 in Hsp90, and Ser-82 in shepherdin[79–83] hydrogen bonded with Asn-51 and Phe-138 in Hsp90. In the most frequently obtained configurations (Fig. 1, D), His-80 in shepherdin[79–83] formed a hydrophobic interaction with Ile-96 in Hsp90 but was also involved in a new hydrogen bonding interaction with the side chain of Asp-54 in Hsp90; Ser-81 interacted with Asp-93 and Asn-106 in Hsp90, and Ser-82 interacted with Asn-106 and Asp-102 in Hsp90. Consistent with these molecular dynamics predictions, shepherdin[79–83] efficiently displaced ATP binding from the N-domain of recombinant Hsp90, whereas the scrambled peptide was ineffective (Fig. 1, E).

### Anti-AML Activity of Shepherdin[79–83]

Endogenous survivin was highly expressed by AML cell lines, including the myeloblastic cell line HL-60, the acute monocytic cell line THP-1, the monoblast leukemia cell line U937, and K562, the cell line from a blast transformation of chronic myelogenous leukemia (Fig. 2, A). Within 30 minutes after addition, cell-permeable variants of shepherdin[79–83], shepherdin[79–83], or their scrambled sequences accumulated equally well in all AML cell lines tested (Fig. 2, B, and data not shown). Under these experimental conditions, addition of shepherdin[79–87] or shepherdin[79–83] resulted in the rapid (within 30 minutes) and concentration-dependent (with concentrations inducing 50% cell death of 24–35 μM) complete killing of all AML cell lines (Fig. 2, C). In contrast, cell-permeable scrambled peptides did not essentially decrease AML cell viability in 30 minutes (Fig. 2, C). In addition, comparable concentrations of shepherdin[79–83] or shepherdin[79–83] but not their scrambled peptides, killed HeLa or MCF-7 cells, which are derived from epithelial tumor types, with equal activity (Fig. 2, D).

### Mechanisms of Shepherdin[79–83] Anti-AML Activity

The speed of shepherdin-mediated cell killing was further investigated. A 30-minute exposure of various AML cell types to shepherdin[79–83] (75 μM) was sufficient to induce high levels of caspase activity and the loss of plasma membrane integrity in all cells treated, as demonstrated by multiparametric flow cytometry (Fig. 3, A). In contrast, the corresponding cell-permeable scrambled peptide did not induce caspase activity or disrupt the integrity of the plasma membrane, as compared with untreated cultures (Fig. 3, A). Under these experimental conditions, treatment of HL-60 cells with shepherdin[79–83] resulted in the rapid discharge of mitochondrial cytochrome c in the cytosol, in a reaction that was completed within 10 minutes after peptide addition, as measured by western blot analysis (Fig. 3, B). To further characterize the speed at which shepherdin induced mitochondrial dysfunction, control or treated HL-60 cells were evaluated for changes in the membrane potential of mitochondria. A 2-minute exposure of HL-60 cells to shepherdin [79–83] resulted in collapse of mitochondrial membrane potential,
we used a suboptimal concentration (20 μM) of shepherdin[79–83] peptide and then analyzed after 2 minutes for differential ratios in red or green fluorescence changes that are indicative of loss of transmembrane potential, by flow cytometry. Cells treated with carboxyl cyanide 3-cholorphenyl hydrazone (CCCP) were used as a control. D) Loss of Hsp90 client proteins. HL-60 cells were left untreated or incubated with a suboptimal concentration (20 μM) of shepherdin[79–83] (K83-G83) or its scrambled (Sc) peptide (K83-G83 Sc), harvested after 12 hours, and examined by western blot analysis for the expression of survivin, Akt, cyclin-dependent kinase 6 (CDK-6), and β-actin with corresponding antibodies. Bands were visualized by chemiluminescence. E) Kinetics of Hsp90 inhibition. HL-60 cells were incubated with shepherdin[79–83] (K83-G83) or its scrambled peptide (K83-G83 Sc), harvested at the indicated times, and examined by western blot analysis for the expression of Akt with its corresponding antibody. Bands were visualized by chemiluminescence.

as demonstrated by JC-1 staining and flow cytometry, whereas such an exposure to the control scrambled peptide had no effect (Fig. 3, C).

We next investigated whether shepherdin treatment altered the expression levels of Hsp90 client proteins. For these experiments, we used a suboptimal concentration (20 μM) of shepherdin[79–83] that would not cause rapid cell killing. Overnight treatment of HL-60 cells with 20 μM shepherdin[79–83] resulted in the complete loss of expression of many Hsp90 client proteins, including survivin, Akt, and CDK-6, as compared with control untreated cultures or cells incubated with scrambled peptide (Fig. 3, D). In time-course experiments, treatment of HL-60 cells with shepherdin[79–83], but not scrambled peptide, induced loss of Akt expression as early as 30 minutes after treatment (Fig. 3, E); the loss of Akt expression may further contribute to the rapid kinetics of cell death induced by shepherdin, given the broad survival functions of this kinase.

Functional Diversity Between Shepherdin and 17-AAG

Because of the unique structure of the shepherdin–Hsp90 complex and because of the speed with which shepherdin can kill AML cells, we next investigated whether the anticancer mechanisms used by shepherdin and by 17-AAG (a clinically available prototype Hsp90 inhibitor) differed. Treatment of HL-60 cells with 17-AAG resulted in the time-dependent increased expression of Hsp70, as shown by western blot analysis (Fig. 4, A). In contrast, a 30-minute treatment of HL-60 cells with shepherdin[79–87], shepherdin[79–83], or their corresponding scrambled peptides did not alter the expression of Hsp70 (Fig. 4, B). Consistent with the data presented above, a 30-minute exposure of HL-60 cells to shepherdin[79–83], but not its corresponding scrambled peptide, was sufficient to produce concentration-dependent loss of cell viability in the entire cell population, as determined by a MTT assay (Fig. 4, C). In contrast, treatment with 17-AAG did not substantially affect HL-60 cell viability, even after 24 hours of treatment (Fig. 4, C). However, a 48-hour exposure of HL-60 cells to 17-AAG resulted in a 60%–65% decrease in cell viability (Fig. 4, C), which is consistent with its anticancer activity (39).

Selectivity of Shepherdin Anti-AML Activity

Mitogenic stimulation of PBMCs with phytohemagglutinin resulted in cell cycle progression and the increased expression of survivin; the reaction peaked 3 days after stimulation (Fig. 5, A), as previously observed (40). Shepherdin[79–87], shepherdin[79–83], or their scrambled sequences efficiently accumulated intracellularly in phytohemagglutinin-stimulated PBMCs, as assessed by fluorescent microscopy (Fig. 5, B). Treatment of phytohemagglutinin-stimulated PBMCs with shepherdin[79–87] or shepherdin[79–83] at concentrations sufficient to induce complete AML cell killing did not decrease the viability of PBMCs, as compared with control cultures treated with scrambled peptides (Fig. 5, C). In contrast, shepherdin[79–83] induced extensive killing of patient-derived AML peripheral blasts within 30 minutes of treatment, whereas a scrambled peptide was ineffective (Fig. 5, D).
Anti-AML Activity of Shepherdin[79–83] In Vivo

We injected HL-60 cells subcutaneously into both flanks of immunocompromised SCID/beige mice to give rise to exponentially growing tumors. When tumors reached a volume of 100–150 mm³, animals (three animals per group per experiment) were used in the following experiments. Within 1 hour after treatment of tumor-bearing mice, systemically administered shepherdin[79–83] had accumulated within the tumor mass, as detected by immunofluorescence microscopy, but tumors of saline-treated control animals showed no immunofluorescence (Fig. 6, A). Treatment of tumor-bearing animals with shepherdin[79–83] (50 mg/kg per day, intraperitoneally) for 11 days completely suppressed tumor growth (mean of control group = 1698 mm³, mean of treated group = 232 mm³; difference = 1466 mm³, 95% confidence interval = 503.8 to 2426; P = .008), whereas treatment with saline for 11 days had no inhibitory effect on tumor growth (Fig. 6, B). Immunohistochemical analysis of AML tumors revealed that shepherdin treatment induced a high level of apoptosis in tumor cells, as determined by nuclear fragmentation and the loss of expression of Hsp90 client protein survivin and Akt (Fig. 6, C). In addition, examination of kidney, liver, and lung tissue collected from shepherdin-treated animals at the end of treatment was unremarkable, compared with the saline group, indicating that prolonged shepherdin administration did not induce organ toxicity (Fig. 6, D).

DISCUSSION

In this study, shepherdin[79–83], a novel peptidyl antagonist of the survivin–Hsp90 interaction, was shown to act as an inhibitor of Hsp90, with strong and selective anti-AML activity both in vitro and in xenograft tumors. We also found unexpected molecular and functional diversity between the Hsp90 inhibitors shepherdin[79–83] and 17-AAG in their binding to Hsp90, their speed of tumor cell killing, and their ability to quickly disrupt mitochondrial function.

Our results reinforce previous observations that shepherdin acts as a genuine Hsp90 inhibitor (28), in that it binds to the ATP pocket of Hsp90, it can displace Hsp90-bound ATP, and it destabilizes multiple Hsp90 client proteins in vitro and in vivo. Compared with the earlier prototype shepherdin[79–87] (28), the novel variant shepherdin[79–83] has improved solubility at concentrations greater than 2 mM, does not require chemical retro-inversion (which uses d-amino acids), has a shorter primary sequence of only five amino acid residues, and has similar anticancer activities for epithelial and hematopoietic tumor cells. Thus, shepherdin[79–83] is a good lead compound for the development of novel human anticancer drugs (41).

We found unexpected diversity in the anticancer activities of the Hsp90 inhibitors shepherdin and 17-AAG. First, shepherdin and geldanamycin are predicted to use different contact sites in the Hsp90 ATPase pocket (37). Residue Asp-93 in Hsp90, which coordinates the binding of geldanamycin to the ATP pocket of the chaperone, is only marginally involved in the contacts made by shepherdin[79–83] (or its longer variant shepherdin[79–87]). In contrast, Hsp90 residues that are peripherally implicated or not implicated at all in geldanamycin binding, including Asn-51, Ile-96, Asp-102, and Phe-138 (37), make up most of the contact sites for shepherdin[79–83]. Second, shepherdin had more potent anticancer activity than 17-AAG. A 30-minute treatment of cells from a heterogeneous panel of AML cell types with shepherdin[79–83], but not with 17-AAG, was sufficient to kill all AML cells tested. A 48-hour treatment with 17-AAG was required to reduce AML cell viability by 50%–60%.

Third, shepherdin appeared to have at least two anticancer mechanisms. One mechanism induced the loss of expression of multiple Hsp90 client proteins involved in cell proliferation and cell survival, probably through their destabilization and proteasome-dependent destruction, which is reminiscent of the mechanism used by 17-AAG. The other anticancer mechanism used by shepherdin included the fast disruption of mitochondrial function, involving dissipation of the mitochondrial membrane potential within 2 minutes of treatment and release of mitochondrial cytochrome c within 10 minutes. These events were followed within 30 minutes by increased activity of the effector caspases (42,43). Although 17-AAG also disrupts mitochondrial functions, more time is required, possibly because of the delayed loss of Hsp90 client protein(s) such as Akt (39), which ultimately results in mitochondrial dysfunction.

Fourth, shepherdin treatment did not alter Hsp70 levels in AML cells, unlike 17-AAG treatment, which consistently increases the...
increased Hsp70 expression may be detrimental to the anticancer activity of 17-AAG because Hsp70 can suppress the mitochondrial permeability transition (46), inhibit apoptosome-associated caspase 9 processing (47), and preserve the integrity of lysosomes (48). The fact that shepherdin does not affect Hsp70 levels, which may be due to the extremely rapid induction of expression of Hsp70. The increased expression of Hsp70 that is observed after treatment with 17-AAG (44) may reflect release of heat shock factor 1 (Hsf1) from an inactive state maintained by Hsp90 (45), so that Hsf1 can induce downstream gene expression. Although increased Hsp70 expression is considered a surrogate biomarker for Hsp90 inhibition in human trials (7–10), increased Hsp70 expression may be detrimental to the anticancer activity of 17-AAG because Hsp70 can suppress the mitochondrial permeability transition (46), inhibit apoptosome-associated caspase 9 processing (47), and preserve the integrity of lysosomes (48). The fact that shepherdin does not affect Hsp70 levels, which may be due to the extremely rapid induction of

Fig. 5. Selectivity of shepherdin. A) Time course of survivin expression. Peripheral blood mononuclear cells (PBMCs) were stimulated with phytohemagglutinin (PHA), and extracts that were harvested at the indicated times were examined by western blot analysis for the expression of Hsp90 and survivin with corresponding antibodies. β-Actin was used as a loading control. Bands were visualized by chemiluminescence. B) Intracellular penetration of shepherdin. PBMCs were incubated with cell-permeable biotinylated shepherdin[79–87] (K79-L87), shepherdin[79–83] (K79-G83), or their corresponding scrambled peptides (K79-L87 Sc and K79-G83 Sc, respectively), labeled with streptavidin–phycoerythrin, and analyzed by phase-contrast or fluorescence microscopy. Original magnification was ×100. Scale bars are 30 μm. C) PBMC viability after shepherdin treatment. PHA-stimulated PBMCs (harvested at day 3 after PHA stimulation) were incubated with shepherdin[79–87] (K79-L87), shepherdin[79–83] (K79-G83), or their corresponding scrambled peptides (K79-L87 Sc and K79-G83 Sc, respectively) for 4 hours and analyzed for cell viability with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are the means from one representative experiment of at least two determinations with three replicates per point. D) Shepherdin activity on acute myeloid leukemia (AML) blasts. Patient-derived AML blasts were treated with shepherdin[79–87] (K79-L87), shepherdin[79–83] (K79-G83), or their corresponding control scrambled peptides (K79-L87 Sc and K79-G83 Sc, respectively) for 30 minutes (squares) or 1 hour (circles) and analyzed for cell viability by the MTT assay. Data are the means of a representative experiment with three replicates per point.

Fig. 6. Anti-acute myeloid leukemia (AML) activity of shepherdin in vivo. A) Intratumor accumulation of shepherdin. CB17 SCID/beige mice (three mice per group) with superficial HL-60 cell tumors (two tumors per mouse) were injected with saline or shepherdin[79–83] (50 mg/kg intraperitoneally) and then killed 1 hour later. Tumors were excised, and frozen sections of these tumors were stained with streptavidin conjugated to phycoerythrin and analyzed by phase-contrast (upper panel) or fluorescence (lower panel) microscopy. B) Inhibition of tumor growth. SCID/beige mice with HL-60 superficial xenograft flank tumors (six tumors per group of three mice, and two tumors per mouse) were treated daily with phosphate-buffered saline or shepherdin[79–83] at 50 mg/kg intraperitoneally. Data are the mean tumor volumes in the two groups, and values were corrected to account for within-animal correlations by averaging the within-animal volumes and calculating a best estimate of the tumor volume for each mouse. The means and SEM were then calculated with mice as the data units. C) Tumor histology. Sections of HL-60 tumors from saline- or shepherdin[79–83]-treated animals were recovered at the end of the experiment (day 12 after treatment) and stained with control IgG or antibodies against survivin or Akt followed by peroxidase-conjugated species-specific secondary antibodies and immunohistochemical detection with 3,3-diaminobenzidine as the chromophore. D) Organ histology. The indicated organs from saline- or shepherdin[79–83]-treated mice were harvested at the end of treatment (day 12 after treatment) and analyzed by staining with hematoxylin–eosin. C and D) Original magnification was ×400. Scale bars are 30 μm.
cell death by shepherdin in cancer cells, may indicate that shepherdin can be administered for a long time in vivo without Hsp70-associated side effects.

A limitation of this study is that the molecular differences in the mechanisms of action for shepherdin and 17-AAG have not been fully elucidated. Previous experiments validated the specificity of shepherdin for Hsp90 and ruled out its interaction with Hsp70 (28). The unique properties of shepherdin may reflect its structurally distinctive binding to the ATP pocket of Hsp90, which might affect the recruitment of cochaperones, such as p50ecd (49) or the stability or maturation of client proteins that are not affected by treatment with 17-AAG. Because of the rapid onset of shepherdin-induced disruption of mitochondrial permeability, it is also possible that shepherdin may directly target a specialized subcellular pool of Hsp90 associated with mitochondrial homeostasis. This mechanism is consistent with a role for Hsp90 in the importation of proteins by mitochondria through the Tom70 receptor (50) and the ability of an Hsp90–Hsp70 complex to counteract discharge of cathepsin G by lysosomes; cathepsin G, in turn, can mediate the mitochondrial permeability transition (48).

These data support the development of shepherdin or its advanced derivatives for use in novel anticancer strategies. When tested in a xenograft AML model, shepherdin had strong single-agent activity that suppressed tumor growth, induced apoptosis in the tumor, and decreased the expression of Hsp90 client proteins (e.g., survivin and Akt); these results indicate that shepherdin functions as an Hsp90 inhibitor in vivo. Similar to earlier results with survivin (28) in solid tumors (28), prolonged systemic administration of shepherdin was well tolerated by mice. Preliminary analyses of toxicity and histologic examination of the kidneys, lungs, and liver were unremarkable, which is in keeping with the safety of shepherdin for normal cells, including hematopoietic progenitors (28) or actively proliferating, survivin-expressing PBMCs (Fig. 5). This degree of selectivity may reflect the higher affinity with which Hsp90 binds ATPase pocket antagonists in tumor cells as opposed to normal tissues, a result that has also been previously validated for shepherdin in affinity chromatography experiments (28).

In summary, shepherdin belongs to a novel class of Hsp90 inhibitors whose members have a dual mechanism of anticancer activity involving the rapid disruption of mitochondrial function and decreased expression of Hsp90 client proteins, such as survivin, Akt, and CDK-6. The combined molecular properties of shepherdin, its selectivity for transformed cells, and its safety in mice indicate that shepherdin may be a good lead prodrug for the development of therapies for AML and other epithelial malignancies.

References


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NOTES

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