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Inhibition of gastric tumor growth by a novel HSP90 inhibitor

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Abstract

Heat shock protein 90 (Hsp90) is a molecular chaperone engaging multiple cellular signaling by stabilizing oncoproteins (*e.g.*, Akt and c-Raf) in tumor cells. Whereas Hsp90 inhibitors such as 17-AAG exert promising antitumor effects in clinical trials, current efforts focus on developing agents targeting Hsp90 with improved efficacy and lower toxicity. Using a fluorescence polarization assay, we screened over a hundred of synthetic small molecules and identified a resorcinol derivative LD053 that bound the Hsp90 ATP-binding pocket. The binding of LD053 to Hsp90 dissociated the co-chaperone protein cdc37 from Hsp90, resulting in destabilization of Akt and c-Raf and subsequent inhibition of PI3K/Akt and c-Raf/Mek/Erk signaling in BGC823 gastric cancer cells. As a consequence, LD053 decreased cancer cell viability and induced apoptosis evidenced by increased subG0/G1 cell population and increased cleavage of caspase 3 and PARP. Interestingly, normal human cells appeared insensitive to LD053 treatments. Consistent with its *in vitro* anticancer activities, LD053 significantly inhibited growth of BGC823 xenografts in nude mice without apparent body weight loss. These results thus demonstrate that LD053 is a novel Hsp90 inhibitor and has potential to be used to treat gastric cancer.

Keywords

Hsp90; Hsp90 inhibitor; gastric cancer; cancer therapy; molecular chaperone

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1. Introduction

Cancer cells often harbor abnormalities in multiple cellular signaling pathways. It has become increasingly clear that these wide-range aberrations collectively contribute to the genesis and/or maintenance of transformed, cancerous phenotypes. Therefore, there is a frequent need of targeting multiple tumor-associated signaling pathways in order to abrogate malignant phenotypes of tumors and overcome drug resistance. One approach to interfere with multiple signaling pathways with one single agent is to target the molecular chaperone heat shock protein 90 (Hsp90)[1]. Hsp90 harbors ATPase activity in its N-terminus, and act alone or in concert with co-chaperones (*e.g.*, cdc37) to govern maturation and promote stabilization and activation of a large number of client proteins that often mediate essential cellular signaling[2]. Since many client proteins (*e.g.*, Akt, c-Raf, ErbB2) are oncogenic, Hsp90 increases tumor cell proliferation and promotes tumor cells to evade apoptosis, and accordingly, Hsp90 is often found to be overexpressed in human cancers[3]. Conversely, inhibition of Hsp90 chaperone activity by geldanamycin (GA) or its derivative 17-allylamino-17-demethoxyl-geldanamycin (17-AAG) results in simultaneous degradation of multiple oncoproteins and subsequent inhibition of various signaling pathways essential for the maintenance of growth and survival of cancer cells[4]. The Hsp90 inhibitor 17-AAG was also recently shown to alter actin dynamics leading to inhibition of migration and invasion of cancer cells[5]. It is thus not surprising that Hsp90 has emerged as a promising anti-cancer drug target. Indeed, 17-AAG exhibits potent anticancer activity in preclinical experiments and has entered phase II/III clinical trials for treatments of various human cancers[4, 6].

Binding the N-terminal ATP-binding pocket of Hsp90, GA and 17-AAG represent the first-in-class Hsp90 inhibitors that competitively inhibit Hsp90 chaperone activity resulting in depletion of a wide range of oncogenic client proteins through ubiquitin-mediated proteasomal degradation[4]. Whereas GA has significant toxicities precluding its clinical development, 17-AAG also has limitations including its low oral bioavailability, poor aqueous solubility, potential hepatotoxicity and dependency on quinone reductase NQO1[7]. 17-AAG was also shown to be a substrate of the P-glycoprotein multidrug resistance efflux pump and thus may be sensitive to multidrug resistance[8]. Given these limitations, many other Hsp90 inhibitors that bind the Hsp90 ATP-binding site and harbor diverse pharmacophores have been developed[4, 9]. These inhibitors include synthetic purine-based compounds (*e.g.*, PU3) and pyrazole-resorcinol derivatives (*e.g.*, CCT018159). CCT018159 was identified through high-throughput drug screening using an ATPase assay, and can deplete Hsp90 client proteins (*e.g.*, c-Raf, CDK4, ErbB2) resulting in growth inhibition in a broad range of cancer cell lines[10, 11]. Crystallographic studies reveal that the two phenolic hydroxyls in the resorcinol ring and the adjacent pyrazole N-atom of CCT018159 bind the Hsp90 N-terminal residues[12]. Subsequent structure-based design generated several more potent resorcinol derivatives that dramatically decrease levels of Hsp90 client proteins including Raf-1[10, 13]. The discovery of these synthetic Hsp90-binding molecules also allows for the development of cost-effective fluorescence polarization assays (FPA) applicable for searching for novel Hsp90 inhibitors in a high-throughput manner[14].

Here we report the identification of a novel resorcinol derivative LD053 that efficiently bound the Hsp90 N-terminus. This novel Hsp90 inhibitor dissociated cdc37 from Hsp90, resulting in inhibition of PI3K/Akt and c-Raf/Mek/Erk signaling essential for growth and survival of gastric cancer cells. Indeed, LD053 induced apoptosis and inhibited gastric tumor growth in nude mice. This small molecule thus represents a new class of anti-gastric cancer agents targeting a wide range of oncogenic signaling through inhibiting Hsp90 molecular chaperone activity. Given that gastric cancer is the second leading cause of

cancer-related deaths worldwide and no effective therapeutics is currently available[15], LD053 has a potential to be further developed into a therapeutic agent for gastric cancer.

2. Materials and Methods

2.1. Cell culture and chemicals

Human cancer cell lines A549, A2780, BGC823, BGC803, MGC803, N87, HCT8, HepG2, Bel7402 and MDA-MB-231 were purchased from ATCC or Cell Culture Center at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Immortalized normal human retinal pigmented epithelium cells (RPE-hTERT) and lung fibroblasts (BJ-hTERT) were obtained from Dr. Todd Waldman at Georgetown University. RPE-hTERT, BJ-hTERT and MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics, while other cancer cell lines were cultured in supplemented RPMI-1640 medium. LD053 was synthesized through an intramolecular dipolar cycloaddition reaction (detailed method is available upon request). For *in vitro* experiments, LD053 and GA (Sigma, St. Louis, MO, USA) were dissolved in DMSO (Sigma, St. Louis, MO, USA), and stored in aliquots at -20°C for no more than 1 month before use. The vehicle (DMSO) was used as a control in *in vitro* experiments at a maximum concentration of 0.1%. For animal experiments, LD053 was solubilized in 15% PEG (Beijing Chemical works, Beijing, China) to yield stock solutions of 10 and 15mg/ml, and stored at -20°C before use.

2.2. Fluorescence Polarization assay (FPA)

FPA was used to screen for Hsp90 inhibitors based on displacement of a fluorescent compound (VER00051001) that bind specifically to the Hsp90 ATP-binding site[13], and was carried out as described[14]. Briefly, reaction mixes (100 μl) containing 100 mM Tris-HCl (pH7.4), 20mM KCl, 6mM MgCl_2 , 2 mM DTT, 5 $\mu\text{g/ml}$ BSA, 80 nM of recombinant Hsp90 protein (Sigma, St. Louis, MO, USA), 80nM of VER00051001, and varying concentrations (10^{-5} , 10^{-6} , 10^{-7}M) of test compounds or GA were added in black 96-well plates. Controls that do not contain tested compounds, or Hsp90, were also included in each plate. After incubation for 24 h, plates were read on Spectra Max M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) with excitation at 485/20nm and emission at 535/25nm. Polarization values (mP) were used to calculate competitive effects, which were represented as percentage of control (%Control) and calculated following the formula: $\% \text{control} = 100 \times (\text{mPc} - \text{mPf}) / (\text{mPb} - \text{mPf})$, where mPc, mPb, and mPf are recorded mP values from wells containing test compounds, control wells containing both VER0051001 and Hsp90, and wells containing only the fluorescent probe, respectively.

2.3. Molecular modeling/docking

Docking simulations were carried out based on the crystal structure of the HSP90-CCT018159 complex (PDB code: 2CCS)[10] and using Induced Fit Docking implemented in the Schrödinger software suite as previously described[16, 17]. Essentially, the protein preparation wizard was first used to add hydrogens and charges, correct structural errors and minimize the protein complex. All water molecules were deleted except three near Asp 93. The grid for docking was defined using CCT018159 in the crystal structure encompassing a 15-Å cubic box. We set H-bond constraints at Asp93 to maintain the H-bonding interactions with the hydroxyl groups of compounds. The residues within 5 Å were refined for each compound pose. The top 20 structures for each compound were redocked using Glide Extra Precision (XP) docking, and structures were ranked with respect to their induced fit docking (IDF) score[16]. In order to validate the docking protocol and software, we extracted CCT018159 from the pdb structure and docked it back into the protein, and compared with

its original pose and interactions. The docked CCT018159 had the same pose and conformation as that shown in the crystal structure.

2.4. Western blotting and co-immunoprecipitation (co-IP) assay

For Western blotting, cells were lysed in RIPA buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1mM EDTA) containing proteinase inhibitor cocktails (aMResco, Solon, OH, USA). Equal amounts of protein were resolved by SDS polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore, Darmstadt, Germany). After blocking for 2 h in TBST (20mM Tris-HCl, pH7.5, 137mM NaCl, 0.05% Tween-20) containing 5% (w/v) dry skim milk powder (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature, membranes were subsequently incubated with primary antibodies and secondary antibodies. Proteins were then visualized using an enhanced chemiluminescence detection kit (APPLYGEN Technologies Inc., Beijing, China) following the manufacturer's recommendation. For co-IP assays, cell lysates (1 mg) were incubated with 1.5 µg of anti-Hsp90 antibody or control IgG at 4°C overnight, and then precipitated with 30µl of Protein A/G PLUS-Agarose (Santa Cruz, Dallas, Texas, USA). After extensive washes, proteins were eluted by boiling in 2×SDS loading buffer for 5 min and subjected to Western blotting. The Hsp90 and β-actin antibody were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and Santa Cruz, respectively. Other antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.5. MTT and colony formation assays

MTT assays were performed as previously described[18]. Briefly, cells in 96-well plates were incubated with 100µL of 0.5 mg/mL MTT solution (aMResco, Solon, OH, USA) for 4 h. MTT was then dissolved in DMSO, and absorbance at 570 nm was measured using a microplate reader. IC50 (50% inhibitory concentration) was calculated using the software GraphPad Prism 5.0. For colony formation assays, cells (300/well) in 6-well plates were treated with or without LD053 for 14 days. Cells were then fixed in 4% formaldehyde (Beijing Chemical works, Beijing, China) for 15 minutes, washed with PBS, and stained with 0.1% of crystal violet (Sigma, St. Louis, MO, USA) for 15 minutes at room temperature. After extensive washes, colonies were counted for each group.

2.6. Flow cytometry

Cells with or without LD053 treatments were dissociated from culture flasks using 0.5% trypsin/EDTA solution. After fixed in cold 70% ethanol at 4°C overnight, cells were stained with a solution containing 0.1 mg/ml propidium iodide (Sigma, St. Louis, MO, USA), 0.02 mg/ml RNase A (Sigma, St. Louis, MO, USA), 1mg/ml sodium citrate and 0.3% Triton X-100 at 37°C for 30 min. DNA contents were then measured using a Beckman flow cytometer.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed using the RT-PCR kit (Takara, Mountain View, CA, USA) following the manufacturer's protocol. Briefly, total RNA was extracted by TRIZOL (Invitrogen, Grand Island, NY, USA), reverse transcribed, and then subjected to PCR. The following primers were used: AKT, 5'-TTTATTCTCGGGTGCAT-3' (forward) and 5'-CATTTCCCTACGTGAATCGG-3' (reverse); c-Raf, 5'-AACTTGCTCGGAAGACGTT-3' (forward) and 5'-GAAACAGACTCTCGCATACGAC-3' (reverse); β-actin, 5'-ATGACTTAGTTGCGTTACACC-3' (forward) and 5'-GACTTCCTGTAAACAACGCATC-3' (reverse). The sizes of amplicons for Akt, c-Raf and

β -actin were 202 bp, 248 bp and 275 bp, respectively. Numbers of PCR cycles were empirically determined for each primer pair to ensure amplification occurring at exponential growth phases.

2.8. Xenografts and animal experiments

Animal experiments were performed following a protocol approved by the Animal Ethics Committees of the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Female nude mice (5~6 week old, BALB/c Nu) were purchased from Vital River Laboratories, and injected subcutaneously with 1×10^7 BGC823 gastric carcinoma cells at dorsa. 4 weeks later, tumors were chopped into $3 \times 3 \times 3$ mm³ pieces and implanted subcutaneously at dorsa of female nude mice with a trocar. Animals implanted with xenografts were randomized into 4 groups one week later when tumors reached to an average volume of 100 mm³, and were injected peritoneally with 100, 150 mg/kg of LD053, or vehicle, in a volume of 10 ml/kg, daily for 3 weeks. Mice were monitored daily for signs of toxicity, and tumor diameters were measured 3 times per week with a caliper. Tumor volumes were calculated using the formula: Volume = (width)² $\times$ length/2. Mice were sacrificed 3 weeks later, and tumors were weighed and fixed in 10% formaldehyde for histological examinations.

2.9. Histology and immunohistochemical staining

Tumors were embedded in paraffin for sectioning. For histological examinations, 5 μ m serial sections were stained by H&E. Immunohistochemical staining was carried out as described previously[19]. Briefly, sections were de-paraffinized and rehydrated followed by antigen retrieval using hot citrate buffer. Sections were then blocked in 5% of normal horse serum and 1% of normal goat serum, and incubated with the Ki-67 antibody (1:400, Abcam, Cambridge, MA, USA) for 2 h. The ABC Elite Kit and the DAB Kit (Vector, Burlingame, CA, USA) were used to visualize Ki-67 positive cells according to the manufacturers' recommendations.

3. Results

3.1. LD053 binds the Hsp90 N-terminal domain

Resorcinol derivatives are a major class of small molecules that bind the Hsp90 N-terminal ATP-binding pocket and inhibit Hsp90 molecular chaperone activity[4, 9]. To develop novel Hsp90 inhibitors, we synthesized over a hundred compounds containing the resorcinol scaffold and subjected these compounds (0.1, 1 and 10 μ M) to a FPA-based screen. This fluorescence polarization assay takes advantage of VER00051001, a fluorescent compound which strongly bind the Hsp90 N-terminal ATP-binding site[13], and evaluates whether test compounds compete with VER0051001 binding to Hsp90 resulting in decreased polarization[14]. Out of 101 screened compounds, we identified 15 compounds (14.8%) decreased the binding between the fluorescent probe and Hsp90 by more than 1 fold at 10 μ M (Fig 1A), indicating that these hits likely competitively bind the Hsp90 ATP-binding pocket. LD053(4-chloro-6-(5-(2-(p-tolyloxy)ethyl)-4,5,6,7-tetrahydro-[1,2,3]triazolo[1,5-a]pyrazin-3-yl)benzene-1,3-diol, Fig 1B) was one of the most potent compounds, and was further shown to competitively inhibit VER0051001 binding to Hsp90 in a dose-dependent manner (Fig 1C). The EC₅₀ of LD053 equaled 0.278 μ M, which is similar to other resorcinol-class Hsp90 inhibitors (*e.g.*, CCT018159)[20] but lower than GA (EC₅₀ = 0.028). Interestingly, this novel resorcinol derivative contains a N-atom adjacent to the resorcinol hydroxyls (Fig 1B) - a structure shown to mediate the binding of CCT018159 to the Hsp90 ATP-binding pocket[12]. Indeed, molecular modeling analysis showed that LD053 docked at the Hsp90 ATP-binding pocket with a good IDF docking score and reranking docking score of -428.099 and -12.979, respectively (Fig 1D). This small molecule appears to bind

to the Hsp90 N-terminus in a similar orientation with CCT018159. The resorcinol moiety of LD053 was in the same pocket and engages in hydrogen-bonding with Asp93, Asn51 and H-bonding with a water molecule bridging it with Asp93 and Leu48. The fused triazolopiperazine ring system of LD053 also hydrogen bonded with a water molecule bridging it to Asp93. Therefore, LD053 is a new resorcinol derivative that can bind the Hsp90 N-terminal ATP-binding site.

3.2. LD053 dissociates the Hsp90-cdc37 complex and promotes proteosomal degradation of Akt and c-Raf

Hsp90 promotes tumorigenesis mainly through activating multiple pro-proliferative/pro-survival signaling as a consequence of preventing oncogenic protein kinases (*e.g.*, Akt and c-Raf) from ubiquitin-mediated proteosomal degradation [21, 22]. Hsp90-mediated activation of protein kinases often requires the co-chaperone protein cdc37 which binds the Hsp90 N-terminal ATP-binding domain and targets protein kinases to the Hsp90-based chaperone machinery [21, 22]. Since LD053 bound the Hsp90 N-terminus, we tested a possibility whether this compound dissociates the Hsp90-cdc37 complex leading to destabilization of Akt and c-Raf. Towards this end, we treated BGC832 gastric carcinoma cells with LD053 and subjected cell lysates to co-IP assays. Indeed, the Hsp90-specific antibody, but not the normal IgG, precipitated cdc37 (Fig 2A, lane 2 vs. lane 1), confirming the interaction between Hsp90 and cdc37. Importantly, while LD053 had little effect on cdc37 expression, this novel Hsp90-binding compound dramatically decreased the amounts of cdc37 precipitated by the Hsp90 antibody (Fig 2A, lanes 3 and 4 vs. lane 2), indicating that LD053 dissociated cdc37 from Hsp90. Consistent with these results, LD053 decreased Akt and c-Raf protein levels in BGC832 cells as early as 12 h (Fig 2C). Treating cells with 10 μ M of LD053 for 48 h decreased the Akt/c-Raf level by more than 90% (Fig 2B). The decrease in Akt/c-Raf protein level was not due to inhibition of Akt/c-Raf gene transcription, since their mRNA levels were not altered by LD053 as measured by semi-quantitative RT-PCR (Fig 2D). Rather, LD053 promoted proteosomal degradation of c-Raf/Akt proteins as pre-treating cells with a proteasome inhibitor MG132 diminished the LD053-caused decrease in the c-Raf/Akt level (Fig 2E, lane 3 vs. lane 2). In line with these results, LD053 dramatically increased cellular levels of low-migratory, ubiquitin-conjugated Akt/c-Raf proteins (Fig 2F, lane 3 vs. lane 2), indicating that LD053 promoted Akt/c-Raf ubiquitination, particularly polyubiquitination. Therefore, our results demonstrated that LD053 dissociated cdc37 from the Hsp90 molecular chaperone machinery thereby promoting ubiquitin-mediated proteosomal degradation of Akt and c-Raf. Of note, in addition to Akt and c-Raf, LD053 also decreased levels of other Hsp90 client proteins including cyclin D, cyclin E, CDK4, CDK6, Wee1 and Myt1 [23] (Fig 3A). Moreover, LD053 treatments resulted in dose-dependent increased in Hsp70 level (Fig 3B) – a well established biochemical marker for Hsp90 inhibition [24]. These results are in line with the notion that LD053 inhibits Hsp90 chaperone activity in general.

3.3. LD053 inhibits the oncogenic signaling mediated by c-Raf and Akt

Akt and c-Raf mediate two major signaling pathways leading to increased proliferation and enhanced survival of a broad range of cancer cells (Fig 4A). We therefore tested whether LD053-caused destabilization of Akt/c-Raf impairs the PI3K/AKT and the Raf/MEK/ERK signaling. As expected, LD053 decreased c-Raf and Akt phosphorylation levels as a consequence of reduced c-Raf/Akt expression level in a dose-dependent (Fig 4B and 4D) and time-dependent manner (Fig 4C and 4E). Decreased phosphorylated c-Raf levels resulted in dramatic inhibition of MEK phosphorylation and subsequent activation - evidenced by significant down-regulation of ERK phosphorylation (Fig 4B and 4C). Similarly, LD053 inhibited phosphorylation of the Akt substrate GSK3 β in a dose-dependent (Fig 4D) and time-dependent manner (Fig 4E). These results are in line with a

notion that LD053 abrogates pro-proliferative/pro-survival signaling by destabilizing c-Raf/Akt through inhibiting Hsp90 chaperone activity.

3.4. LD053 induces BGC823 cells to undergo apoptosis

Depending on cell context, the outcome of Hsp90 inhibition in cancer cells could be cytoskeleton arrest or apoptosis[23, 25–28]. The Hsp90 inhibitor GA induced accumulation of BGC823 cells in the subG0/G1 stage (Fig 4A), suggesting that the main consequence of Hsp90 inhibition might be apoptosis in cultured gastric carcinoma cells. To test whether LD053 exerts similar effects, we treated BGC823 cells with LD053 and measured percentages of subG0/G1 cells with flow cytometry. We found that LD053 treatments resulted in significant accumulation of subG0/G1 cells (Fig 5A and 5B). Such apoptosis-inducing effect became apparent when the cells were treated with LD053 for 48 h. Treatments with 15 μ M of LD053 for 72 h induced more than 45% of cells to apoptosis (Fig 5A and 5B). To corroborate these results, we determined effects of LD053 on cleavage of caspase 3 and PARP, two well-established biochemical markers for apoptosis. Indeed, LD053 increased the levels of cleaved caspase 3/PARP while decreasing amounts of full-length caspase 3/PARP (Fig 5C). These results thus indicate that, like GA, the novel Hsp90 inhibitor LD053 can induce apoptosis in gastric carcinoma cells.

3.5. LD053 inhibits cancer cell growth

Consistent with its ability to induce apoptosis, LD053 decreased the viability of BGC823 cells in a dose-dependent manner (Fig 6A). The IC₅₀ value was 2.187 μ M, similar to other resorcinol Hsp90 inhibitors. We also measured cell growth potentials using colony formation assays. Numbers of surviving colonies of BGC823 cells were dramatically decreased by LD053 treatments (Fig 6B). 7.5 μ M of LD053 completely inhibited colony formation under our experimental conditions (Fig 6B). These results indicate that LD053 inhibited growth of BGC823 cells. In addition to BGC823 cells, LD053 efficiently inhibited growth of a broad range of cancer cells, including A549 lung, A2780 ovary, Bel7402 and HepG2 hepatocellular carcinomas, and other gastric carcinoma cells (*i.e.*, BGC803 and MGC803) (Fig 6C), with IC₅₀ values falling in the lower micromolar range (Table 1). LD053 also inhibited growth of HCT8 colorectal, MDA-MB-231 breast and N87 gastric carcinoma cells (Fig 6C), but the IC₅₀ values ranged from 25 to 47 μ M (Table 1). Interestingly, the latter 3 cell lines were also less sensitive to growth inhibition induced by the well-known Hsp90 inhibitor GA (Fig 6D and Table 1), consistent with the notion that LD053 and GA inhibited cell growth through the same mechanism, *i.e.*, inhibiting Hsp90 activity. However, while GA also efficiently inhibited the growth of RPE-hTERT and BJ-hTERT normal human cells (Fig 6D and Table 1), these normal epithelial/stromal cells were insensitive to LD053 (Fig 6C, Table 1), indicating that LD053 might preferably inhibit cancer cell growth and thus would have a better therapeutic index.

3.6. LD053 inhibits tumor growth in animals

To evaluate its therapeutic potential, we determined whether LD053 inhibits BGC823 tumor xenograft growth in nude mice. BGC823 cells were derived from a human gastric tumor and grow rapidly when subcutaneously implanted in nude mice[29]. After tumors had established, we treated mice with 100 and 150 mg/kg of LD053 (6 days per week) through intraperitoneal injection for three weeks. Consistent with its *in vitro* anti-tumor activity, LD053 inhibited tumor growth resulting in significantly-reduced tumor volumes (Fig 7A). Indeed, the tumors grew in LD053-treated mice were significantly smaller (Fig 7B), and 100 and 150 mg/kg of LD053 decreased the tumor mass by 20.95% and 50.84%, respectively (Fig 7C). We also examined the tumors by immunohistochemical staining of proliferative (Ki-67-positive) and apoptotic (cleaved caspase 3-positive) cells. We found that LD053 significantly decreased numbers of Ki-67 positive cells in tumor masses (Fig 7D and 7E).

However, LD053 treatments did not significantly increase the number of apoptotic cells in tumors (data not shown). These results indicate that LD053 mainly inhibited proliferation of tumor cells *in vivo*. Interestingly, LD053 did not decrease mouse body weights (Fig 6F) - a result consistent with the notion that LD053 preferably targets tumor cells and thus exhibited little toxicity to the animals. Taken together, we demonstrated that LD053 inhibited tumor growth *in vivo*.

4. Discussion

Pharmacologic inhibition of the Hsp90 molecular chaperone activity has emerged as a promising therapeutic strategy that provides an effective means to simultaneously abrogate multiple oncogenic signaling thereby inhibiting cancer cell growth, induce apoptosis, and/or increase vulnerability of malignant cells to chemotherapeutic interventions[1, 30]. Although structurally-diverse Hsp90 inhibitors have been developed in the past decades[4], many of these small molecules have limitations including poor bioavailability and toxicity due to low selectivity towards tumor cells. In this study, we developed a novel Hsp90 inhibitor LD053 and found that it inhibited growth of a variety of cultured cancer cells partly through disrupting the oncogenic signaling mediated by Akt and c-Raf. Importantly, LD053 inhibited growth of a gastric carcinoma xenograft in nude mice. Given that gastric cancer is the second leading cause of cancer-related deaths worldwide and that current treatment options for this deadly disease are very limited[15], our results suggest that LD053 has a potential to be further developed into a therapeutic agent greatly benefiting patients with gastric cancer. In spite of the increasing awareness of the important role of Hsp90 in maintaining the malignant phenotype of gastric cancer, it is important to point out that preclinical and clinical testing of Hsp90 inhibitors for their anti-gastric cancer activities just start to emerge[31, 32]. These Hsp90-targeting agents as well as LD053 hold strong promise for decreasing the high mortality caused by gastric cancer[15].

LD053 is a resorcinol derivative and belongs to the new generation of Hsp90 inhibitors with improved aqueous solubility and independent of metabolic activation[11]. The anti-tumor activities of GA, 17-AGG and other quinone-type Hsp90 inhibitors are known to be dependent on expression of NQO1/DT-diaphorase, which often varies in patients [33]. In addition to the resorcinol ring, LD053 contains a fused triazolopiperazine (Fig 1B) that might increase Hsp90-binding affinity and enhance solubility (Fig 1D). Similar to the pyrazole-resorcinol-class Hsp90 inhibitors like CCT018159[12], the two resorcinol hydroxyls together with the adjacent N-atom likely mediate binding to the residues in the Hsp90 ATP-binding pocket (Fig 1D). In spite of the structure similarity, however, CCT018159 is unlike LD053 and appears to inhibit growth of normal epithelial cells and cancer cells at equal efficiencies[11]. NVP-AUY922, a synthetic isoxazole resorcinol which is the most potent Hsp90 inhibitor reported so far, inhibits growth of a normal epithelial cell line at an IC₅₀ value that is 3 to 10 folds higher than gastric cancer cells[31]. In contrast, LD053 appeared to preferably target cancer cells as the IC₅₀ value for two tested normal cell lines was about 50–70 folds higher than that for BGC823 cells (Fig 6C and Table 1). Consistently, LD053 did not cause apparent weight loss of animals at dosages effectively inhibiting tumor growth (Fig 7D). These results suggest that LD053 would provide a wide therapeutic window for cancer treatments.

As a molecular chaperone, Hsp90 physically bind immature client protein kinases and subsequently stabilize and increase their activities[22, 34, 35]. This chaperone activity is often regulated by Hsp90-bound co-chaperone proteins (*e.g.*, cdc 37), which not only serve as a structural bridge between Hsp90 and protein kinases but also respond to nucleotide-regulated conformational switching of Hsp90[36]. In addition to destabilizing a number of Hsp90 client proteins (*e.g.*, Cyclin D, CDK4, Wee1) known to regulate cancer cell

proliferation and survival (Fig 3A), LD053 dissociated cdc37 from Hsp90 (Fig 2A) leading to proteosomal degradation of Akt and c-Raf (Fig 2E and 2F). Akt and c-Raf mediates activation of two major oncogenic signaling pathways that regulate a large variety of cellular functions including proliferation, survival, apoptosis, and migration (Fig 4A)[37–40]. Accordingly, LD053-caused destabilization of Akt/c-Raf would result in inhibition of Akt/c-Raf-mediated signaling frequently elevated in various human cancers[37]. Indeed, we found that LD053 inhibited phosphorylation of the Akt/c-Raf targets GSK3 β and Mek/Erk, and thus disrupted the oncogenic signaling required for the maintenance of malignancy (Fig 4A). The observations that LD053 simultaneously targeted multiple oncogenic proteins and signaling pathways suggest that LD053-based therapeutics would be more effective and unlikely succumbed to drug resistance - a major factor limiting the success of current cancer therapies[1, 2].

Like GA, LD053 was found to dramatically induce BGC823 gastric cancer cells to undergo apoptosis (Fig 5). While LD053 might promote apoptosis by inducing endoplasmic reticulum (ER) stress [41], our results suggest that the apoptosis-inducing effects of LD053 were also likely caused by Akt destabilization, given that Akt activation is known to promote cell survival by phosphorylating and inactivating pro-apoptotic proteins including Bad and FKHRL1[42] and that BGC823 gastric cancer cells harbor a high level of Akt activity[42]. Indeed, we found that LD053 treatments resulted in Akt inhibition and subsequent down-regulation of the GSK3 β phosphorylation level in BGC823 cells (Fig 4D and 4E). The latter effect is expected to activate GSK3 β , which might induce cancer cell apoptosis by phosphorylating the antiapoptotic protein Mcl-1 and promoting its degradation[43]. Although the induction of apoptosis was more apparent in cultured BC823 cells under our experimental conditions, LD053 might also inhibit cancer cell proliferation through targeting cell cycle regulators that are either Hsp90 clients (Fig 3A) or components of the Ras/Raf/Mek/Erk and the PI3K/Akt pathway. Indeed, we found that LD053 treatments dramatically decreased the number of Ki-67-positive proliferative cells in BGC823 xenografts. However, LD053 did not appear to induce apoptosis in the BCG823 xenografts at tested dosages. While higher doses of LD053 might confer apoptosis, our results argue for the notion that the outcome of pharmacological inhibition of Hsp90 activity is likely context dependent[23, 25–28].

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Abbreviation

17-AAG	17-allylamino-17-demethoxyl-geldanamycin
co-IP	co-immunoprecipitation
GA	geldanamycin
Hsp90	heat shock protein 90
IDF	induced fit docking

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Fig 1. LD053 binds the Hsp90 N-terminal domain

(A) The fluorescence polarization assays (FPA) were carried out to screen for small molecules competitively binding the Hsp90 ATP-binding site. The competitive effect of each compound was expressed as polarization relative to control (% control), and a representative screening result is shown here. (B) The chemical Structure of LD053. (C) Varying amounts of GA or LD053 were incubated with VER0051001 and Hsp90 for 24h and subjected to FPA. EC_{50} values were calculated using a nonlinear curve-fitting program (Prism 5.0). (D) LD053 docks at the Hsp90 ATP-binding pocket. The protein is shown as ribbons with compound-interacting amino acids displayed as wires with white labels. LD053 is shown in bold sticks and balls, while water molecules are displayed as thin sticks and balls. Atom colors are: C, white; H, cyan; N, blue; S, yellow; O, red; Cl: green.

Fig 2. LD053 dissociates cdc37 from Hsp90 and destabilizes Akt and c-Raf

(A) BGC823 cells were treated with or without 10 μ M of LD053 for 12 h and 24h. Cell lysates were subjected to co-IP assays using an Hsp90 antibody. Bound proteins were eluted and visualized by Western blotting (WB) for Hsp90 and cdc37. Amounts of cdc37 were quantified by densitometry, normalized to the precipitated Hsp90 levels, and shown in the histogram. (B, C) BGC823 cells were treated with varying amounts of LD053 for 48 h, or 10 μ M of LD053 for different time, and subjected to Western blotting for Akt and c-Raf expression. Histograms under each panel show relative protein levels quantified by densitometry analyses. (D) BGC823 cells were treated with varying amounts of LD053 for 48 h, or 10 μ M of LD053 for different time. Cells were lysed, and total RNA was subjected to semi-quantitative RT-PCR to measure Akt/c-Raf mRNA levels. Relative protein levels were quantified by densitometry and indicated under each blot. (E) BGC823 cells were pretreated with or without 1 μ M of MG132 for 3 h, and then exposed to 10 μ M of LD053 for 48h. Akt and c-Raf levels were measured by Western blotting. Relative protein levels were quantified by densitometry and indicated under each blot. (F) BGC823 cells were treated with or without 10 μ M of LD053 for 48h. Cell lysates were then immunoprecipitated with the Akt or c-Raf antibody, and subjected to Western blotting to detect ubiquitinated Akt/c-

Raf using an ubiquitin antibody. Relative protein levels were quantified by densitometry and shown in the histogram.

Fig 3. LD053 decreases expression levels of other Hsp90 client proteins and increases Hsp70 expression

BGC823 cells were treated with indicated amounts of LD053 for 48 h. Cell lysates were subjected to Western blotting. Relative protein levels were quantified by densitometry and shown in the histograms.

Fig 4. LD053 inhibits oncogenic signaling mediated by Akt and c-Raf

(A) Schematic representative of the oncogenic signaling mediated by c-Raf and Akt. (B, C) BCG823 cells were treated with varying amounts of LD053 for 48 h, or 10 μM of LD053 for different time, and subjected to Western blotting for phosphorylation levels of proteins involved in the c-Raf pathway. (D, E) BCG823 cells were treated as in (B, C), and tested for proteins involved in the Akt pathway. Relative protein levels were quantified by densitometry and indicated under each blot. Relative protein levels were quantified by densitometry and shown in the histograms.

Fig 5. LD053 induces BGC823 cells to undergo apoptosis

(A, B) BGC823 cells were treated with 1 μ M of GA, or varying amounts of LD053 for 24, 48 and 72 h, and then stained with propidium iodide for flow cytometry analyses. Percentages of SubG0/G1 (apoptotic) cells were quantitated using FlowJo. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, Student t-test ($n = 3$). (C) BGC823 cells were treated with indicated concentrations of LD053 for 48h and subjected to Western Blotting to measure full-length and cleaved caspase 3/PARP levels. Relative protein levels were quantified by densitometry and shown in the histogram.

Fig 6. LD053 inhibits growth of BGC823 and various cancer cells

(A) BGC823 cells were treated with LD053 for 96 hours for MTT assays. (B) BGC823 cells were plated in 6 well plates and then treated with indicated concentration of LD053 for 14 days. Colony numbers were counted after crystal violet staining. **, $p < 0.01$; ***, $p < 0.0001$, Student t-test ($n = 3$). (C, D) Indicated cancer cells and normal human cells were treated with varying amounts of LD053 (C) or GA (D) for 96 h and subjected to MTT assays.

Fig 7. LD053 inhibits growth of BGC823 xenografts in nude mice

(A, B, C) Female nude mice implanted with BGC823 xenografts were treated with 50, 100, 150 mg/kg of LD053 for 3 weeks. Tumor diameters were measured and used to calculate tumor volumes (A). 21 days later, the mice were sacrificed and individual tumors were photographed (B) and weighed (C). *, $p < 0.05$, Student t-test ($n = 6$). Bar equals 1 cm. (D, E) Individual tumors were sectioned and subjected to immunohistochemical staining for Ki-67 expression (D). At least 1,500 cells were counted for Ki-67 positivity under a microscope (random $\times 40$ fields) and positive rates are shown in (E). *, $p < 0.05$; **, $p < 0.01$, Student t-test. Bar equals 10 μm . (F) Animals were weighed during treatments to evaluate potential toxicity.

Table 1

Effects of LD053 on cell growth

Cell lines	Origin	Growth inhibition(IC ₅₀)	
		LD053 ×10 ⁻⁶ M	GA ×10 ⁻⁶ M
Bel7402	Hepatocellular carcinoma	8.49±0.43	0.96±0.24
HepG ₂	Hepatocellular carcinoma	3.42±0.12	0.89±0.06
BGC823	Gastric carcinoma	2.19±0.04	0.33±0.11
BGC803	Gastric carcinoma	2.89±0.67	0.42±0.09
MGC803	Gastric carcinoma	5.47±0.51	0.96±0.48
A2780	Ovary carcinoma	6.29±0.62	0.53±0.28
A549	Lung carcinoma	4.88±0.51	0.67±0.03
HCT8	Colorectal carcinoma	25.88±1.65	6.72±0.44
MDA-MB-231	Breast carcinoma	27.06±2.31	7.25±0.37
N87	Gastric acarcinoma	47.13±5.38	9.87±1.05
RPE-hTERT	Retinal pigmented epithelium	158.78±19.15	0.27±0.13
BJ-hTERT	Lung fibroblasts	113.63±11.74	1.04±0.09