Hsp90 Inhibitor Geldanamycin Enhances the Antitumor Efficacy of Enediyne Lidamycin in Association with Reduced DNA Damage Repair

Fei-Fei Han, Liang Li, Bo-Yang Shang, Rong-Guang Shao, Yong-Su Zhen

Abstract

Inhibition of heat shock protein 90 (Hsp90) leads to inappropriate processing of proteins involved in DNA damage repair pathways after DNA damage and may enhance tumor cell radio- and chemo-therapy sensitivity. To investigate the potentiation of antitumor efficacy of lidamycin (LDM), an enediyne agent by the Hsp90 inhibitor geldanamycin (GDM), and possible mechanisms, we have determined effects on ovarian cancer SKOV-3, hepatoma Bel-7402 and HepG2 cells by MTT assay, apoptosis assay, and cell cycle analysis. DNA damage was investigated with H2AX C-terminal phosphorylation (γH2AX) assays. We found that GDM synergistically sensitized SKOV-3 and Bel-7402 cells to the enediyne LDM, and this was accompanied by increased apoptosis. GDM pretreatment resulted in a greater LDM-induced DNA damage and reduced DNA repair as compared with LDM alone. However, in HepG2 cells GDM did not show significant sensitizing effects both in MTT assay and in DNA damage repair. Abrogation of LDM-induced G2/M arrest by GDM was found in SKOV-3 but not in HepG2 cells. Furthermore, the expression of ATM, related to DNA damage repair responses, was also decreased by GDM in SKOV-3 and Bel-7402 cells but not in HepG2 cells. These results demonstrate that Hsp90 inhibitors may potentiate the antitumor efficacy of LDM, possibly by reducing the repair of LDM-induced DNA damage.

Keywords: Geldanamycin - DNA damage repair - lidamycin - enediyne - ATRIP
pretreatment potentiated the cytotoxicity of LDM and decreased repair of LDM-induced DNA damage in SKOV-3 and Bel7402 cells. To our knowledge, this is the first report that Hsp90 inhibition can sensitize tumor cells to LDM by decreasing DNA damage repair.

Materials and Methods

Cell culture
SKOV-3 cells, Bel-7402, and HepG2 cells were maintained in monolayer culture in DMEM medium with 10% fetal bovine serum in a humidified atmosphere of 5% CO2.

Chemicals and reagents
LDM (provided by Professor Lian-fang Jin of our institute) was prepared at 10 μM in 0.9% NaCl solution and stored at -80°C until use. GDM was purchased from Sigma and reconstituted according to the manufacturer’s instructions. All other analytical grade chemicals were purchased from Sigma. Antibodies respectively against ATM, phospho-ATM, ATR, phospho-Chk1, phospho-Chk2, ATR interaction protein (ATRIP), and γH2AX were purchased from Cell Signaling. Anti-actin antibody was from Santa Cruz.

Cell proliferation assay
Cells were seeded at 4000 cells/well in 96-well tissue culture plates (Costar, Cambridge, Massachusetts, USA). After overnight incubation, triplicate wells were treated with varying concentrations of LDM for 48h with or without 16h pretreatment with 100 nM GDM. Then, 20 μl MTT solutions (5mg/ml in PBS) were added to each well and incubated for 4h at 37°C. The MTT formazan was dissolved in 150 μl DMSO and absorbance at a wavelength of 570 nm was measured by a multiskan MK3 microplate reader (Thermo Labsystem, USA).

Immunofluorescence
Cells were exposed to LDM for various periods of time following a 16 h pretreatment with GDM. Cells grown on tissue culture slides were fixed with 4% paraformaldehyde, permeabilized with 0.2% NP40, and blocked with 1% bovine serum albumin (BSA) in PBS. The slides were incubated with primary antibodies overnight at 4°C and with secondary antibodies (DyLight goat anti-rabbit IgG; 1:500) and Hoechst in PBS with 1% BSA. The primary antibody used for immunostaining was phospho-H2AX (Cell Signaling, USA) at 1:200.

Cell cycle analysis
After 16h GDM pretreatment, cells were exposed to LDM. Then cells were fixed in ice-cold 70% ethanol and stored at -20°C for 24h before analysis. For cell cycle analysis, cells were washed twice in PBS and stained with 50mg/ml propidium iodide and 200mg/ml RNase A for 30 min. The samples were analyzed on a fluorescence-activated cell sorter.

Apoptosis assay by annexin V-FITC staining
To quantify apoptosis, cells were stained with annexin V and PI using an Annexin V-FITC apoptosis kit (Biovision Inc., CA, USA) following the protocol provided by the manufacturer. Briefly, cells were harvested by trypsinization and washed once with cold PBS. Cell pellets were resuspended in 500 μl binding buffer. Then, 5 μl of annexin V-FITC and 10μl PI working solution were added into 500 μl of cell suspension. The cells were incubated at room temperature for 10 min in the dark, and then analyzed for apoptosis by flow cytometry.

Immunoblot analysis
Cells were exposed to LDM for 24h after 16h pretreatment of GDM. Cell culture monolayers were washed twice with ice-cold PBS and lysed with the lysis buffer containing 50 nM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.1% SDS, protease inhibitors (1 mM PMSF, 10mg/ml aprotinin, 10mM leupeptin and 10mM pepstatin) and phosphatase inhibitors (20mM β-glycerophosphate, 50mM NaF and 1mM Na3VO4). Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, Illinois, USA). Equal amounts of protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, Massachusetts, USA) which were then incubated in blocking solution (5% bovine serum albumin in 20mM Tris-HCl, 150mM NaCl, 0.1% Tween-20) (TBS-T), followed by incubation with the indicated antibodies at 4°C overnight. Membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. The antibody detection was performed by the enhanced chemiluminescence technique (Millipore).

Results

GDM sensitizes SKOV-3 cells and Bel-7402 cells to LDM
MTT results showed that LDM markedly inhibited cell growth in a dose dependent manner. At the concentration of 100nM, GDM showed moderate inhibition of cell proliferation. Therefore, in subsequent experiments, 100 nM of GDM was chosen as an appropriate concentration for combination with varying concentrations of LDM to determine the augmentation effects on the three cell types under study.

After pre-treatment with GDM for 16h, the cells were exposed to different concentrations of LDM for 48h. As shown in Figure 1, LDM induced growth inhibition was enhanced in GDM-pretreated SKOV3 cells (Figure 1A, B) and Bel-7402 cells (Figure 1C, D). The potentiation of LDM cytotoxicity by GDM Hsp90 inhibition was more marked in Bel-7402 cells than in HepG2 cells (Figure 1E, F).

Table 1 showed the IC50 values of LDM at different GDM doses in the three cell lines. GDM pretreatment decreased the IC50 value of LDM in SKOV-3 (LDM/LDM+GDM 100 nM, P=0.0087; LDM/LDM+GDM 500 nM, P=0.0028) and Bel-7402 cells (LDM/LDM+GDM 100 nM, P=0.0150; LDM/LDM+GDM 500 nM, P=0.0156), but not in HepG2 cells (LDM/LDM+GDM 100 nM, P=0.1339; LDM/LDM+GDM 500 nM, P=0.6727).
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GDM potentiates LDM-induced apoptosis

The augmentation of LDM-induced apoptosis by Hsp90 inhibitor was examined with annexin V–PI staining assay. Consistent with above results from MTT assay, LDM induced much higher apoptotic rates in GDM pretreated cells than in non-pretreated cells. Bel7402 and HepG2 cells were treated with 0.01 nM LDM with fresh GDM-free medium for 24h after exposed to GDM (100 nM) for 16 hours, and the annexinⅤ positive cell were determined. As shown in Figure 2, cells treated with LDM plus GDM had more percent of annexinⅤ positive cell than with LDM or GDM alone in Bel7402 cells (Figure 2A). However, in HepG2 cells the percent of annexinⅤ positive cell by GDM did not significantly increase in as Bel-7402 cells (Figure 2B).

Table 1. Determined IC<sub>50</sub> Values on Different Cancer Cell Lines

<table>
<thead>
<tr>
<th>Groups</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td></td>
<td>LDM</td>
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<tr>
<td>SKOV-3</td>
<td>0.363 ± 0.041</td>
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<tr>
<td>Bel-7402</td>
<td>0.19 ± 0.044</td>
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<tr>
<td>HepG2</td>
<td>0.143 ± 0.051</td>
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Figure 1. Effect of GDM on LDM-Induced Cytotoxicity in SKOV-3 and Bel-7402 Cells. Shown is the cytotoxicity of the GDM–LDM combination relative to the effect of the two agents alone using the combination index (CI), which determines whether the cytotoxicity for the combination is greater than (CI<1), equal to (CI=1), or less than (CI>1) the additive effect of the individual agents (McCollum et al., 2008). Cells were exposed to GDM at 100 nM GDM (circle) and 500 nM (triangle) separately for 16 h, and then to various concentrations of LDM for 48 h or exposed to LDM alone for 48 h. Cell viability was determined by MTT assay. A) SKOV-3 cell viability curves, bars, SE, and combination index values for SKOV-3 cell. B) Bel-7402 cell viability curves, and combination index values for Bel-7402 cells. C) Effect of GDM on LDM-induced cytotoxicity in HepG2 cells

Figure 3. LDM-induced DNA damage was enhanced by GDM. A) Bel7402 cells were exposed to GDM (100 nM), fed fresh GDM-free medium with 0.01 nM LDM, and the number γH2AX foci were observed by confocal microscopy; γH2AX (green) and nucleus (red) were shown. B) Analysis γH2AX foci for Bel7402 cell, columns, mean; bars, SE. #p=0.002757, *p=0.002049, **p=0.001114

Pretreatment with GDM enhance LDM-induced DNA damage

DNA damages caused by IR, UV or radiomimetic agents result in rapid phosphorylation of the histone H2A family member, H2AX, at Ser139, also known as γH2AX (Dziegielewski and Beerman, 2002; Ward et al., 2004; Clingen et al., 2008; Yajima et al., 2009). Cells were treated with 0.01 nM LDM with fresh GDM-free medium for 24h after exposed to GDM (100 nM) for 16h, and the number γH2AX foci were determined. In Bel7402 cells the exposure to GDM and LDM alone had no great effects on the number of γH2AX foci. For cells exposed to the combination of GDM and LDM, there was great difference in the level of γH2AX foci compared with GDM or LDM alone (Figure 3A, 3B), suggesting that Hsp90 inhibition enhance the DNA damage effect of LDM.

Pretreatment with GDM impairs LDM-induced DNA repair in SKOV-3 and Bel7402 cells

Cells were exposed to GDM (100 nM) for 16h, then
The 0.01 nM LDM alone induced an increase in the number of γH2AX positive cells at 1h, and did not reduce at 6h (Figure 4C).

We further investigated the mechanism that GDM interferes with the repair kinetics of LDM-induced DNA damage. Both ATM and ATR pathways play significant roles in the response to LDM-mediated DNA damage. Western blot analysis was used to identify the effect of Hsp90 inhibition on ATM and ATR pathways of DNA damage repair. As shown in Figure 4D, the activity of ATM was decreased in both SKOV-3 and Bel-7402 cells pretreated with GDM, suggesting that GDM interfered with the activation of ATM-mediated DSB repair in response to LDM exposure. ATR and ATRIP are also important response pathways involved in DNA damage repair (Dziegielewski and Beerman, 2002). Figure 4D shows that ATM and ATRIP expression were dramatically decreased after 16h GDM treatment.

GDM abrogates LDM-induced G₂/M arrest in SKOV-3 but not in HepG2

Cell cycle progression was evaluated with PI staining and FACS analysis. The number of cells in the G₂/M phase increased from 15% (control) to 77% upon treatment with 0.05nM LDM in SKOV-3 cells. Treatment with 100 nM GDM alone did not significantly induce G₂/M phase arrest. However, pretreatment with GDM reduced the number of G₂/M phase cells from 77% to 29% (Figure 5A). In HepG2 cells we did not found this result (Figure 5B). According to Western blot analysis, the LDM-induced phosphorylation of Chk2 and the total expression of Chk1 were both decreased by GDM pretreatment in SKOV-3 cells.

Discussion

Previous reports have established that Hsp90 inhibitors enhance the cytotoxicity of a variety of chemotherapeutic
In this study, we have shown that the potent cytotoxicity of LDM was enhanced by Hsp90 inhibitor, GDM. This was accompanied by the abrogation of cell cycle arrest and a significant increase in apoptosis induction. Because the anticancer effect of LDM is mainly dependent on DNA damage and previous studies show that Hsp90 inhibitors decrease DNA damage repair responses (Dote et al., 2006; Koll et al., 2008), we hypothesize that Hsp90 inhibitors may enhance LDM cytotoxicity by decreasing DNA damage repair, resulting in augmentation of DNA damage. The present study confirms that Hsp90 inhibitor GDM enhance the antitumor efficacy of LDM in association of reduced DNA damage repair in cancer cells.

C-terminally-phosphorylated histone H2AX (γH2AX) plays an important role in the cellular response to DNA damage and is evident following both DSBs and SSBs. We measured γH2AX as a marker for DNA damage and found that GDM significantly enhanced LDM-induced DNA damage. As shown in Bel7402 and SKOV-3 cells, LDM induced a transient surge and subsequent decline in DNA damage. The delayed dispersal of γH2AX suggested that GDM inhibited the repair of LDM-induced DNA damage in Bel7402 and SKOV-3 cells. In HepG2 cells the γH2AX level did not reduce at 6 hours LDM treatment. This suggested that the DNA damage repair did not work in HepG2 cells and the effect of GDM potentiation in HepG2 was not as sensitive as in Bel7402 and SKOV-3 cells.

The cellular response to LDM-induced DNA damage is dependent on ATM and ATR (Dziegielewski and Beerman, 2002; Kennedy and Beerman, 2006; Kennedy et al., 2007). And inhibition of ATM and ATR (Sangster-Guity et al., 2011 ) expression has been demonstrated to increase sensitivity to chemotherapeutic drugs. In this research, we found that GDM decreased the LDM-induced G/M phase arrest in SKOV-3 cells but not in HepG2 cells. In HepG2 cells, the inhibition of ATM did not as strong as in SKOV-3 and Bel7402 cells. The augmented effects of LDM by GDM were more potent in SKOV-3 and Bel7402 cells than that in HepG2 cells. This suggested that GDM can not inhibit LDM induced DNA damage repair sufficiently. Taken together, these data suggested that GDM could increase the effect of LDM by decreasing the DNA damage repair pathways. Results indicate that the synergistic effect is, at least in part, cell type-dependent and related to the DNA damage response induced by LDM.

In summary, we found that Hsp90 inhibitors could enhance the antitumor efficacy of LDM, an enediyne agent with extremely potent cytotoxicity against cancer cells. The molecular mechanism of the combination of Hsp90 inhibitor and enediyne LDM is highly related cellular DNA damage. There occurs a potentiation of DNA damage associated with a reduction of DNA damage repair.

Recent years the application of Hsp90 inhibitors were gradually went into the bottleneck, the failure of the clinical trials gradually reveal the defects of these drugs. However, the unique mechanism of Hsp90 inhibitors has been the ideal medicine against cancer or virus. Some researchers concluded that combination of chemotherapy drug and chemo-radiation was a safe and effective regimen in management of cancer, meriting further investigation in randomized clinical trials (Jamshed et al., 2014). LDM in our research was a radiomimetic enediyne anticancer drug and showed great effect in combination with Hsp90 inhibitor which was also a good selection in the drug combination. The combination of Hsp90 inhibitor and enediyne agent might be potentially effective in cancer chemotherapy.

Acknowledgement

The authors declare that there are no conflicts of interest.

References


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