Targeting Heat Shock Response to Sensitize Cancer Cells to Proteasome and Hsp90 Inhibitors

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Abstract

Novel classes of anticancer drugs, including proteasome inhibitors and Hsp90 inhibitors, potently induce heat shock proteins (Hsps). Because Hsps show antiapoptotic activities, we suggested that suppression of such induction may sensitize cancer cells to these drugs. Here, we knocked out the major heat shock transcription factor HSF-1 in several cancer cell lines using small interfering RNA and showed that such cells, which can no longer induce Hsps in response to proteasome and Hsp90 inhibitors, become more sensitive to these drugs. Furthermore, we developed a high-throughput screen for small molecules that inhibit induction of Hsps. The first step was a cell-based screen for inhibitors of Hsps-mediated luciferase refolding followed by a counterscreen for toxicity. The second step was a direct testing for inhibition of Hsp induction by immunoblotting with anti-Hsp72 antibody. After screening of 20,000 compounds from several diversity libraries, we focused on a compound we called NZ28, which potently inhibited induction of Hsps by heat shock, proteasome, and Hsp90 inhibitors in a variety of cell lines, and showed no significant toxicity. After testing of a set of analogues of NZ28, we identified a structural element that was critical for the activity. We also identified another inhibitor of the Hsp induction that was practically nontoxic. This compound, which we called emunin, strongly sensitized myeloma cells to proteasome and Hsp90 inhibitors and prostate carcinoma cells to proteasome inhibitors. This work indicates that targeting the heat shock response may facilitate use of proteasome and Hsp90 inhibitors for cancer treatment.

Introduction

Recently, a number of novel anticancer therapeutics have been developed that specifically target signaling pathways. Among these newly developed drugs are inhibitors of the proteasome, such as Velcade (1), that indirectly activate c-Jun NH2-terminal kinase signaling pathway, resulting in apoptosis of cancer cells (2). Proteasome inhibitors were found to be quite potent agents in targeting multiple myeloma, and Velcade has already been introduced in clinical practice (3).

Inhibitors of Hsp90, such as geldanamycin, 17-allylamino-17-demethoxygeldanamycin (17-AAG), or radicicol, have also been studied for their anticancer activities (4). Because Hsp90 plays a critical role in folding, maturation, and stability of several important signaling proteins, including IKK, Act, Raf, and many others, inhibition of Hsp90 by small molecules leads to degradation of these proteins and inactivation of the corresponding signaling pathways (5–7). This, in turn, results in activation of the apoptotic machinery and specific killing of cancer cells (8). The reason why cancer cells are more sensitive to proteasome inhibitors and Hsp90 inhibitors than normal cells is poorly understood. In addition, resistant clones emerge occasionally, and the mechanisms of such resistance are unclear. Although both Velcade and 17-AAG are well tolerated, the toxicity becomes an issue upon dose escalation. Therefore, sensitizers to these drugs may become quite beneficial.

A potentially powerful approach towards development of sensitizers to these classes of drugs would be to target their major side effect activity in induction of the heat shock response. In fact, both proteasome inhibitors and Hsp90 inhibitors are potent inducers of heat shock proteins (Hsps). Because Hsp72, Hsp27, and other Hsps show strong antiapoptotic potential (9–12), induction of Hsps counterbalances the proapoptotic activities of these drugs, thus leading to enhanced resistance (13, 14). In fact, additional induction of Hsps by pretreatment of cells with mild heat shock led to increased cell resistance to proteasome inhibitors (15). Furthermore, resistance to proteasome inhibitors in a certain lymphoid lines was attributed to increased expression of Hsp27 (13). Moreover, inactivation of HSF-1, the main transcription factor that controls induction of Hsps, led to enhanced sensitivity of primary murine embryonic fibroblast (MEF) cells to inhibitors of Hsp90 (16).

Based on these findings, we have hypothesized that inactivation of the heat shock response in cancer cells may significantly enhance tumor cell sensitivity to proteasome and Hsp90 inhibitors. Interestingly, there have been previous attempts to target the heat shock response for cancer therapy. In fact, a flavonoid quercetin that inhibits activation of HSF-1 was successfully tested as a sensitizer to hyperthermia in animal models (17). However, hyperthermia has a limited potential as a treatment modality; in addition, quercetin has many side effects in addition to inhibiting Hsp expression. Therefore, we have investigated whether specific induction of the heat shock response can sensitize cancer cells to proteasome inhibitors and Hsp90 inhibitors. Based on this work, we have developed a high-throughput screen to identify chemical compounds that inhibit the heat shock response. The selected compounds were tested for their ability to sensitize cancer cells to proteasome and Hsp90 inhibitors.

Materials and Methods

Cell cultures and materials. MM.1S myeloma, PC-3, and DU-145 prostate carcinoma cells were grown in RPMI 1640 with 10% fetal bovine
serum (FBS), HCT-116 colon carcinoma cells were grown in McCoy medium with 10% FBS. MEF cells and CHO-Luciferase tet-off cells were grown in DMEM with 10% FBS. For Chinese hamster ovary (CHO) cells, gentamicin (100 μg/mL), hygromycin (100 μg/mL), and tetracycline (1 μg/mL) were added. All cells were grown at 37°C in an atmosphere of 5% CO2.

The following antibodies were used for immunoblotting: SPA-901 for HSF-1; SPA-810 for Hsp72; SPA-800 for Hsp27 (all from Stressgen, Victoria, British Columbia, Canada), anti-poly(ADP-ribose) polymerase (anti-PARP; PharMingen, San Diego, CA), anti-luciferase (Sigma, St. Louis, MO), anti-green fluorescent protein (anti-GFP; Clontech, Palo Alto, CA); anti-β-actin (Sigma) and anti-γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA).

Chemical libraries were provided by the National Cancer Institute (NCI) and the Harvard Institute of Chemistry and Cell Biology (ICCB); emetine, quercetin, and cisplatinum were from Sigma. MG132, radicicol, doxorubicin, and 17-AAG were from Biomol (Plymouth Meeting, PA).

**Small interfering RNA, retrovirus infection, and transfection.** For knocking down HSF-1, we used RNAi-Ready pSilRen-RetroQ vector with puromycin resistance (BD Biosciences, San Jose, CA). Target sequence for HSF-1 small interfering RNA (siRNA) was 5'-TATGGACTCACCCTG-GATAA-3'.

Retroviruses were produced, and cells were infected according to standard protocols. PGL.hsp70B plasmid with luciferase promoter regulated by the HSF70B gene was described previously (18).

**High-throughput screening.** Chemical compounds from various libraries were dissolved in DMSO at concentration of 1 mmol/L and distributed in 384-well master plates. CHO cells were plated in 384-well plates to assay plates using an automated pin-based compound transfer robot to final concentration of 2 μmol/L. In each plate, one column was without chemical compound but with DMSO as a negative control. Four plates were prepared for each set of compounds, two plates for the inhibitor assays, and two for toxicity assays. Sixteen hours after incubating cells with compounds, plates were immersed in 45°C water bath for 10 minutes, kept for 6 hours at 37°C, and exposed again to 45°C for 50 minutes, and after 70 minutes at 37°C, luciferase assay was done. For luciferase assay, cells were washed twice with PBS and lysed with cell lysis reagent (Promega, Madison, WI), 10 μL for each well in 384-well plate. Samples were frozen at −70°C and thawed before checking luciferase activity; 20 μL for 384 well plate of luciferase reagent (Promega) were dispensed per well, and luminescence was read by luminometer (Bio-Rad Hercules, CA) or Analyst L.jL.

**Semiquantitative reverse transcription-PCR.** Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was done using RETROscript kit (Ambion, Austin, TX) following factory protocol. For HSP70A, the forward primer was 5'-TGTTCCGTTCGATCAGCCCTTACCAGCCA'A-3' and the reverse was 5'-GGGCTTGCTTCCGT-CGTGTGAT-5' to give 359 bp. β-Actin forward primer was 5'-CAGCTCACCATGGATGATGAT-3' and the reverse was 5'-CTCGGCCGTGGTGGTGTAAGCT-3' to give 626 bp. Amplification by PCR instrument (Mastercycler gradient, Eppendorf, Hamburg, Germany) was done by 3 minutes at 95°C for denaturation, and 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds. The final extension was carried at 72°C for 5 minutes.

**Results**

**Inhibition of the heat shock response sensitizes cancer cells to proteasome and Hsp90 inhibitors.** Novel classes of anticancer drugs (Hsp90 and proteasome inhibitors) are potent inducers of the heat shock proteins. Because Hsps, especially Hsp72 and Hsp27, have strong antiapoptotic activities, we hypothesized that inhibition of the heat shock response may promote the cytotoxic effects of these drugs, thus enhancing their anticancer activities. To address this possibility, we tested whether prevention of induction of the Hsps can sensitize cancer cells to these drugs. Because expression of Hsps is regulated by the major heat shock transcription factor HSF-1, depletion of HSF-1 must make cells unable to induce Hsps, as was previously shown with the HSF-1−/− MEF cells (19). To deplete HSF-1 in cancer cells, prostate carcinoma PC-3 cells were infected with retrovirus encoding siRNA against HSF-1 (si-HSF-1) or with a control virus (RetroQ).

To confirm that depletion of HSF-1 suppresses the stress response, PC-3 cells infected with either si-HSF-1 or RetroQ were heated for 20, 30, and 40 minutes at 45°C and then recovered for 24 hours at 37°C, and the levels of Hsp72 were measured by immunoblotting. In RetroQ cells, heat treatment led to a robust induction of Hsp72, whereas no changes of Hsp72 levels occurred in si-HSF-1 cells, indicating the lack of the heat shock response (not shown).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of HSF-1 depletion on sensitivity of PC-3 cells to heat shock, proteasome, and Hsp90 inhibitors. Two days after PC-3 cells were infected with retroviral vectors expressing siRNA to HSF-1 (si-HSF-1) or empty vector (RQ) cells were selected with puromycin (0.5 μg/mL). After 2 days of selection, cells were exposed to stresses. A, depletion of HSF-1 by siRNA sensitizes cells to apoptosis by a proteasome inhibitor MG132. HSF-1 levels were tested in RQ and sihsf1 cells by immunoblotting. si-HSF-1 and RQ cells were exposed to MG132 at indicated concentrations, and after 16 hours of incubation, Hsp72 levels were measured by immunoblotting. After 48 hours of incubation with MG132, apoptosis was measured by monitoring PARP cleavage. 8, quantification of apoptosis measured by PARP cleavage in cells exposed to heat shock, proteasome inhibitor MG132, and Hsp90 inhibitor 17-AAG. PARP cleavage 24 hours after heat shock or 17-AAG was quantified by Quantity One software (Bio-Rad). This experiment was repeated three times. Quantification of a typical experiment is presented. C and D, effect of HSF-1 depletion on overall clonogenic survival of cells exposed to MG132 (C) or 17-AAG (D) for 24 hours.
When these cells were incubated with Hsp90 inhibitor 17-AAG (data not shown) or proteasome inhibitor MG132 (Fig. 1A) for 24 hours, Hsp72 was strongly induced in RetroQ cells but not in si-HSF-1 cells. Interestingly, the background levels of Hsp72 were not significantly altered upon depletion of HSF-1, indicating that another transcription factor is responsible for maintaining elevated levels of Hsp72 in PC-3 cells. Such an alternative activator of the Hsps transcription in cancer cells could be an isoform of p63, as suggested previously (20).

To test for the effects of suppression of the heat shock response on drug sensitivities of cells, si-HSF-1 cells were exposed to heat shock, MG132, or 17-AAG, and degrees of apoptosis were tested by monitoring cleavage of PARP, a substrate of caspase-3. Exposure of control RetroQ cells to 45°C for 20 minutes led to about 20% of PARP cleavage, whereas si-HSF-1 cells showed dramatically increased PARP cleavage (about 50%). Similarly, effect was shown after 40 minutes of heat shock (Fig. 1B). These results with prostate carcinoma PC-3 cells are in line with previous results using noncancerous primary MEF cells, showing that HSF-1 knockout cells are more sensitive to heat shock and an Hsp90 inhibitor than control MEFs (16, 19).

When PC-3 cells were exposed to the proteasome inhibitor MG132 at concentration of 0.125 and 0.25 μmol/L for 48 hours, PARP cleavage was about twice higher in si-HSF-1 compared with control RetroQ cells (Fig. 1A and B). Similarly, depletion of HSF-1 led to increase in PARP cleavage by 100% when PC-3 cells were exposed to the Hsp90 inhibitor 17-AAG at concentrations of 0.25 μmol/L for 24 hours (Fig. 1B).

To assess the overall drug sensitivity of si-HSF-1 and RetroQ PC-3 cells, we monitored the colony-forming ability following exposure to drugs. After treatments with various concentrations of either MG132 or 17-AAG, cells were diluted in a medium and plated. After 10 days, colonies of surviving cells were stained and counted. When treated with MG132, at concentrations between 0.25 and 2 μmol/L, a very strong enhancement of sensitivity was seen with si-HSF-1 cells (Fig. 1C). Similarly, with 17-AAG, about 5-fold sensitization was seen at a wide range of concentrations (Fig. 1D).

Sensitization to apoptosis in response to anticancer drugs by suppression of the heat shock response was relatively specific, because we observed little or no sensitization by si-HSF-1 in cells exposed to a distinct anticancer drug doxorubicin that does not activate the heat shock response (data not shown).

Sensitization to proteasome and Hsp90 inhibitors was seen when we tested additional cancer cells lines. In fact, depletion of HSF-1 by treatment with siRNA in either a different prostate carcinoma line DU-145 (data not shown) or colon carcinoma HCT-116 cells led to suppression of the heat shock response and increased sensitivity to MG132 and 17-AAG (Fig. 2). Interestingly, in contrast to PC-3 and DU-145 cells, the high endogenous levels of Hsp72 in HCT-116 cells seemed to be dependent on HSF-1. Depletion of HSF-1 in these cells led to a dramatic reduction of the constitutive Hsp72 levels (Fig. 2A) and very strong sensitization to the drugs (Fig. 2B and C). Effects on sensitivity to Hsp90 inhibitor radicicol was most dramatic, because control cells were resistant to this drug, whereas si-HSF-1 cells were quite sensitive, suggesting that the resistance of original cells was due to the endogenous expression of one or several of the Hsps.

Our experiments therefore suggest a novel approach towards sensitization of cancer cells to proteasome and Hsp90 inhibitors. Therefore, we screened for small molecules to identify inhibitors of induction of Hsps by these drugs.

Figure 2. Effect of HSF-1 depletion on sensitivity of HCT-116 cells to heat shock, proteasome, and Hsp90 inhibitors. Infection of HCT-116 cells by retrovirus expressing si-HSF-1 was done as described in Fig. 1. A, expression of HSF-1 and Hsp72 in si-HSF-1 cells. B, effects of HSF-1 depletion on sensitivity to apoptosis of cells exposed to heat shock at 45°C for the indicated time, proteasome inhibitor MG132, or Hsp90 inhibitor radicicol at the indicated concentrations. Cells were kept for overnight after heat shock and kept overnight with the drug. C, PARP cleavage was quantified by Quantity One software (Bio-Rad). This experiment was repeated three times. Quantification of a typical experiment is presented.

Screening chemical libraries for inhibitors of Hsp induction. We have developed a two-stage, high-throughput screen for inhibitors of Hsp expression. The first stage involved a cell-based screen for Hsp-mediated refolding of heat-denatured luciferase. Firefly luciferase expressed in mammalian cells is very sensitive to denaturation upon exposure of cells to severe but nonlethal heat insults. On the other hand, pretreatment of cells with milder heat shock leads to induction of Hsps, which protect luciferase from further exposure to denaturing heat insults, and facilitates luciferase refolding. Therefore, exposure of cells to a potential inhibitor of Hsp induction would be predicted to suppress the protective effects of mild heat shock and result in reduced luciferase activity after the second denaturing insult.

For the high-throughput assay, we used CHO cells expressing luciferase under the control of the tet-off promoter. Cells were seeded in 96-well or 384-well plates with medium that lacks tetracycline to allow induction of luciferase, and after 4 hours, chemical compounds were added. Following overnight incubation at 37°C, cells were exposed to heat shock at 45°C for 10 minutes to induce Hsps. After an additional 6 hours at 37°C, the plates were exposed to severe denaturing heat shock at 45°C for 50 minutes followed by recovery for 70 minutes at 37°C to allow
luciferase refolding. The cells were then lysed, and luciferase activity was measured (see Materials and Methods). Exposure of cell to severe heat shock without pretreatment with mild heat shock led to unrepairable damage of luciferase. On the other hand, induction of Hsps after mild heat shock allowed rapid refolding of about 50% of luciferase after 70 minutes of recovery. Compounds that inhibit induction of Hsps must prevent luciferase refolding, and to select the inhibitors, we established a cutoff line at 70% of inhibition of luciferase activity. As a counterscreen against toxic chemicals, the compounds were

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>IC50 HSP72 induction inhibition, μmol/L (CHO cells)</th>
<th>IC50 toxicity, μmol/L (CHO cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emetine</td>
<td></td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
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<tr>
<td>Isocephaeline, NCS-32944</td>
<td></td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
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<tr>
<td>Dehydroemetine, NCS-129414</td>
<td></td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
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<tr>
<td>NZ60, NCS-134757</td>
<td></td>
<td>&gt;10</td>
<td>&lt;2</td>
</tr>
<tr>
<td>NZ28, NCS-134754</td>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>NZ62, NCS-134759</td>
<td></td>
<td>&gt;10</td>
<td>&lt;2</td>
</tr>
<tr>
<td>NZ71, Emunin, NCS-113238</td>
<td></td>
<td>5</td>
<td>&gt;10</td>
</tr>
<tr>
<td>NZ61, NCS-134758</td>
<td></td>
<td>&gt;10</td>
<td>&lt;2</td>
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<td>&gt;10</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Tubulosine, NCS-131547</td>
<td></td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>NZ72, NCS-131548</td>
<td></td>
<td>2</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>
transcription-PCR was done as described in Materials and Methods. Compounds in the NCI chemical library using a Similarity Engine. are critical for inhibition of induction of Hsps, we obtained 15 compounds from the Harvard ICCB, were used for the screen. From about 20,000 chemicals, 40 compounds were found to inhibit luciferase refolding without showing significant toxicity in the luciferase test.

The compounds that were selected at the first step were directly tested for inhibition of Hsp induction by immunoblotting with an anti-Hsp72 antibody in the second step of the screening. Compounds at final concentration of 2 μmol/L were added to CHO cells, and the cells were exposed to 45°C for 10 minutes and then incubated at 37°C for 6 hours to allow accumulation of Hsp72. Cells were lysed, and Hsp72 levels were measured.

Ten of 40 originally selected suppressors of luciferase refolding were found to inhibit Hsp72 induction. Three of these compounds, including emetine, tubulosine, and NZ28, have significant structural similarity. Among these compounds, emetine and tubulosine, while passing the toxicity test at the first step of the screen, nevertheless showed toxicity in an apoptotic assay (data not shown). In contrast, NZ28 did not show toxicity in CHO cells but rather slight growth inhibition (data not shown).

To identify structural elements in this family of compounds that are critical for inhibition of induction of Hsps, we obtained 15 compounds in the NCI chemical library using a Similarity Engine. These compounds were tested directly for inhibition of Hsp72 induction at concentrations between 2 and 10 μmol/L (some of these compounds are shown in Table 1). Four compounds, including NZ71, NZ72, dehydroemetine, and isocephaeline, were found to inhibit Hsp72 induction by heat shock at this range of concentrations, whereas the rest of compounds were found to be inactive. Based on this analysis, we defined the elements of the structure that are essential for the activity (i.e., 2H-benzo[a]quinolizine tricyclic ring). Furthermore, we were able to identify another compound that we called emunin (NZ71) that inhibited Hsp72 induction (Table 1), was nontoxic and showed very little cell growth inhibition.

Characterization of novel inhibitors of the stress response. Both NZ28 and emunin were much more potent inhibitors of the stress response than a previously described inhibitor, the bioflavonoid quercetin, which works through inhibition of HSF-1. In fact, in CHO cells, the IC50 for inhibition of Hsp induction for quercetin was ~50 μmol/L, whereas for emunin, it was 5 μmol/L, and for NZ28, it was 1 μmol/L (Fig. 3A). In all the following experiments, emunin and NZ28 were used at concentrations of 10 and 2 μmol/L, respectively. The inhibitory activities of the selected compounds were not limited to Hsp72. In fact, we observed that induction of another heat shock protein (Hsp27), which also has an antipapoptotic activity, was strongly inhibited (see Fig. 4C).

To assess the specificity of the selected inhibitors, we tested whether they have general inhibitory effects on protein synthesis. This was especially important because emetine, a structural
analogue of NZ28 and emunin, inhibits protein synthesis at concentrations above 10 μmol/L. Therefore, we tested whether emunin and NZ28 can inhibit induction of two unrelated reporter proteins (luciferase and GFP) under the control of tet and cytomegalovirus (CMV) promoters, respectively. To investigate the effects on luciferase induction, the compounds were added to CHO cells, which express luciferase under the tet-off promoter, simultaneously with the removal of tetracycline, and after 24 hours, cells were lysed, and the luciferase induction was assayed by immunoblotting with anti-luciferase antibody. Neither NZ28 (Fig. 3B) nor emunin (data not shown) inhibited luciferase activity.

To test for the effects of the compounds on the distinct reporter protein GFP, CHO cells were infected with retrovirus that encodes CMV-driven GFP gene. At 16 hours after infection (i.e., at the time where no GFP is expressed yet), the compounds were added, and after additional 24 hours, the GFP expression was assayed after overnight incubation. Immunoblotting with anti-tubulin antibody was used as a loading control.

Strong induction of Hsp72 was seen under these conditions, suggesting that the major effect of the compounds is at the post-transcriptional level.

To test whether NZ28 and emunin can inhibit induction of Hsp72 in response to proteasome and Hsp90 inhibitors, MM.1S cells were exposed to proteasome inhibitors, Velcade, or MG132 for 16 hours. Strong induction of Hsp72 was seen under these conditions, whereas keeping the cells with either NZ28 or emunin during the course of the experiment almost completely inhibited Hsp72 induction (Fig. 4). Similarly, these compounds inhibited induction of Hsp72 by the proteasome inhibitors in PC-3 cells (Table 2). These effects were seen 6 hours after addition of the proteasome inhibitors, but inhibition was relieved after 20 hours. Such transient inhibition of the stress response was nevertheless sufficient to sensitize these cells to proteasome inhibitors (see below).

Table 2. Hsp72 inhibition by NZ28 and emunin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Stress</th>
<th>Stress conditions</th>
<th>Hsp72 inhibition, by NZ28 (2 μmol/L), %</th>
<th>Hsp72 inhibition, by emunin (10 μmol/L), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Heat Shock</td>
<td>45°C, 10 min</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td>MM.1S</td>
<td>Heat Shock</td>
<td>45°C, 4 min</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>PC-3</td>
<td>Heat Shock</td>
<td>45°C, 20 min</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>MM.1S</td>
<td>Proteasome Inhibitor</td>
<td>Velcade, 5 μmol/L</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>PC-3</td>
<td>Proteasome Inhibitor</td>
<td>MG132, 1 μmol/L</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>MEF</td>
<td>Proteasome Inhibitor</td>
<td>Velcade, 10 μmol/L</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>MM.1S</td>
<td>HSP90 Inhibitor</td>
<td>Radicicol, 0.8 μmol/L</td>
<td>90</td>
<td>90</td>
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<tr>
<td>PC-3</td>
<td>HSP90 Inhibitor</td>
<td>17-AAG, 2 μmol/L</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>MEF</td>
<td>HSP90 Inhibitor</td>
<td>Radicicol, 0.2 μmol/L</td>
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</tbody>
</table>
Similarly, emunin and NZ28 inhibited induction of Hsp72 in response to Hsp90 inhibitors radicicol and 17-AAG. As with the proteasome inhibitors, the response of multiple myeloma MM.1S cells (Fig. 4B) to Hsp90 inhibitors was blocked by the selected compounds stronger than in PC-3 cells (Table 2). In fact, in PC-3 cells effects of NZ28 on inhibition of Hsps by 17-AAG were significant but transient, whereas emunin showed only weak inhibition of Hsp72 induction by 17-AAG (Table 2).

**NZ28 and emunin sensitize cancer cells to proteasome and Hsp90 inhibitors.** As described above, inhibition of the heat shock response by depletion of HSF-1 sensitized various cancer cells to proteasome and Hsp90 inhibitors. Here, we used MM.1S cells to evaluate whether NZ28 and emunin that inhibit the stress response can sensitize cells to these novel classes of drugs. The degree of caspase-dependent apoptosis in response to proteasome and Hsp90 inhibitors was assessed by PARP cleavage.

MM.1S cells were incubated with Velcade at a concentration of 5 nmol/L with or without emunin. After overnight incubation, 30% cleavage was detected with Velcade alone, whereas incubation with emunin and Velcade together led to 70% of PARP cleavage (Fig. 5A). It is important to note that emunin alone did not cause PARP cleavage. To test for sensitization to Hsp90 inhibitors, MM.1S cells were incubated with radicicol with or without emunin for 24 hours. Sensitization of cells to radicicol was seen at a wide range of concentrations. For example, 0.5 μmol/L radicicol caused 10% of PARP cleavage in control cells, whereas incubation with both radicicol and emunin led to about 50% cleavage (Fig. 5B). NZ28 similarly sensitized MM.1S cells to radicicol (Fig. 5C).

Similar experiments were done with prostate carcinoma PC-3 cells. These cells were treated with a proteasome inhibitor MG132 with or without emunin. After 2 days of incubation, ~10% of PARP cleavage was detected with MG132 treatment alone, whereas incubation with both MG132 and emunin led to 50% of PARP cleavage (Fig. 5D). Similarly, addition of emunin very significantly reduced the colony-forming ability of cells treated with MG132 (Fig. 5E). At the same time, treatment with emunin alone did not affect the colony-forming ability of these cells. On the other hand, we observed little sensitization of PC-3 cells by emunin to radicicol (data not shown). That was probably because emunin was not efficient in inhibiting induction of Hsps in these cells upon exposure to Hsp90 inhibitors.

If emunin-mediated sensitization to proteasome inhibitors is related to suppression of induction of Hsps, we expected that this compound would not further sensitize cells after depletion of HSF-1. Accordingly, to test for the specificity of emunin, we investigated its effects on sensitivity of si-HSF-1 PC-3 cells to MG-132. In contrast to RetroQ cells, no apparent sensitization was seen under these conditions (data not shown), indicating that in fact, the activity of emunin in cell sensitivity to the proteasome inhibitors is mostly related to suppression of the heat shock response.

Therefore, novel inhibitors of the stress response that we have identified may be used as sensitizers of cancer cells to novel classes of drugs, proteasome, and Hsp90 inhibitors and could play a role in combination chemotherapy approaches.

**Discussion**

We have developed drugs that interact with a novel target in cancer cells, after transcriptional regulation of Hsp expression.

We have also shown how these drugs can be effectively deployed in molecular target-specific chemotherapy approaches. As discussed in the Introduction, two novel classes of drugs (proteasome and Hsp90 inhibitors), although highly effective, are limited in effectiveness by off-target induction of heat shock proteins. A number of indirect indications suggested that this side effect of the inhibitors could significantly enhance resistance of cancer cells towards them. Here, we clearly showed that specific inhibition of the heat shock response in cancer cells using siRNA against the transcription factor HSF-1 strongly enhances killing of cells by proteasome and Hsp90 inhibitors. These experiments become the proof of principle for further development of small molecular
weight inhibitors of the heat shock response to sensitize cells towards these novel classes of drugs.

Interestingly, during the course of this work, we found that high background levels of Hsp72 that are often seen in cancer cell lines and in biopsies (11) are maintained due to the activation of HSF-1 (e.g., PC-3 or DU-145) and are probably dependent on specific isoform of p65, ΔNp63α (20). In addition, HSF-1 and Hsp72 expression are induced by the transforming factor heregulin B in breast cancer cells (24). These observations indicate that enhanced constitutive expression of Hsp72 and probably certain other heat shock proteins play an important role in cancer cells specifically, and various mechanisms serve to maintain such an expression. In cancer lines, where the background levels of Hsp72 are reduced by inhibition of the heat shock response, sensitivity to proteasome and Hsp90 inhibitors became especially high. In fact, almost complete resistance of HCT-116 cells to the Hsp90 inhibitor radicicol was reversed after depletion of HSF-1 by siRNA (Fig. 2). Furthermore, recently we showed that reduction of the background levels of Hsp72 makes cancer cells sensitive to other conventional drugs and radiation therapy (14). Therefore, with cancers where the background levels of Hsps are HSF-1 dependent, inhibition of the heat shock response in combination with radiation and other drugs may be especially beneficial.

Very importantly, even in cells where the background levels of Hsps are independent on HSF-1, sensitivity to Hsp90 and proteasome inhibitors is enhanced significantly by inhibition of de novo induction of Hsps. Therefore, this approach of targeting the heat shock response to enhance sensitivity of cancer cells to proteasome and Hsp90 inhibitors seems to be quite general.

An important finding in this work is that we were able to develop a high-throughput chemical screen for inhibitors of the heat shock response and identify novel potent inhibitors. An extensive analysis of various analogues of the original inhibitors allowed us to identify a common structure that is essential for the inhibition of the Hsp induction (i.e., 2H-benzo[a]quinolizine tricyclic ring system found in the naturally occurring bis-benzyloquinoline alkaloid emetine). Furthermore, among the inhibitors, we were able to find two compounds that were nontoxic, the dehydroemetine derivative NZ28 and emunin, the latter a glycine amide conjugate of emetine (25). Our inhibitors are significantly more potent than previously described inhibitors, such as the bioflavonoid drugs Quercetin and genistein. Furthermore, the new inhibitors seem to be relatively specific because they do not inhibit expression of two unrelated reporter genes, while strongly inhibiting expression of Hsp72 and Hsp27 (Fig. 4). Moreover, if the heat shock response was suppressed by an alternative mechanism, depletion of HSF-1 expression, these inhibitors did not further sensitize cells to the proteasome inhibitors. These data indicate that the activity of the inhibitors on cell sensitization to drugs is directly related to suppression of Hsp induction.

Effects of these novel inhibitors on sensitization of various cell lines to the proteasome and Hsp90 inhibitors were different in degree. Most sensitization was seen with the multiple myeloma cells MM.1S, whereas the prostate carcinoma PC-3 cells showed less sensitization to the proteasome inhibitors, and almost no sensitization was seen with the Hsp90 inhibitors (Fig. 5). This difference in part could be because these cells may have different permeability for the novel compounds. In addition, PC-3 cells have higher background levels of the Hsps than MM.1S cells. Accordingly, the proteasome and Hsp90 inhibitors induced Hsps in PC-3 cells to a lesser extend; therefore, the role of such induction in cell protection may be less significant than in the MM.1S cells.

The novel inhibitors described here are unique, because they do not affect the transcriptional activity of HSF-1. Neither do they affect the rate of degradation of Hsp mRNA or proteins (data not shown). Therefore, these compounds seem to have a relatively specific regulatory effect on translation of the major Hsps. It is not clear by which mechanisms Hsp translation is specifically targeted, although Hsp translation can occur by CAP-independent mechanisms in contrast to most proteins in unstressed cells. However, this finding opens an interesting opportunity for pharmacologic intervention in that heat shock protein expression can be modulated specifically either at translational (as here) or transcriptional levels, and chemical screens for inhibitors of the heat shock response can be designed accordingly.

An important drawback in using proteasome inhibitors in anticancer therapy is the development of resistant clones. Little is known about the nature of such a resistance, except that in one lymphoid line the resistance was associated with overexpression of Hsp27 (13). If this is a general phenomenon, compounds that inhibit the heat shock response may restore the sensitivity of such clones to the drugs.

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