Zinc ribbon domain-containing 1 (ZNRD1) mediates multidrug resistance of leukemia cells through regulation of P-glycoprotein and Bcl-2

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Abstract
Here, we investigated the role of zinc ribbon domain-containing 1 (ZNRD1) in multidrug resistance (MDR) of leukemia cells and the possible underlying mechanisms. ZNRD1 was found overexpressed in the vincristine-induced MDR leukemia cell HL-60/vincristine moreso than its parental cell HL-60. Up-regulation of ZNRD1 expression could confer resistance of both P-glycoprotein (P-gp)-related and P-gp-nonrelated drugs on HL-60 cells and suppress Adriamycin-induced apoptosis accompanied by decreased accumulation and increased releasing amount of Adriamycin. ZNRD1 could significantly up-regulate the expression of P-gp, Bcl-2, and the transcription of the MDR1 gene but not alter the expression of MDR-associated protein, glutathione S-transferase activity, or intracellular glutathione content in leukemia cells. In addition, inhibition of ZNRD1 expression by RNA interference or P-gp inhibitor could partially reverse ZNRD1-mediated MDR. The further study of the biological functions of ZNRD1 may be helpful for understanding the mechanisms of MDR of leukemia and developing possible strategies to treat leukemia. [Mol Cancer Ther 2005;4(12):1936–42]

Introduction
Multidrug resistance (MDR) is a major impediment to the effective chemotherapy of many human malignancies.

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and β-actin (5'-AGCCGGAAAATCGTCGTCG-3' and 5' CAGGGTACATGGTGCC-3'). The PCR conditions of ZNRD1 were 93°C for 30 seconds, 54°C for 30 seconds, and 72°C for 60 seconds. Thirty-four cycles were done. All of the PCR products were separated on ethidium bromide-stained agarose and visualized with UV.

**Western Blot Analysis**

The total cellular proteins were prepared and then quantified by Bradford method. A measure of 80 μg lysates were electrophoresed in 12% SDS-PAGE and blotted on a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked with 5% fat-free milk powder at room temperature for 2 hours and incubated overnight with primary antibody at 4°C. After three washes for 15 minutes in PBS-Tween 20, the membrane was incubated with the horseradish peroxidase–conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. The membrane was washed again in PBS-Tween 20; enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ) was added and monitored for the development of color. The following antibodies were used: anti-P-gp, anti-MRP, anti-Bcl-2, and anti-Bax polyclonal antibodies (Santa Cruz Biotechnology); anti-Bcl-xL and anti-Bak (PharMingen, San Diego, CA); anti-ZNRD1 (prepared by our laboratory; ref. 14); and anti-β-actin (Sigma Chemical Co., St. Louis, MO).

**Cell Transfection**

Cells were planted in six-well plates and cultured in drug-free medium. At 90% to 95% confluence, cells were washed twice with PBS and grew in 2 mL DMEM without antibiotics. Using LipofectAMINE 2000 reagent (Invitrogen, Inc., Carlsbad, CA), 2 μg pcDNA3.1 (Invitrogen) that included the full-length cDNA for ZNRD1 was transfected into HL-60 cells, and 2 μg mU6pro-ZNRD1 small interfering RNA (siRNA) plasmids were transfected into HL-60/vincristine cells as described previously (15). The cells transfected with mU6pro vector alone or pcDNA3.1 vector alone were served as negative control. Forty-eight hours later, cells were placed in growth medium containing G418 (Life Technologies) for clone selection. The expression levels of ZNRD1 in G418-resistant stable clones were evaluated by Western blot analysis.

**In vitro Drug Sensitivity Assay**

Vincristine, Adriamycin, cisplatin (CDDP), 5-fluorouracil (5-FU), and mitomycin C were all freshly prepared before each experiment. Drug sensitivity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (16). Briefly, cells were cultured with culture medium to the seeding density (1 × 10^4/well), suspended in 96-well flat-bottomed plates (200 μL/well; Costar, Cambridge, MA), and incubated at 37°C for 24 hours. Cells were then incubated for 72 hours in the absence or presence of various concentrations of the anticancer agents in 200 μL medium. Each condition was assayed in triplicate. After cells being cultured for 72 hours, 50 μL of 2 mg/mL MTT (Sigma) was added into each well, and cells were cultured for another 4 hours. Then, supernatants were discarded and 150 μL DMSO (Sigma) was added into each well to dissolve crystals. Absorbance at 490 nm was measured with a microplate reader BP800 (Biohit, Helsinki, Finland). Cell survival rates were calculated according to the formula: survival rate = (mean A_{490} of treated wells / mean A_{490} of untreated wells) × 100%. Finally, dose-effect curves of anticancer drugs were drawn on semilogarithm coordinate paper and IC_{50}s were determined.

**Intracellular Adriamycin Concentration Analysis**

Fluorescence intensity of intracellular Adriamycin was determined by flow cytometry. Briefly, leukemia cells were seeded into six-well plates (1 × 10^4/well) and cultured overnight at 37°C. After addition of Adriamycin to the final concentration of 5 μg/mL, cells continued to be cultured for 1 hour. Cells were then harvested (for detection of Adriamycin accumulation) or cultured in drug-free RPMI 1640 for another 1 hour followed by harvesting (for detection of Adriamycin retention). Then, cells were washed with PBS and the mean fluorescence intensity of intracellular Adriamycin was detected using flow cytometry with an exciting wavelength of 488 nm and emission wavelength of 575 nm. The experiment was independently done thrice. Finally, the Adriamycin releasing index of leukemia cells was calculated according to the formula: releasing index = (accumulation value − retention value) / accumulation value.

**Glutathione S-Transferase Activity**

Total glutathione S-transferase (GST) activity of leukemia cells was evaluated by conjugation of reduced glutathione to 1-chloro-2,4-dinitrobenzene as described previously (17). Briefly, cells were resuspended in tetramethylsilane buffer and sonicated at 4°C for 30 seconds. After centrifuging, 100 μg cellular fractions were added to cuvettes containing 1.0 mmol/L 1-chloro-2,4-dinitrobenzene and 1.0 mmol/L reduced glutathione at pH 6.5, and the final volume was adjusted to 1.0 mL by addition of PBS. Change in absorbance (340 nm) was measured over a span of 3 minutes to calculate the rate of conjugation of 1-chloro-2,4-dinitrobenzene.

**Reduced Glutathione Measurements**

Intracellular reduced glutathione contents of leukemia cells were measured using the GSH-400 assay kit from OXIS International, Inc. (Portland, OR). In brief, cells in log phase were harvested and resuspended in freshly prepared 5% metaphosphoric acid. The suspension was sonicated for 30 seconds and centrifuged at 3,000 × g for 10 minutes at 4°C. The supernatant (100 μL) was used in the assay for the detection at 400 nm of thione formed from the conjugation of reduced glutathione to 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methysulfate.

**Reporter Gene Assay**

HL-60 cells were plated in six-well dishes and grown in maintenance medium. The pGL3-MDR1 vector (promoter of MDR1, −136 to +10) and the control vector were established previously by Dr. Changcun Guo in our laboratory (18). Briefly, cells were cotransfected with indicated amounts of pcDNA3.1/ZNRD1 plasmids (0.3, 0.5, and...
0.8 μg), pGL3-MDR1, and pRL-TK vector using the Fugene transfection reagent (Roche, Indianapolis, IN) as described previously (18). After 48 hours, luciferase reporter assays were done following the protocol of Promega (Madison, WI) as described previously (19). Cells were then processed for β-galactosidase staining with the PanVera β-Galactosidase Staining kit according to the manufacturer’s protocols.

A total of 300 cells per well were counted, and the percentage of blue cells was determined.

**DNA Fragmentation Assay**

After incubation with 1.5 μg/mL Adriamycin for 36 hours, cells were collected and washed twice with ice-cold PBS (20). Cell pellets were resuspended in lysis buffer and incubated overnight at 50°C with continuously shaking. The nucleic acids were extracted with phenol-chloroform, precipitated with ethanol-sodium acetate, and redissolved in deionized water containing 100 mg/mL RNase A. After incubation in a water bath at 37°C for 30 minutes, the DNA samples were analyzed on 1.5% agarose gel containing 0.1 μg/mL ethidium bromide.

**Annexin V Staining**

Cells were washed twice with cold PBS and resuspended in 100 μL binding buffer at a concentration of 1 × 10⁶/mL. Then, 5 μL Annexin V-FITC (PharMingen) and 10 μL of 20 μg/mL propidium iodide (Sigma) were added to these cells. After incubation at room temperature for 15 minutes, 400 μL Annexin-binding buffer was added to each sample, and the samples were kept on ice for counting the stained cells by flow cytometry. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane (21).

**Statistical Analysis**

The data were expressed as the means ± SD. ANOVA and Student’s-Newman-Keuls’ test were done to evaluate the changes of IC₅₀s and Adriamycin fluorescence intensity. All data were analyzed using the SPSS software package (SPSS, Inc., Chicago, IL). P < 0.05 was considered significant.

**Results**

**Expression of ZNRD1 in Leukemia Cell Lines**

The expression of ZNRD1 in HL-60/vincristine and HL-60 cells was detected by reverse transcription-PCR and Western blot. Figure 1A showed that the mRNA and protein levels of ZNRD1 were both higher (2.7- and 3.6-fold) in HL-60/vincristine than in HL-60 cells, indicating that ZNRD1 might be related to drug-resistant phenotype of leukemia cells.

**In vitro Drug Sensitivity Assay**

HL-60 cells were transfected with the recombinant plasmids containing the full open reading frame of wild-type ZNRD1, and two stable effective clones (HL-60-Z1 and HL-60-Z2) were selected with significantly increased ZNRD1 expression (2.1- and 1.5-fold) compared with control cells (Fig. 1B). As shown in Table 1, the IC₅₀s of HL-60-Z1 and HL-60-Z2 cells for vincristine, Adriamycin, 5-FU, and CDDP were significantly increased compared with empty vector transfected control cells. We also found that ZNRD1 siRNA can effectively reduce the endogenous level of ZNRD1 protein with the inhibitory rate of ~81%, and down-regulation of ZNRD1 partially reversed the resistance of HL-60/vincristine cells toward chemical therapeutic drugs. However, no significant differentiation was found in the IC₅₀ of cells treated with mitomycin C. Taken together, the expression of ZNRD1 affected the sensitivity of cells not only to P-gp-related drugs vincristine and Adriamycin but also to P-gp-nonrelated drugs 5-FU and CDDP.

**Adriamycin Content in ZNRD1-Related Transfectants**

Because MDR of cancer is mainly due to alterations of drug influx and efflux, Adriamycin intracellular accumulation and releasing were explored. Adriamycin is fluorescent and this attribute provides easy monitoring of its intracellular accumulation and retention by flow cytometry. Figure 2A showed the intracellular Adriamycin content in ZNRD1-related transfectants and their controls after exposed to Adriamycin for 1 hour. Decreased accumulation of Adriamycin of HL-60-Z1 cells and increased accumulation of Adriamycin in HL-60/vincristine-siRNA cells were observed compared with that of their corresponding controls (P < 0.01). Consistent with this, HL-60-Z1 cells showed increased releasing index, whereas HL-60/vincristine-siRNA cells decreased (Fig. 2B).

**Effect of ZNRD1 on Classic MDR Molecules**

To study the possible molecular mechanisms involved in ZNRD1-related MDR of leukemia, P-gp and MRP, two well-characterized drug transporters, were examined in leukemia cells (Fig. 3). The relative expression level of P-gp to β-actin was markedly higher in HL-60-Z1 cells (3.2-fold)
Table 1. IC₅₀s (µg/mL) of anticancer drugs for leukemia cells

<table>
<thead>
<tr>
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<th>P-gp-related drugs</th>
<th>P-gp-nonrelated drugs</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vincristine</td>
<td>Adriamycin</td>
</tr>
<tr>
<td>HL-60</td>
<td>0.34 ± 0.11</td>
<td>0.17 ± 0.22</td>
</tr>
<tr>
<td>HL-60-vector</td>
<td>0.42 ± 0.03</td>
<td>0.21 ± 0.02</td>
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<tr>
<td>HL-60-Z1</td>
<td>6.45 ± 0.79</td>
<td>3.42 ± 0.78</td>
</tr>
<tr>
<td>HL-60-Z2</td>
<td>5.08 ± 0.53</td>
<td>2.67 ± 0.48</td>
</tr>
<tr>
<td>HL-60/vincristine</td>
<td>44.24 ± 0.21</td>
<td>7.16 ± 0.12</td>
</tr>
<tr>
<td>HL-60/vincristine-vector</td>
<td>42.33 ± 0.18</td>
<td>5.06 ± 0.23</td>
</tr>
<tr>
<td>HL-60/vincristine-siRNA</td>
<td>10.32 ± 0.17</td>
<td>1.29 ± 0.15†</td>
</tr>
</tbody>
</table>

NOTE: Survival rates of leukemia cells to anticancer drugs were evaluated by MTT assay as described in Materials and Methods. The dose-effect curves of anticancer drugs were drawn on semilogarithm coordinate paper; thus, IC₅₀s were determined. Data are mean ± SD of four independent experiments.

*P < 0.01 versus HL-60-vector and HL-60.
† P > 0.05 versus HL-60/vincristine-vector and HL-60/vincristine.
‡ P < 0.05 versus HL-60/vincristine-vector and HL-60/vincristine.

and HL-60-Z2 cells (1.8-fold) and decreased in HL-60/vincristine-siRNA cells (65%) compared with empty vector transfected control cells. Meanwhile, all these cell lines exhibited no obvious difference of MRP expression.

Partial Reversal Effects of Verapamil on ZNRD1-Related MDR

To further investigate the possible role of P-gp in ZNRD1-related MDR, we examined whether resistance of leukemia cells to drugs could be modulated by verapamil. The results of MTT assay showed that verapamil could partially reverse the drug resistance in HL-60-Z1 cells (Fig. 4), indicating that P-gp may play a certain role in ZNRD1-related MDR.

Up-Regulation of ZNRD1 Increases MDR1 Transcriptional Activity

To elucidate the regulatory effects of ZNRD1 on the promoter activity of MDR1, luciferase reporter assays were done. As shown in Fig. 5, cotransfection of the MDR1 reporter gene with increasing amounts of ZNRD1 expression vector resulted in an essentially linear increase in MDR1 promoter activity, suggesting that ZNRD1 might be involved in regulation of MDR1 transcription.

GST Activity and Intracellular Reduced Glutathione Content

GST-mediated drug-detoxifying systems were detected in the transfected cells. Compared with control cells, the transfected cells showed slightly altered GST activity and intracellular reduced glutathione concentration (Table 2). However, statistical analysis revealed that these changes were not significant (P > 0.05).

Effects of ZNRD1 on Apoptosis

As the blockade of apoptosis is another important mechanism of MDR, we investigated the capacity of HL-60-Z1 cells to undergo Adriamycin-induced apoptosis by DNA fragmentation assay and Annexin V staining. Treatment of HL-60-vector cells with 1.5 µg/mL Adriamycin for 36 hours resulted in internucleosomal DNA fragmentation, evidenced by the formation of DNA ladders on agarose gels (Fig. 6A), a hallmark of cells undergoing apoptosis. No DNA ladders were detected in the sample from HL-60-Z1 cells. Similarly, results of Annexin V staining suggested that up-regulation of ZNRD1 could protect cells from Adriamycin-induced apoptosis and the apoptotic rate of HL-60-Z1 cells was significantly lower than that of control cells (Fig. 6B).

Effect of ZNRD1 on Proteins Regulating Apoptosis

To gain insight into the molecular mechanisms involved in ZNRD1-mediated apoptosis, the expressions of Bcl-2, Bax, Bcl-xL, and Bak were assessed in ZNRD1-related transfectants. As shown in Fig. 3, the expression of Bcl-2 protein was increased in response to up-regulation of ZNRD1 (2.2- and 1.6-fold) and decreased in response to...
down-regulation of ZNRD1 (71%). However, relatively equal levels of Bax, Bcl-xL, and Bak were detected in all HL-60-derived cell lines. These data strongly suggested that overexpression of ZNRD1 might suppress drug-induced apoptosis by enhancing the Bcl-2/Bax ratio in leukemia cells.

Discussion

In the present study, ZNRD1 was found overexpressed in the vincristine-resistant leukemia cell line HL-60/vincristine compared with its parental cell HL-60. Then, its potential role in MDR of leukemia cells and the possible underlying mechanisms were further investigated.

To obtain a better model in which cells of the same origin could be compared, we transfected HL-60 cells with the recombinant plasmids containing the full open reading frame of wild-type ZNRD1 and the effective stable transfectants were selected. MTT assay revealed that ZNRD1 had different effects on drug sensitivity depending on the drug used. HL-60-Z1 and HL-60-Z2 cells showed a >12-fold increased resistance to vincristine and Adriamycin and a >5-fold increased resistance to 5-FU and CDDP compared with control cells (P < 0.01). We also transfected HL-60/vincristine cells with the siRNA vectors of ZNRD1, and HL-60/vincristine-siRNA showed significantly increased sensitivity to Adriamycin, vincristine (>4-fold), and 5-FU and CDDP (>2-fold). It was interesting that HL-60/vincristine, selected by drug resistance to vincristine, showed resistance not only to P-gp-related vincristine and Adriamycin but also to 5-FU, CDDP, and mitomycin C (Table 1). This was consistent with previous report that vincristine-induced resistant gastric cancer cell line SGC7901/vincristine and Adriamycin-induced resistant gastric cancer cell line SGC7901/Adriamycin showed resistance not only to typical MDR drugs but also to non-P-gp-related drugs (11, 12, 20).

Adriamycin was used as probe to evaluate drug accumulation and retention in leukemia cells. As shown by the results, up-regulation of ZNRD1 in leukemia cells was accompanied with significantly decreased Adriamycin accumulation and retention and increased Adriamycin releasing index. Consistent with this, HL-60/vincristine-siRNA cells showed increased Adriamycin accumulation and retention and decreased Adriamycin releasing index. The results indicated that ZNRD1 had a direct or indirect function of pumping drug out of cells.

It should be noted that vincristine and Adriamycin are the common substrates for P-gp and MRP. To clarify the association of P-gp and MRP with ZNRD1-related MDR, we investigated the effects of ZNRD1 on expression of them. The results showed that P-gp might mediate the ZNRD1-related MDR of leukemia. We further observed the effects of verapamil on ZNRD1-related MDR. Verapamil has three domains that seem to be important for its ability to interact with P-gp and modulate MDR: two aromatic rings and a basic nitrogen atom (22). As the first-generation P-gp inhibitor, verapamil showed poor P-gp selectivity and might be complicated by metabolic drug interactions with the anticancer drug regimen (23). Verapamil had profound effects on the pharmacokinetics of oncolytic drugs in addition to their ability to inhibit P-gp, and as a result, the dose of the agent was limited by toxicity (23–26). Gaitanos et al. (27) and Du et al. (20) described that coadministration of verapamil could overcome drug resistance to both P-gp-related and non-P-gp-related drugs due to a direct toxic effect of verapamil or induction of redistribution of drugs from cytoplasm to nucleus (28). Their results agree with our data that verapamil could partially reverse the effects of ZNRD1 on drug sensitivity in leukemia cells, especially for vincristine and Adriamycin,
suggesting that regulation of P-gp might be one of the mechanisms by which ZNRD1 mediated MDR. However, verapamil only partially inhibited the MDR phenotype of leukemia cells, perhaps because the drug-resistant phenotype of the cells may be multifactorial.

Each case of P-gp-related MDR has been related to an increased human MDR1 mRNA level that can be linked to either gene amplification and/or increased gene transcription (29). Alterations in MDR1 promoter are important for P-gp function (30). In this report, MDR1 gene, which encodes P-gp that functions as an ATP-dependent drug-efflux pump, was further analyzed by reporter gene assay. Analysis of the MDR1 revealed putative ZNRD1-binding sites located among −136 to +10 in the MDR1 promoter (11). Cotransfection of HL-60 cells with MDR1 reporter gene and increasing amounts of ZNRD1 expression vectors resulted in a linear increase in MDR1 promoter activity, and the data were confirmed by transfection experiments in leukemia cells. The increase in the steady-state levels of the P-gp protein in ZNRD1 transfected cells and the ability of ZNRD1 to induce the MDR1 reporter gene in transient transfections argue that ZNRD1 is a transcriptional regulator of the MDR1 gene. We assumed that the role of ZNRD1 might depend not only on the promoter sequences but also on the association of ZNRD1 with different cofactors. The precise mechanism by which ZNRD1 influences MDR1 gene expression is the subject of future experimental work.

However, how HL-60-Z1 and HL-60-Z2 cells resisted to 5-FU and CDDP could not be explained by up-regulation of P-gp, which suggested that other mechanisms might exist. Thus, we further tested whether the GST-mediated drug-detoxifying system was involved in ZNRD1-related MDR. GST catalyzed the conjugation of reduced glutathione to some electrophilic anticancer drugs, which deprived these drugs of the possibility to reach their cellular targets (31). It has been reported that increased GST activity and reduced glutathione content in drug-resistant tumor cells were associated with resistance to nitrogen mustards, melphalan, and CDDP (32–35). We detected total GST activity and intracellular GST content in leukemia cells. However, GST-mediated drug-detoxifying system was not found significantly involved in ZNRD1-mediated MDR.

Apoptosis was a common pathway that finally mediated the killing functions of anticancer drugs, which was an...
important cause of MDR. HL-60-Z1 cells displayed an impaired capacity to undergo Adriaycin-induced apoptosis, whereas control cells displayed a higher proportion of apoptosing cells after Adriamycin treatment. Bcl-2 family, including Bcl-2, Bcl-xl, Bax, Bad, and Bak, were a rapidly expanding family of proteins involved in apoptosis and responses of tumor cells to chemotherapy (36). These proteins were believed to modulate apoptosis by forming homodimers or heterodimers with other Bcl-2 family members (36, 37). A wide variety of human cancers, with poor clinical response to chemotherapy, exhibited high levels of Bcl-2 expression. It has been implied that Bcl-2 family expression provided resistance to a wide variety of cell death stimuli, including classic chemotherapeutic drugs and radiation (38). The changed levels of Bcl-2 caused by ZNRD1 might also contribute to suppression effects of ZNRD1 on apoptosis and thus ZNRD1-related MDR of leukemia cells.

In conclusion, we clearly showed for the first time that ZNRD1 might play certain roles in MDR of leukemia through P-gp signal pathway and/or cooperation with Bcl-2. Further analysis of the mechanism of biological actions of ZNRD1 in MDR of leukemia might help to further understand the mechanisms of MDR in leukemia and generate a new approach to reverse MDR.

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Molecular Cancer Therapeutics

Zinc ribbon domain-containing 1 (ZNRD1) mediates multidrug resistance of leukemia cells through regulation of P-glycoprotein and Bcl-2

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