Expression of Siva-1 Protein or Its Putative Amphipathic Helical Region Enhances Cisplatin-Induced Apoptosis in Breast Cancer Cells: Effect of Elevated Levels of BCL-2

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Abstract
cis-Diaminedichloroplatinum (II) (cisplatin) is routinely used to treat various types of cancers; however, a significant number develop resistance. One of the underlying factors that contribute to cisplatin resistance is the elevated level of BCL-2 and/or BCL-XL, which promotes cell survival. A potential method of overcoming such resistance is to use a potentiatior that is capable of neutralizing the antiapoptotic effects of BCL-2/BCL-XL, such as Siva-1. We previously cloned the proapoptotic protein Siva-1 and showed a possible role for it in both extrinsic and intrinsic apoptosis. Using an adenovirus-based expression system, we now show that Siva-1 can synergize with cisplatin in inducing apoptosis in MCF7 and MDA-MB-231 breast cancer cells. In an anchorage-independent clonogenicity assay, MCF7/caspase-3 cells stably expressing Siva-1, but not the controls, showed a dramatic decrease in the number of colonies formed on one-time cisplatin treatment. Further, we show that the unique putative amphipathic helical region (SAH) in Siva-1 (amino acid residues 36-55) is necessary and sufficient for the observed enhancement in cisplatin-induced apoptosis by Siva-1. Although cisplatin treatment results in significant elevation in the expression of Fas ligand and intracellular p21 levels, expression of Siva-1 has no additional benefit. Instead, the enhancement in cisplatin-induced apoptosis seems to be due to activation of intrinsic pathway that involves caspase-9 activation. Moreover, Siva-1 augments cisplatin-mediated cell death in MCF7 cells stably expressing BCL-2. We therefore propose that Siva-1 or its SAH region can be used as a potentiatior of cisplatin-based chemotherapy. (Cancer Res 2005; 65(12); 5301-9)

Introduction
Cisplatin, a chemotherapeutic agent, is known to cause DNA damage by forming DNA-DNA or DNA-protein adducts that trigger p53/p73-mediated apoptosis (1, 2). Apart from toxicity, a major hurdle to successful use of cisplatin (and other chemotherapeutic agents in general) is the invariable development of resistance (1), and an important contributory factor is the elevated level of the antiapoptotic proteins BCL-2 and/or BCL-XL (3-6). A molecule that can overcome such resistance can therefore be used to potentiate cisplatin-induced apoptosis. Hence, it is not surprising that targets directed against specific players of apoptosis are currently being used to enhance the efficacy of various chemotherapeutic treatments (7). Siva-1, a proapoptotic protein we previously cloned, using the cytoplasmic tail of CD27 [a tumor necrosis factor (TNF) receptor family member] as the bait in the yeast two-hybrid system, definitely seems to be one such molecule (8).

Siva-1 is relatively short (175 amino acids) and has a unique putative 20-amino-acid amphipathic helical region (SAH) towards the NH₂ terminus and pure-cysteine Zn fingers towards the COOH terminus. Unlike other intracellular molecules that mediate signaling of some of other TNF receptor family members, Siva-1 has neither a death domain nor a TRAF domain. It also lacks any of the known BCL-2 homology regions despite its role in some of the receptor (extrinsic) and nonreceptor (intrinsic) mediated cell death pathways (8-18). The minor alternate splice form, Siva-2, lacks the exon 2 region that codes for the SAH region and is much less toxic (8, 16). We have previously shown that the SAH region can interact with the antiapoptotic members of the BCL-2 family, such as BCL-XL and BCL-2, and sensitize cells stably expressing BCL-XL or BCL-2 to UV radiation–induced apoptosis (16, 18). Moreover, the SAH region is sufficient to interact with BCL-XL and BCL-2 and inhibit the antiapoptotic activity of BCL-XL and BCL-2 (18).

Transcription of Siva-1 seems to be significantly decreased along with p53 in colorectal cancer (19). A recent study by Daoud et al. (20) showed that colon cancer cells treated with topotecan increase their Siva-1 transcripts in a p53-dependent manner. Subsequently, both p53 and E2F1, two transcription factors that are potent tumor suppressors, were found to directly activate in neuronal cells the transcription of Siva gene of which the main transcript is Siva-1 (21). Incidentally, cancer cells treated with cisplatin also resulted in enhanced expression of Siva-1 (22). This is not surprising because cisplatin mainly targets genomic DNA thereby triggering p53-mediated DNA repair and apoptosis. We therefore investigated further to see if Siva-1 could potentiate cisplatin-induced apoptosis in breast cancer cells such as MCF7 and MDA-MB-231. In this article, we show that adenoviral-mediated expression of Siva-1 acts synergistically with cisplatin in inducing apoptosis in breast cancer cells. The SAH region in Siva-1 is necessary and sufficient to augment cisplatin-induced apoptosis. Using MDA-MB-231 cells that stably express BCL-2 as a model for cisplatin resistance, we further show that Siva-1 can significantly enhance cisplatin-induced cell death. We therefore propose that Siva-1 or its SAH region can be used as a potentiatior of cisplatin-based chemotherapy with the added advantage of curbing BCL-2-mediated resistance.

Materials and Methods
Reagents and cells. Cisplatin and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, St. Louis, MO); fetal calf serum (FCS), cell culture media such as RPMI and DMEM, antibodies for selection such as G418 and Zeocin (Invitrogen, Carlsbad, CA); Hoechst 33342

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(Molecular Probes, Eugene, OR) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit (Roche, Nutley, NJ) are commercially available. Antibodies against caspase 3, caspase 8, caspase 9, actin, green fluorescent protein (GFP), BCL-2, Fas ligand (Fasl), p21, and the corresponding horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Generation of anti-Siva rabbit antisera and the breast cancer cell line MCF-7 stably expressing caspase 3 under the control of G418 selection has been previously described (8, 23). MDA-MB-231 cells were a kind gift from Dr. Das Gupta (University of Illinois at Chicago, Chicago, IL).

**Generation of adeno-Siva-1 virus.** The pAd-CMV5-IRES-GFP recombinant virus was constructed as previously described (24, 25). Monoclonal recombinant adenovirus (adeno-Siva-1) was propagated in 293A cells and purified by CsCl gradient centrifugation. Concentrated viral preparations were titrated by limiting dilution assay and had titers in the 10^10 to 10^12 range. Viral stocks were stored at −80°C until use. As a control vector, we used the adenovirus that expresses GFP (adeno-null). Equivalent amounts of adeno-null and adeno-Siva viruses were used to infect MCF7/caspase-3 and MDA-MB-231 cells. Twenty-four hours later, more than 90% of cells were observed to fluoresce green and Siva-1 was only expressed in adeno-Siva–infected cells as evidenced by immunoblotting. Microscopic examination revealed normal morphology, indicating that the virus itself is not toxic (data not shown).

**Generation of stable cell lines.** MDA-MB-231 cells were either transiently transfected with empty pCDNA or pCDNA-BCL-2 using lipofectamin (Invitrogen) and G418 was used for selection.

Final G418 concentration used for maintenance was 500 μg/mL and the selected cells were maintained as a polyclonal mixture. Intracellular expression of BCL-2 was confirmed in whole cell lysates separated on a SDS-PAGE, followed by transfer and immunoblotting with anti-BCL-2 antibody. Empty pEF4 or pEF4-Siva-1 plasmids (16, 18) were transiently transfected into MCF7/caspase-3 cells. Transfectants were selected using G418 and Zeocin. The polyclonal mixture of cells was cultured in medium containing G418 (500 μg/mL) and Zeocin (250 μg/mL). The expressions of Siva-1 and procaspase 3 were both confirmed by immunoblotting.

**Cell viability and specific apoptosis.** Viability of cells was determined using the MTT assay as per instructions of the manufacturer (Sigma). The absorbance value of untreated cells was considered 100% viability. Each experiment was done in triplicate and repeated thrice. The percentage of specific apoptosis in green fluorescing cells was assessed by fluorescent microscopy as previously described using Hoechst 33342 (16, 18). In all, seven different fields were randomly chosen to determine the percentage of cells with condensed chromatin (apoptotic nuclei) in the cells expressing GFP. Specific apoptosis was also determined by the TUNEL method based on the protocol of the manufacturer (Roche).

**Anchorage-independent clonogenicity assay.** Soft agar solution [final 0.7% agarose in 2 × MEM (containing 20% FCS, 2 × Pen/Strep, 2 × G418/ Zeocin)] was used to line the bottom of each well in a six-well plate. Five-thousand cells diluted in 1 mL of 0.7% agarose in MEM were then used to form the top layer. After 21 days, the number of colonies per unit field was determined by using a low-power microscope. Seven fields were counted to obtain the average number of colonies and the experiment was repeated thrice.

**Immunoblotting.** Cell lysates were prepared as described (16, 18). Equivalent amounts of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with the following primary antibodies: anti-Siva (1:500), anti–BCL-2 (1:1,000), anti–GFP (1:1,000), anti–Fasl (1:1,000), anti–p21 (1:1,000), anti–caspase 3 (1:1,000), and anti–caspase 9 (1:5,000), and anti–caspase 8 (1:2,000).

**Statistical analysis.** All values were expressed as means ± SD. Student’s t test was used to evaluate differences in cytotoxicity in the different treatment groups. A P value of ≤0.05 was considered statistically significant. CalcuSyn program was applied to determine synergy between Siva-1 expression and cisplatin treatment in killing breast cancer cells. The method is based on the principle of Chou and Talay (26) and a combination index (CI) value of <1, 1, and >1 represents synergy, additivity, and antagonism, respectively.

**Results**

**Effect of Siva-1 on cisplatin IC50.** Cisplatin is known to induce apoptosis in various types of cancer cells including that of breast cancer (27–29). To determine the added effect of Siva-1 expression, we first determined the IC50 dose of cisplatin in MCF7/caspase-3 cells treated with the null or the Siva-1 adenovirus. The viability of cells was determined using the MTT assay (Fig. 1) and independently confirmed using trypan blue (data not shown).

The curves generated by cells infected with adeno-null and noninfected cells were almost superimposable, suggesting that the adenovirus expressing GFP per se was not toxic and the IC50 ranged from 40 to 70 μg/mL. In contrast, the curve for adeno-Siva-1–treated cells significantly shifted to the left of adeno-null–infected or noninfected cells. IC50 in the presence of expressed Siva-1 was significantly lower and ranged from 15 to 20 μg/mL. Similar data for MDA-MB-231 cells were also observed (data not shown) and the IC50 values for both MCF7/caspase 3 and MDA-MB-231 cells are summarized in Table 1. Statistical analysis and comparison of the dose-response curves revealed that expressed Siva-1 acts synergistically with cisplatin in inducing apoptosis in the above two breast cancer cell lines.

**Siva-1 expression enhances cisplatin-induced apoptosis in MCF7/caspase-3 and MDA-MB-231 cells.** We next determined the effect of adenviral-mediated expression of Siva-1 on cisplatin-mediated apoptosis using the Hoechst stain in the two breast cancer cell lines MCF7/caspase-3 and MDA-MB-231. The experiment consisted of two sets, each containing three groups of cells. One was infected with adeno-null, the second with adeno-Siva-1, and the third served as control uninfected group. After 24 hours, one of the two sets was treated with cisplatin (15 μg/mL). Percentage of specific apoptosis was determined 24 hours later using the Hoechst stain and the data are presented in Fig. 2A and B.

![Figure 1. Dose-response curve for cisplatin in MCF7/caspase-3 breast cancer cells](Image)
Infecting the cells with adeno-Siva-1 generally resulted in a slight increase in apoptosis as compared with adeno-null–infected or noninfected MCF7/caspase 3 or MDA-MB-231 breast cancer cells. The cisplatin-induced apoptosis observed in adeno-null–infected cells was comparable to that of uninfected cells and was significantly higher than that observed with any of the untreated groups. In adeno-Siva-1–infected cells, cisplatin treatment resulted in a further 2- to 3-fold increase in cell death as compared with those infected with the adeno-null virus or noninfected cells and treated with cisplatin.

The expression of GFP and Siva-1 in the three groups of MCF7/caspase-3 cells is shown in Fig. 2C and similar results were obtained using MDA-MB-231 cells (data not shown). The noninfected cells neither express GFP nor Siva-1, however, the levels of GFP expression were comparable in both adeno-null– and adeno-Siva-1–infected cells and only adeno-Siva-1–infected cells express Siva-1. The Siva-1–mediated enhancement in cisplatin-induced apoptosis was also confirmed in MDA-MB-231 cells using the TUNEL method (Fig. 2D).

Effect of Siva-1 expression and cisplatin treatment on anchorage-independent colony formation. To better judge the effect of cisplatin treatment and expression of Siva-1 on colony formation ability, which is a reflection of the combined effects of

| Table 1. Range of IC_{50} values obtained for MCF-7/caspase 3 and MDA-MB-231 cells for cisplatin treatment and cell viability |
|-----------------|-----------------|-----------------|
| IC_{50} (μg/mL) | MCF-7/caspase 3  | MDA-MB-231       |
| Uninfected      | 40-70           | 60-90           |
| Adeno-null      | 40-70           | 60-90           |
| Adeno-Siva-1    | 15-20           | 20-30           |

Figure 2. Siva-1 significantly enhances cisplatin-induced apoptosis in breast cancer cells. A, two sets of MCF7/caspase-3 cells were infected with equivalent amounts of adeno-null or adeno-Siva-1 virus and uninfected cells served as control. After 24 hours, one set was treated with cisplatin (15 μg/mL) for an additional 24 hours and percentage of specific apoptosis was determined using Hoechst stain. Expression of Siva-1 alone did result in slightly higher percentage of apoptosis as compared with adeno-null–infected cells or control cells; however, the differences were not significant. Cisplatin treatment, however, resulted in significantly higher percentage of cell death in Siva-1–expressing cells as compared with adeno-null or uninfected cells (P < 0.01). B, MDA-MB-231. The experiment is similar to the one above and the results obtained were also very similar, suggesting that the observed potentiation between Siva-1 and cisplatin is not restricted to any one cell type (P < 0.01). C, expression of Siva-1 as well as GFP in the whole cell lysates of MCF7/caspase-3 cells was determined by immunoblotting with anti-Siva and anti-GFP antibodies. D, the percentage of specific apoptosis was also determined by TUNEL assay in MDA-MB-231 cells. The difference between adeno-Siva-1 and adeno-null cells treated with cisplatin was statistically significant (P < 0.04). The trend observed was very similar to that seen in A and B.
loss in cell growth and death, we used MCF7/caspase-3 cells stably expressing Siva-1 (pEF4-Siva-1) or harboring the empty plasmid (pEF4). The experiment had two identical sets of three groups each, consisting of untransfected cells, cells stably transfected with the empty plasmid, and cells expressing Siva-1, plated on soft agar. The second set was similar to the first except that the cells were one-time treated with cisplatin (15 μg/mL) and then plated. Colonies were allowed to develop for 21 days and representative colonies are shown in Supplement 1 and the quantitative data are summarized in Table 2. Colony-forming ability of parental MCF7/caspase-3 cells and cells harboring the empty plasmid decreased by more than 50% on one-time treatment with cisplatin (compare 23.4% with 10.0% and 20.3% with 8.8%). In comparison, the ability of MCF7/caspase-3 cells expressing Siva-1 cells to form colonies dropped dramatically from 18.6% to 2.2% after the drug treatment. In general, the average colony size of untreated cells stably expressing Siva-1 was not much different from that of its controls; however, cisplatin treatment did result in significant decrease in colony size (see Supplement 1).

**Siva-1 putative amphipathic helical region is responsible for the observed potentiation between Siva-1 and cisplatin.** Because our previous work showed that the SAH region in Siva-1 is necessary and sufficient to abrogate the antiapoptotic activity of BCL-2 and BCL-XL (16, 18), we therefore investigated its role in cisplatin/Siva-1–mediated apoptosis. MCF7/caspase-3 cells were transiently transfected with a combination of plasmids that express GFP along with glutathione S-transferase (GST), GST-Siva-1, GST-Siva-2, GST-Siva-1Δ136–55 (a deletion mutant that lacks the SAH region), or GST-Siva-1Δ130–149 (a deletion mutant that lacks an equivalent unrelated downstream region). One day later, they were treated with cisplatin (15 μg/mL). After 24 hours, percentage of specific apoptosis was determined in green fluorescing cells. As shown in Fig. 3A, expression of GST-Siva-1, but not of the tag GST or GST-Siva-2, significantly enhanced cisplatin-induced apoptosis. Also, Siva-1 mutant that lacked the SAH region failed to increase cisplatin-induced apoptosis, suggesting that it is necessary to significantly enhance cisplatin-mediated cell death. This claim is further substantiated by the fact that an equivalent deletion of amino acids 130 to 149 did not have a similar effect. The corresponding whole cell lysates were analyzed for the expression of various GST-tagged proteins (Fig. 3B) and the expression levels of Siva-1 and its mutants further support the above contention. We also carried out a similar experiment using MDA-MB-231 cells and obtained comparable results, further demonstrating the importance of the SAH region in the observed synergy between Siva-1–mediated and cisplatin-mediated cell death (Fig. 3C). The expression levels of various proteins were similar to those shown in Fig. 3B (data not shown).

To ensure that the expression of SAH region alone is sufficient to enhance cisplatin-induced apoptosis, we transiently transfected pEF-Siva-1-SAH (amino acid residues 36–55 of Siva-1) and its control plasmid (pEF-Siva-1-130–149) along with pEGFP into MCF7/caspase-3 cells followed by treatment with cisplatin for 24 hours. As shown in Fig. 3D, cells transfected with the SAH peptide–expressing plasmid, but not those transfected with the control peptide–expressing plasmid, showed a significant increase in cisplatin-induced cell death similar to that observed in cells expressing Siva-1 and also expressed to cisplatin (compare Fig. 3D with Fig. 3A and C). As noted in our previous study, similar levels of peptide expression were observed as judged by fluorescent microscopy and immunoblotting (18).

**Mechanism underlying Siva-1/cisplatin–mediated cell death.** Cisplatin is known to enhance FasL expression in target cells and thus induce apoptosis through Fas receptor clustering (24). One of the hallmarks of Fas-mediated cell death is activation of the initiator caspase 8 (28). In addition, the data presented here (Table 2 and Supplement 1) and previous reports support a role for cisplatin growth arrest, and one of the mediators is p53-induced p21, a cyclin-dependent kinase inhibitor (1). To investigate the underlying mechanism of Siva-1–enhanced cisplatin-mediated apoptosis, we determined the relative levels of FasL, procaspase 8, and p21 in whole cell lysates prepared from cisplatin-treated MCF7 cells infected with either adeno-null or adeno-Siva-1 virus or left alone by immunoblotting (Fig. 4A). Similar to previous reports (1), cisplatin treatment resulted in the up-regulation of FasL levels; however, expression of Siva-1 did not seem to have any additional effect. The procaspase-8 levels in samples representing cisplatin-treated cells were slightly lower compared with their respective controls; however, expression of Siva-1 per se or in combination with cisplatin had no discernable effect. In addition, our attempts to detect active form of caspase 8 in the above samples failed (data not shown). As expected, cisplatin treatment resulted in significantly enhanced expression of p21 in all three groups; however, there was no additional effect due to Siva-1 expression thereby confirming our earlier observation (Table 2 and Supplement 1) that Siva-1 mainly affects apoptosis. The actin levels served as loading control (Fig. 4A, bottom).

We previously observed that Siva-1–mediated abrogation of antiapoptotic activity of BCL-2/BCL-XL is via activation of caspase 9 (18). We therefore determined the status of caspase 9 by carrying out experiments similar to the one described above using an anti-caspase-9 rabbit polyclonal antibody that mainly detects the 12 kDa active fragment of caspase 9; the results are shown in Fig. 4B. In contrast to uninfected cells, slight activation of caspase 9 in cisplatin-treated MDA-MB-231 cells infected with adeno-null virus was observed. In comparison, dramatic activation of caspase 9 was seen in adeno-Siva-1–infected cells that were also treated with cisplatin, clearly supporting our contention that Siva-1 enhances cisplatin-mediated cell death by probably targeting endogenous BCL-2 and BCL-XL.

### Table 2. Effect of one-time cisplatin treatment on the number of colonies formed by MCF-7/caspase-3 cells stably expressing Siva-1 or harboring the empty plasmid

<table>
<thead>
<tr>
<th>Colonies (mean ± SD)</th>
<th>% Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MCF7/caspase 3</td>
<td>1.168 ± 0.36</td>
</tr>
<tr>
<td>2. MCF7/caspase 3/pEF4</td>
<td>1.014 ± 0.63</td>
</tr>
<tr>
<td>3. MCF7/caspase 3/pEF4-Siva-1</td>
<td>0.931 ± 0.78</td>
</tr>
<tr>
<td>4. MCF7/caspase 3/cisplatin</td>
<td>0.502 ± 0.70</td>
</tr>
<tr>
<td>5. MCF7/caspase 3/pEF4/cisplatin</td>
<td>0.438 ± 0.59</td>
</tr>
<tr>
<td>6. MCF7/caspase 3/pEF4-Siva-1/cisplatin</td>
<td>0.109 ± 0.14</td>
</tr>
</tbody>
</table>

NOTE: Various types of cells were plated on soft agar after one-time treatment with cisplatin (15 μg/mL) for 24 hours. The differences between 1 and 4, 2 and 5, 3 and 6, and 5 and 6 are statistically significant (P ≤ 0.01).
Activated caspase 9, in turn, is known to target effector caspase 3. We therefore determined the status of caspase-3 activation in the above experiment. MDA-MB-231 cells were infected with adenovirus-null and adenovirus-Siva-1 viruses. After 24 hours, the cells were then treated with cisplatin and equivalent whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody that recognizes the procaspase 3. As shown in Fig. 4C (top), there was a profound loss of procaspase 3 in adenovirus-Siva-1–infected cells as compared with adenovirus-null cells, clearly demonstrating that the cisplatin/Siva-1–mediated apoptosis culminates in caspase-3 activation. We also determined actin levels in the above samples and they were comparable (data not shown). In addition, only those cells that were infected with adenovirus-Siva-1 virus expressed detectable levels of Siva-1 (Fig. 4C, bottom). MCF7/caspase-3 cells were stably transfected with the empty pEF4 and pEF4-Siva-1 vector. Polyclonal populations of these stable transfectants were maintained under G418 (selection for caspase 3) and Zeocin (selection for pEF4). These cells were treated with cisplatin for 24 hours and the cell lysates were immunoblotted to determine the expression of Siva-1 (Fig. 4D, bottom) and active caspase 3 (Fig. 4D, top). Because procaspase 3 is stably expressed in these cells at relatively high levels, we did not observe any perceptible drop in the procaspase-3 level even in cells expressing Siva-1 and treated with cisplatin (compare lane 4 with lanes 1-3); however, the cleaved form of caspase 3, indicating that caspase 3 is indeed activated, was only detected in the lane corresponding to cells expressing Siva-1 and treated with cisplatin (compare lane 4 with lanes 1-3; Fig. 4D).

Effect of Siva-1 in combination with cisplatin in a BCL-2 resistance cell model. One of the mechanisms underlying cisplatin-induced resistance in cancer is elevated intracellular

Figure 3. The SAH region in Siva-1 is necessary and sufficient to mediate the observed synergy between Siva-1 and cisplatin in killing breast cancer cells. A, MCF7/caspase-3 cells were transiently transfected with plasmids expressing GFP along with GST, GST-Siva-1, GST-Siva-2, GST-Siva-1Δ36-55, or Siva-1Δ130-149. Twenty-four hours later, they were treated with cisplatin (15 μM/mL) for another 24 hours and the percentage of specific apoptosis was determined. Expression of GST-Siva-1, but not of GST or GST-Siva-2, resulted in a significant increase in cisplatin-induced cell death (P < 0.01). Deletion of the SAH region (GST-Siva-1Δ36-55), but not of the control region (GST-Siva-1Δ130-149), resulted in a loss of the observed synergy between Siva-1 and cisplatin. The differences between cells expressing GST and GST-Siva-1 or between GST-Siva-1 and GST-Siva-2 or between GST-Siva-1Δ36-55 and GST-Siva-1Δ130-149 were statistically significant and the P values ranged from <0.05 to <0.01. B, corresponding protein expression. C, MDA-MB-231 cells were used in the experiment that is similar to the one shown in A. The results once again emphasize the importance of Siva-1 SAH region in the enhancement of cisplatin-induced apoptosis. D, the SAH region in Siva-1 is sufficient to significantly enhance cisplatin-induced apoptosis. Expression of the SAH region (pEF-Siva-1-36-55), but not of the control region (pEF-Siva-1-130-149), in MCF7/caspase-3 cells resulted in significant enhancement of cisplatin-induced apoptosis (P < 0.02).
levels of the antiapoptotic members of the BCL-2 family such as BCL-2 and/or BCL-XL (3–7). To simulate this condition in vitro, we generated MDA-MB-231 cells that stably express BCL-2 and also their controls that harbor the empty plasmid. Once again, we determined the cisplatin dose-response curves using these two cell lines; the results are shown in Fig. 5A. Cells that express BCL-2 are much more resistant to cisplatin-induced cytotoxicity as compared with control cells at every concentration of cisplatin tested and the evidence that the BCL-2 stable transfectants do express relatively higher levels of the antiapoptotic protein is shown in the inset.

To show the potentiation between Siva-1 and cisplatin in the breast cancer cells that stably expressed BCL-2, we used a higher concentration of cisplatin. Figure 4. Mechanism underlying cisplatin/Siva-1–mediated apoptosis in breast cancer cells. A, MDA-MB-231 cells were cultured as such or infected with adeno-null or adeno-Siva-1 virus followed by treatment with cisplatin. Equivalent amounts of whole cell lysates were subjected to immunoblotting using anti-FasL, anti–procaspase-8, anti-p21WAF1, and anti-actin antibodies. Cisplatin treatment resulted in a significant elevation in relative levels of FasL; however, Siva-1 expression had no detectable effect. Although a slight drop in procaspase-8 levels was detected after cisplatin treatment, Siva-1 expression had no added advantage. B, MCF7/caspase-3 cells were left alone (control) or infected with adeno-null or adeno-Siva-1 virus, and 24 hours later they were treated with cisplatin. Equivalent amounts of whole cell lysates were probed for caspase-9 activation (top) and actin levels were determined as loading controls (bottom). Cisplatin treatment did give rise to a very weak active caspase-9 band in adeno-null–infected cells. Maximum activation of caspase 9, however, was seen in adeno-Siva-1–infected cells and treated with cisplatin. C, MDA-MB-231 cells were either infected with adeno-null or adeno-Siva-1 or left alone, and the next day were treated with cisplatin. The whole cell lysates were then immunoblotted with anti–caspase-3 antibody to monitor loss in procaspase 3. Only the cells infected with adeno-Siva-1 and treated with cisplatin showed a significant loss in procaspase 3. D, MCF7/caspase-3 cells were transiently transfected with empty pEF or pEF-Siva-1 plasmids, and after 24 hours the cells were treated with cisplatin for another 24 hours. Equivalent whole cell lysates were immunoblotted with an anti–caspase-3 antibody that also recognizes its active cleaved form. Because these cells stably express caspase 3, we did not observe significant change in the levels of procaspase 3; however, in cells transfected with Siva-1 and treated with cisplatin, the active cleaved form of caspase 3 was apparent. Representative of three independent experiments.
concentration of cisplatin as compared with the previous experiments (25 µg/mL), and the percentage of specific apoptosis was determined at 48 hours instead of the usual 24 hours. Infection of MDA-MB-231/BCL-2 cells with adeno-Siva-1 did result in a slightly higher apoptosis as compared with those cells infected with adeno-null virus or untreated cells (Fig. 5B). Cisplatin treatment naturally resulted in relatively higher percentages of specific cell death in uninfected or adeno-null-infected cells as compared with previous experiments (compare Fig. 5B with Fig. 2A and B) due to the fact that the duration of drug treatment was 48 hours instead of the usual 24 hours and also the drug concentration used here was much higher. Expression of Siva-1 in combination with cisplatin treatment could still overcome the protective effects of elevated BCL-2 in these cells and resulted in almost double the apoptosis seen in adeno-null-infected cells (Fig. 5B). These results thus clearly show that exogenous expression of Siva-1 can potentiate cisplatin-mediated apoptosis in breast cancer cells even under conditions where cisplatin resistance due to elevated levels of BCL-2 prevails.

Discussion

TNF receptor family members are known to regulate cell proliferation and differentiation as well as cell death (29). Using the cytoplasmic tail of CD27 (a member of the TNF receptor family), we previously identified human Siva-1 (8). The Siva gene in humans is localized to chromosome 14q32-33 and its organization is very similar to that of mouse (16). Both generate the predominant full-length Siva-1, which is apoptotic; in addition to a minor shorter alternate splice form, Siva-2, which is significantly less toxic. Siva-2 lacks the exon 2 coding sequence of Siva-1, which harbors the SAH region that is highly conserved between human, mouse, and rat (16). A potential apoptotic role for Siva-1 in various receptor and nonreceptor mediated cell death pathways has been reported (8–18). Interestingly, Siva-1, through its SAH region, can bind to BCL-2 or BCL-XL and abrogate their antiapoptotic activity, thereby sensitizing cells to UV radiation–induced apoptosis. Furthermore, the 20-amino-acid SAH region is also necessary and sufficient to interact with BCL-2/BCL-XL and abrogate their antiapoptotic activity (16, 18). The fact that we could isolate natural complexes of Siva-1 and BCL-XL from T cells suggests that the interaction between Siva-1 and the antiapoptotic BCL-2 family member may be physiologically relevant (16). Because BCL-2 and BCL-XL significantly contribute to the resistance against various cancer therapies in general and cisplatin in particular (3–7), we therefore examined the ability of Siva-1 or its SAH region to enhance cisplatin-induced apoptosis in breast cancer cells.

Cisplatin is known to cause extensive DNA damage, which is known to activate p53 and facilitate enhanced expression of FasL/Fas, thereby triggering apoptosis (1). One of the hallmarks of Fas receptor–mediated cell death is the activation of caspase 8 and, interestingly, cisplatin in some systems is known to activate caspase 8 independent of Fas signaling (1). In our study, at the time points examined, cisplatin treatment of breast cancer cells did enhance significantly FasL expression; however, Siva-1 expression per se or in combination with cisplatin had no additional benefit. Despite the elevated FasL levels, the downstream activation of caspase 8 was not apparent either in cisplatin or cisplatin/Siva-1 cells, clearly suggesting that the extrinsic pathway may not play a major role in cisplatin/Siva-1–mediated apoptosis. In combination with expressed Siva-1, cisplatin treatment instead resulted in substantial activation of the effector caspase 3 (see Fig. 4C and D). The underlying mechanism of Siva-1 enhanced cisplatin-mediated apoptosis seems to involve loss of mitochondrial function as evidenced by the dramatic activation of caspase 9 (Fig. 4B).

As previously shown using UV treatment (18), the SAH region in Siva-1 seems to be necessary and sufficient for the observed enhancement in cisplatin-induced apoptosis by Siva-1. In addition to cisplatin, expression of Siva-1 also significantly enhanced Adriamycin-induced apoptosis in breast cancer cells, and once again, in both cases, expression of the SAH region is sufficient to mimic the effect of full-length Siva-1 (Fig. 3D and Supplement 2C). This strengthens the possibility of utilizing the

Figure 5. Siva-1 expression promotes cisplatin-mediated apoptosis even in breast cancer cells that are engineered to express elevated levels of BCL-2. A, viability was determined in MDA-MB-231 cells stably transfected with empty pCDNA or pCDNA-BCL-2 and treated with various doses of cisplatin for 48 hours. As shown, expression of Siva-1 resulted in increased efficacy of cisplatin at every dose tested. Inset, BCL-2 levels in the whole cell lysates of the two stable transfectants. B, MDA-MB-231/BCL-2 cells were left alone (control) or infected with adeno-null or adeno-Siva-1. Twenty-four hours later, one set of cells was treated with cisplatin (25 µg/mL) for 48 hours and specific percentage of apoptosis was determined. Expression of Siva-1 alone resulted in a slight increase in apoptosis. Although cisplatin treatment of control or adeno-null cells did result in significant cell death, it however more than doubled in cisplatin-treated adeno-Siva-1–infected cells (P < 0.01).
unique 20-amino-acid SAH region as potentiator in various cancer therapies. Interestingly, cisplatin is also known to promote deamidation of the unstructured BCL-XL loop region, resulting in loss of its cell survival function (30). It is therefore possible that both Siva-1 and cisplatin independently target BCL-2 and BCL-XL, resulting in a substantial loss in the ability of cancer cells to survive.

Apart from induction of apoptosis, cisplatin also seems to kill target cells in a caspase-independent manner (30). Jensen and Glazer (31) in a recent article show that the ability of cisplatin to kill transformed fibroblasts and MCF7 cells is directly proportional to cell density, perhaps due to the establishment of intercellular contacts through gap junctions. Cisplatin-mediated cell damage seems to transmit a death signal (molecular weight <1 kDa) through the gap junctions, which requires the kinase activity of Ku70, Ku80, and DNA-PK. Cisplatin treatment in general seems to be responsible for reduction in cell growth as evidenced from the up-regulation of p21 levels (Fig. 4A) and the decrease in the number of colonies (Table 2). In contrast, expression of Siva-1 had no adverse effect on the levels of p21 (Fig. 4A) or on colony-forming ability (Table 2). Therefore, the dramatic decrease in the size and number of colonies formed by cisplatin-treated Siva-1–expressing cells as compared with cells treated with cisplatin alone can be attributed due to the combined effects of growth arrest and enhanced apoptosis. It is also likely that the observed synergy between Siva-1 and cisplatin may also involve the enhancement of the death signal transmitted through the gap junctions to the neighboring cells.

Cisplatin-induced DNA damage is also known to trigger the activity of c-abl tyrosine kinase (32). In this context, we have previously shown that c-abl–related kinase ARG could phosphorylate Siva-1, which dramatically increases its ability to enhance reactive oxygen species–mediated cell death (9). Therefore, we considered whether Siva-1–enhanced cisplatin-induced apoptosis could be mediated by c-abl phosphorylation of Siva-1 at Y34 (9). In the present study, we did not detect any tyrosine phosphorylation of the expressed Siva-1 in cisplatin-treated breast cancer cells (data not shown), suggesting that specific tyrosine phosphorylation of Siva-1 may not be playing a role in the observed synergy between cisplatin and Siva-1.

Cisplatin-induced damage triggers DNA repair and apoptosis, which requires p53. Unfortunately, more than 50% of cancers have mutated nonfunctional p53. Thus, loss of p53 not only contributes to the development of the cancer but also interferes with drug-induced apoptosis. In this context, use of Siva-1 as a potentiator of cisplatin-induced apoptosis would serve a unique purpose. As the transcription of Siva gene is directly activated by p53 and E2F1 (18, 19), by exogenously expressing Siva-1, we can overcome any potential loss of p53 function.

Molecules that are critical to cell proliferation and cell death also indirectly contribute to cancer. Several lines of evidence suggest a role for Siva-1 in the development of cancer. A Japanese study showed a significant decrease in the transcripts of Siva-1 along with p53 in colorectal cancers (19). Incidentally, Siva-1 transcripts are highly expressed in normal human colon (8). In addition, some of the chemotherapeutic drugs, including cisplatin, are known to induce transcription of Siva gene in a p53-dependent and p53–independent manner (20, 21), suggesting that Siva-1 is likely to play a role in drug-induced apoptosis and that it can still be induced even when p53 function is lost. In this study, we have thus taken advantage of one of the components in the cisplatin-mediated cell death pathway. In addition, we tested the combined effects of Siva-1 and cisplatin in a breast cancer cell line that lacks functional p53 (MDA-MB-231; ref. 33) and also in a cell line that harbors functional p53 (MCF7; ref. 33) and obtained similar results clearly supporting our contention that exogenously expressed Siva–mediated apoptosis effects are independent of a functional p53.

One of the major problems of most of the chemotherapeutic drugs, including cisplatin, is the development of drug resistance. In an in vitro chemosensitivity study that involved 177 breast carcinomas and several antineoplastic drugs that included cisplatin, it was observed that increased levels of BCL-2 significantly correlated to enhanced drug resistance (5). In a separate extensive informatics study, it was also observed that the levels of intracellular BCL-XL correlated strongly with drug resistance (6). Clearly, a major component of resistance to chemotherapy and other cancer therapies is the elevated intracellular levels of the antiapoptotic proteins of the BCL-2 family. Both BCL-2 and BCL-XL seem to protect tumor cells from cell death induced by a variety of chemotherapeutic agents (including cisplatin), suggesting that BCL-2 and BCL-XL may pose a significant threat to successful cancer therapy. One way to overcome such a daunting problem is to use a proapoptotic molecule, such as Siva-1, to block the cell survival function mediated by BCL-XL and BCL-2. Although Siva-1 does not belong to the BCL-2 family, it can specifically interact with both BCL-2 and BCL-XL and effectively inhibit their cell survival function (16, 18). In the present study, we clearly show that Siva-1 can indeed potentiate cisplatin-induced killing of two types of breast cancer cells and counteract resistance due to elevated levels of BCL-2. Once the structure of SAH region is determined, we can then use it to model small organic compound agonists that can effectively negotiate the cell membrane barrier and inhibit BCL-2/BCL-XL–mediated survival. Such an approach has been used to develop a small organic compound that acts like a potent BCL-2 homology domain 3–only apoptotic member of the BCL-2 family (34), and similar compounds are being currently tested as potentiators in various cancer therapies (7).

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Expression of Siva-1 Protein or Its Putative Amphipathic Helical Region Enhances Cisplatin-Induced Apoptosis in Breast Cancer Cells: Effect of Elevated Levels of BCL-2

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