Breast cancer cells with acquired antiestrogen resistance are sensitized to cisplatin-induced cell death

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Abstract

Antiestrogens are currently used for treating breast cancer patients who have estrogen receptor–positive tumors. However, patients with advanced disease will eventually develop resistance to the drugs. Therefore, compounds effective on antiestrogen-resistant tumors will be of great importance for future breast cancer treatment. In this study, we have investigated the effect of the chemotherapeutic compound cisplatin using a panel of antiestrogen-resistant breast cancer cell lines established from the human breast cancer cell line MCF-7. We show that the antiestrogen-resistant cells are significantly more sensitive to cisplatin-induced cell death than antiestrogen-sensitive MCF-7 cells and we show that cisplatin induces cell death by activating both the caspase and lysosomal death pathways. The antiestrogen-resistant cell lines express lower levels of antiapoptotic Bcl-2 protein compared with parental MCF-7 cells. Our data show that Bcl-2 can protect antiestrogen-resistant breast cancer cells from cisplatin-induced cell death, indicating that the reduced expression of Bcl-2 in the antiestrogen-resistant cells plays a role in sensitizing the cells to cisplatin treatment. [Mol Cancer Ther 2007;6(6):1869–76]

Introduction

The use of antiestrogens, such as tamoxifen, as therapy for estrogen-responsive breast cancers has been used for almost three decades. However, a major clinical problem with the use of antiestrogens is that most patients with advanced disease eventually develop resistance to the compounds. The exact molecular mechanisms behind development of antiestrogen resistance are still not clear although several mechanisms have been proposed, including loss of estrogen receptor-α expression, posttranslational modifications of the estrogen receptor, and changes in signal transduction (1). In the case of tamoxifen, the partial agonistic properties of the compound have been suggested to play a role in development of resistance to tamoxifen treatment (2). ICI 182,780 (Faslodex; refs. 3, 4) and ICI 164,384 (5) are pure antagonists of estrogen receptor, but these compounds also give rise to outgrowth of resistant cancer cells both in vitro (6–8) and in vivo (9). More and more evidence now points to altered signal transduction as a way for the cancer cells to shift from estrogen receptor–mediated signaling to other cellular pathways (e.g., pathways involving the epidermal growth factor receptor (10) and the phosphatidylinositol 3-kinase-Akt pathway (11)). Therefore, taking advantage of these changed properties of the resistant cells could lead to new strategies for preventing and treating antiestrogen resistance in breast cancer patients.

Cisplatin (cis-diaminedichloroplatinum II) is a platinum compound used for treatment of a variety of cancers. Cisplatin is active in breast carcinoma given alone (12) or in combination with other chemotherapeutic drugs (13–15). In women with HER2-positive metastatic breast cancer, treatment with cisplatin, or the related carboplatin, in combination with taxanes and trastuzumab (Herceptin) shows promising results (16, 17). Cisplatin induces intrastrand and interstrand cross-links in DNA, resulting in DNA adducts, which is followed by induction of cell death. Cisplatin activates caspases through different signaling pathways, including stabilization of tumor suppressor protein p53 and release of cytochrome c from mitochondria (18). Evidence of alternative death programs has emerged during the past few years, where it has become clear that chemotherapeutic compounds, including cisplatin, also can trigger, e.g., lysosomal membrane permeabilization, resulting in release of lysosomal proteins to the cytosol (19–21). Lysosomal proteases, such as cysteine cathepsin B and L, can induce caspase-independent programmed cell death (22) or mitochondrial membrane permeabilization (23), thereby enhancing the caspase-mediated pathway.

Because it was unknown how the response of antiestrogen-resistant cells would be toward cisplatin treatment, our strategy was to compare the cisplatin sensitivity of MCF-7 human breast cancer cells with MCF-7–derived antiestrogen-resistant cell lines. We found clear evidence that acquired antiestrogen resistance sensitized the cells to cisplatin treatment, and we discovered that cisplatin induced increased cell death, mediated by both caspases
and lysosomal proteases. Furthermore, we could show that down-regulation of Bcl-2, one of the key regulators of mitochondrial-mediated cell death, was involved in this sensitization. These results suggest that low doses of cisplatin given in combination with antiestrogens or given as second-line or third-line therapy to patients who have relapsed from endocrine therapy might be a way to overcome or postpone antioestrogen resistance.

Materials and Methods

Cell Cultures
The MCF-7 cell line was originally obtained from the Breast Cancer Task Force Cell Culture Bank (Mason Research Institute, Worcester, MA) and adapted to growth at low serum concentrations (24). Antioestrogen-resistant cell lines MCF-7/TAM<sup>R</sup>-1, MCF-7/182<sup>R</sup>-1, MCF-7/182<sup>R</sup>-6, MCF-7/184<sup>R</sup>-7, MCF-7/182<sup>R</sup>-9, MCF-7/164<sup>R</sup>-4, MCF-7/164<sup>R</sup>-5, and MCF-7/164<sup>R</sup>-7 were established as previously described (6, 25, 26). MCF-7 cells were propagated in growth medium consisting of phenol red–free DMEM/F12 (1:1; Life Technologies, Invitrogen) supplemented with 1% FCS (Life Technologies, Invitrogen), 2.5 mmol/L l-glutamine (Life Technologies, Invitrogen), and 6 ng/mL insulin (Novo-Nordisk). Growth medium was supplemented with 1 μmol/L tamoxifen (Sigma-Aldrich) for growth of tamoxifen-resistant cell lines and with 100 nmol/L ICI 182,780 (ICI 182,780) for growth of ICI 182,780– and ICI 182,780-resistant cell lines and with 13.5 μg/mL digitonin (for extraction of cytoplasmic proteins) or 200 μg/mL digitonin (for total protein extraction), was added to the cells. Protein extraction was done on ice for 10 min. Cysteine cathepsin activity tests of extracts were done in cathepsin reaction buffer (50 mmol/L sodium acetate, 4 mmol/L EDTA, 8 mmol/L DTT, and 1 mmol/L Pefabloc [Roche], supplied with either 13.5 μg/mL digitonin (for extraction of cytoplasmic proteins) or 200 μg/mL digitonin (for total protein extraction), was added to the cells. Protein extraction was done on ice for 10 min. Cysteine cathepsin activity tests of extracts were done in cathepsin reaction buffer (50 mmol/L sodium acetate, 4 mmol/L EDTA, 8 mmol/L DTT, and 1 mmol/L Pefabloc, pH 6.0) by measuring liberation of 7-amino-4-trifluoromethylcoumarin (AFC) from the cathepsin B and L substrate zFR-AFC (50 μmol/L; MP Biomedicals) for 20 min at 30°C with a Spectramax Gemini fluorometer (Molecular Devices Ltd.). NAG activity was measured by incubating extracts for 30 min with 300 μg/mL 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma-Aldrich) in buffer containing 0.2 μmol/L sodium citrate (pH 4.5). Endpoint liberation of methylumbelliferone was measured with the Spectramax Gemini fluorometer.

All cysteine cathepsin and NAG activities were normalized to the activity of the cytosolic protein lactate dehydrogenase of the same extracts to account for variations in cell number between samples. Incubation time and the digitonin concentration for cytoplasmic extraction were optimized to result in total release of cytosolic lactate dehydrogenase without release of lysosomal proteins. Lactate dehydrogenase activity was measured using the Cytotoxity Detection Kit (Roche). Three independent experiments were done with reproducible results. In each experiment, samples were done in triplicate.

Western Blot Analysis

Cells for Western blot analysis were harvested and protein extracts were made in radioimmuno precipitation assay buffer as described before (11). Determination of protein concentration was done using the Bio-Rad protein assay (Bio-Rad Laboratories) and bovine serum albumin as standard. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electrobolting. Membranes were blocked with PBS containing 0.2% casein and 0.1% Tween 20. Immunostaining was done with primary antibodies directed against Bcl-2 (1:200; C-2, Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; 1:1,400; 6639GR, PharMingen), and β-actin (1:10,000; AC-15, Sigma-Aldrich), followed by incubation with alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G (1:20,000; Jackson ImmunoResearch Laboratories). Detection of antibody–protein complexes was done using the chemoluminescence CDP-star (Tropix). Western blot analyses were done on at least two independent sets of lysates with similar results.

Measurement of Cathepsin and β-N-Acetyl-Glucosaminidase Activity

Measurement of cytosolic cysteine cathepsin and β-N-acetyl-glucosaminidase (NAG) activities was done as previously described (28). Briefly, cell medium was removed and extraction buffer containing 250 mmol/L sucrose, 20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EGTA, 1 mmol/L EDTA, 8 mmol/L DTT, and 1 mmol/L Pefabloc (Roche), supplied with either 13.5 μg/mL digitonin (for extraction of cytoplasmic proteins) or 200 μg/mL digitonin (for total protein extraction), was added to the cells. Protein extraction was done on ice for 10 min. Cysteine cathepsin activity tests of extracts were done in cathepsin reaction buffer (50 mmol/L sodium acetate, 4 mmol/L EDTA, 8 mmol/L DTT, 1 mmol/L Pefabloc, pH 6.0) by measuring liberation of 7-amino-4-trifluoromethylcoumarin (AFC) from the cathepsin B and L substrate zFR-AFC (50 μmol/L; MP Biomedicals) for 20 min at 30°C with a Spectramax Gemini fluorometer (Molecular Devices Ltd.). NAG activity was measured by incubating extracts for 30 min with 300 μg/mL 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma-Aldrich) in buffer containing 0.2 μmol/L sodium citrate (pH 4.5). Endpoint liberation of methylumbelliferone was measured with the Spectramax Gemini fluorometer.

All cysteine cathepsin and NAG activities were normalized to the activity of the cytosolic protein lactate dehydrogenase of the same extracts to account for variations in cell number between samples. Incubation time and the digitonin concentration for cytoplasmic extraction were optimized to result in total release of cytosolic lactate dehydrogenase without release of lysosomal proteins. Lactate dehydrogenase activity was measured using the Cytotoxity Detection Kit (Roche). Three independent experiments were done with reproducible results. In each experiment, samples were done in triplicate.

Transfections

Plasmids pCEP4-BCL-2, pCEP4-BCL-nt, and H2B-pEGFP-N1 were kindly provided by Marja Jäättelä (Danish Cancer Society, Copenhagen, Denmark). pCEP4-Bcl-nt plasmid DNA contains a nonfunctional Bcl-2 coding region with a stop codon insertion after amino acid 218 as previously described (29). H2B-pEGFP-N1 is encoding a fusion-protein of histone 2B (H2B) and enhanced green fluorescence protein (eGFP) as described before (30). Cotransfections with H2B-pEGFP-N1/pCEP4-BCL-2 (1:4) or H2B-pEGFP-N1/pCEP4-BCL-nt (1:4) were done with FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions. Transfections were done at least thrice with similar results.
Flow Cytometry

Cells were incubated for 15 min with 0.5 μmol/L SYTOX green nucleic acid stain (Invitrogen), or 2 μg/mL propidium iodide (Sigma-Aldrich), harvested by trypsinization, and combined with floating cells from the medium. The cells were resuspended in PBS containing 1% fetal bovine serum. The fraction of SYTOX green– or propidium iodide–positive cells was measured using a FACSCalibur (Becton Dickinson) flow cytometer. Both SYTOX green and propidium iodide are nucleic acid stains that detect dead cells with disrupted plasma membrane and it was tested that both staining methods gave similar results. This allowed measurement of cell death (propidium iodide; red) together with GFP expression (green) using two-color settings. For SYTOX green measurements, 10,000 cells were analyzed using the FL-1 filter for determination of the fraction of SYTOX-positive cells. For propidium iodide measurements, 10,000 GFP-expressing cells were gated using the FL-1 filter, and these cells were measured at the FL-2 channel for determination of propidium iodide–positive cells. Compensation was done to eliminate overlap between the propidium iodide and GFP emissions. In all cases, the acquired data were analyzed using the Cell Quest Pro software. Fluorescence-activated cell sorting analyses were done thrice with reproducible results.

Figure 1. Effect of cisplatin on cell number in MCF-7 and eight antiestrogen-resistant MCF-7 sublines. Cells were treated with the indicated concentrations of cisplatin and cell number determination was done 24 h after addition of cisplatin by the crystal violet staining method. The results are expressed relative to the corresponding untreated controls. Individual experiments (from 3 to 16 were done for each cell line) were done in triplicate and a representative experiment is shown for each cell line. Bars, SE. The antiestrogen-resistant cell lines were tested against MCF-7 cells at all concentrations. The TAM R-1 cell line was significantly more sensitive to 40 and 60 μmol/L cisplatin, and in 7 of 12 individual experiments the TAM R-1 cell line was significantly more sensitive to 20 μmol/L cisplatin compared with MCF-7 cells. The 182 R-6 cell line was significantly more sensitive than MCF-7 to 20 and 40 μmol/L cisplatin, and in 7 of 12 individual experiments the 182 R-6 cell line was significantly more sensitive to 20 μmol/L cisplatin compared with MCF-7 cells. The 182 R-9 cells were treated with cisplatin for 24 h to determine their response to cisplatin in comparison with MCF-7 cells. The same pattern was seen when treating cells with 40 and 60 μmol/L cisplatin, respectively (i.e., cisplatin was more toxic in the antiestrogen-resistant cell lines than in parental MCF-7 cells).

Table 1. LC50 values for cisplatin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LC50 (±SE), μmol/L</th>
</tr>
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<tbody>
<tr>
<td>MCF-7</td>
<td>55.8 ± 4.5</td>
</tr>
<tr>
<td>TAM R-1</td>
<td>26.8 ± 4.9</td>
</tr>
<tr>
<td>182 R-1</td>
<td>21.3 ± 4.2</td>
</tr>
<tr>
<td>182 R-6</td>
<td>20.6 ± 3.2</td>
</tr>
<tr>
<td>182 R-7</td>
<td>24.7 ± 2.3</td>
</tr>
<tr>
<td>182 R-9</td>
<td>30.5 ± 3.8</td>
</tr>
<tr>
<td>164 R-4</td>
<td>35.7 ± 2.8</td>
</tr>
<tr>
<td>164 R-5</td>
<td>27.5 ± 0.7</td>
</tr>
<tr>
<td>164 R-7</td>
<td>31.9 ± 1.3</td>
</tr>
</tbody>
</table>

NOTE: For each experiment, the LC50 value (lethal concentration resulting in 50% reduction of cells) after treatment with cisplatin for 24 h was calculated with ProStat software (Logit Model). The average LC50 values ± SE are shown. All antiestrogen-resistant cell lines have significantly different LC50 values than MCF-7 (P < 0.05, two-tailed t test).

Results

Cisplatin Is More Efficient in Killing Antiestrogen-Resistant Cell Lines Than Parental Antiestrogen-Sensitive MCF-7 Cells

Antiestrogen-resistant MCF-7 human breast cancer cells were treated with cisplatin for 24 h to determine their response to cisplatin in comparison with MCF-7 cells. The cytotoxicity of cisplatin was measured in dose-response experiments using parental MCF-7 cells and eight different antiestrogen-resistant cell lines. Cisplatin at a concentration of 20 μmol/L did not result in cytotoxicity of MCF-7 cells but resulted in ~40% reduction in cell number in all eight antiestrogen-resistant cell lines. The same pattern was seen when treating cells with 40 and 60 μmol/L cisplatin, respectively (i.e., cisplatin was more toxic in the antiestrogen-resistant cell lines than in parental MCF-7 cells).

The difference in cisplatin effect was statistically significant (P < 0.05) in seven of the eight antiestrogen-resistant cell lines (Fig. 1). However, when comparing LC50 values of cisplatin treatment, 1.5- to 2.7-fold differences between antiestrogen-resistant cells and MCF-7 cells were observed (Table 1), showing that all antiestrogen-resistant cell lines were indeed more sensitive to cisplatin treatment than MCF-7 cells (P < 0.05). Withdrawal of antiestrogens 1 week before cisplatin treatment did not change the effect of cisplatin on the antiestrogen-resistant cell lines (data not shown), showing that it was not the combined effect of cisplatin and antiestrogens in the growth medium that makes cisplatin more cytotoxic to the resistant cells.
To ensure that dose-response curves reflected active cell killing, flow cytometry was conducted for measuring dead cells (SYTOX green–positive cells) after cisplatin treatment. For this and the following experiments, two representative and well-characterized antiestrogen-resistant cell lines were chosen: a tamoxifen-resistant cell line, MCF-7/TAMR-1 (TAMR-1), and an ICI 182,780–resistant cell line, MCF-7/182R-6 (182R-6). Figure 2 shows the fraction of dead cells measured by flow cytometry when treating MCF-7, TAMR-1, and 182R-6 with 40 μmol/L cisplatin. In the case of MCF-7 cells, only 7% cell death was seen after 24 h of cisplatin treatment, and this number increased to 30% after 2 days (Fig. 2). In contrast, almost 30% of the TAMR-1 cells were killed already after 24 h of cisplatin treatment and the fraction of dead cells increased to 57% and 70% after 36 and 48 h, respectively. 182R-6 cells were killed even faster than TAMR-1 cells by cisplatin; the majority of 182R-6 cells were dead after 24 h, and after 36 h, almost all cells were killed (Fig. 2). These results show that the antiestrogen-resistant cells were killed more efficiently by cisplatin treatment than the parental MCF-7 cells.

Involvement of Both Caspases and Lysosomal Proteases in Cisplatin-Induced Cell Death

The broad-range caspase inhibitor zVAD-fmk and the cysteine cathepsin inhibitor zFA-fmk were used to investigate the involvement of caspases and cysteine cathepsins in cisplatin-induced cell death. The cytotoxicity of cisplatin was significantly reduced when treating the cells with zVAD-fmk or zFA-fmk together with cisplatin (Fig. 3A). Combining both inhibitors did not result in further rescue of cells from the cytotoxic effect of cisplatin than obtained with zFA-fmk alone (data not shown). Cell death measured by flow cytometry of SYTOX-stained cells confirmed that zVAD-fmk and zFA-fmk partially rescued the cytotoxic effects of cisplatin on the cells (Fig. 3B).

Western blot analysis of the caspase-3/caspase-7 substrate PARP showed that cisplatin at concentrations up to 60 μmol/L induced cleavage of full-length PARP in TAMR-1 and 182R-6 cells but not in MCF-7 cells (Fig. 4A). PARP cleavage was also induced by cisplatin treatment in the antiestrogen-resistant cell lines 182R-7, 182R-9, 164R-5, and 164R-7 (data not shown). Because MCF-7 cells are lacking caspase-3 (31), we further investigated the involvement of caspases in the PARP cleavage. Western blot analysis of the PARP protein showed that cleavage was inhibited by the caspase inhibitor zVAD-fmk, presumably through inhibition of caspase-7 activity (Fig. 4B). Surprisingly, PARP cleavage was also inhibited by the cysteine cathepsin inhibitor zFA-fmk (Fig. 4B). This, together with the results showing that both zVAD-fmk and zFA-fmk could partially rescue cells from the cytotoxic effect of cisplatin, suggests that the mitochondrial and lysosomal death pathways act in combination to kill the antiestrogen-resistant cells by cisplatin.

To confirm that lysosome membrane permeabilization was induced by cisplatin, release of lysosomal proteins to the cytosol of MCF-7, TAMR-1, and 182R-6 was measured. Treatment with 40 μmol/L cisplatin resulted in increased
cysteine protease cathepsin B and L and NAG activities in the cytoplasm of the antiestrogen-resistant cell lines compared with the parental MCF-7 cells (Fig. 4C and D). These experiments indicate an important role of lysosomal cysteine proteases in mediating cisplatin cell killing of the resistant breast cancer cells.

Decreased Bcl-2 Level in Antiestrogen-Resistant Cell Lines Is Associated with Increased Cisplatin Sensitivity

It has previously been shown that Bcl-2 protein expression is lower in the majority of antiestrogen-resistant MCF-7 cell lines compared with parental MCF-7 cells (32, 33). Figure 5A shows that the Bcl-2 protein level was lower in all eight antiestrogen-resistant cell lines investigated here compared with the MCF-7 cell line. Therefore, we investigated whether ectopic reexpression of Bcl-2 could reduce the cisplatin-induced cell death in antiestrogen-resistant TAMR-1 and 182R-6 cells. MCF-7 cells were not included in the experiment because we had already shown that cisplatin treatment (24 h) only induced a minimum of cell death in these cells (Fig. 2 and 3B). As a control, we used a plasmid encoding a nonfunctional truncated version of Bcl-2, BCL-nt (no tail), which lacks its hydrophobic membrane-anchoring tail thereby preventing its antiapoptotic function (29).

Expression of the two Bcl-2 constructs in TAMR-1 and 182R-6 cells was verified by Western blot analysis (Fig. 5B). As a control, we used a plasmid encoding a nonfunctional truncated version of Bcl-2, BCL-nt (no tail), which lacks its hydrophobic membrane-anchoring tail thereby preventing its antiapoptotic function (29).

Expression of the two Bcl-2 constructs in TAMR-1 and 182R-6 cells was verified by Western blot analysis (Fig. 5B). Because the transfection efficiency was quite low (5–20%; data not shown), cotransfection was done with the Bcl-2 constructs and a H2B-eGFP plasmid. Hereby, the transfected cells could be identified by flow cytometry. Cell death of TAMR-1 and 182R-6 cells, expressing either wild-type Bcl-2 or the nonfunctional Bcl-2 control, was measured by identification of the fraction of propidium iodide–positive cells among the eGFP-positive cells (Fig. 5C and D). Treatment of antiestrogen-resistant cells in the presence of the nonfunctional Bcl-2 led to induction of cell death by cisplatin; at 40 \( \mu \text{mol/L} \) cisplatin, 26% of TAMR-1 cells (Fig. 5C) and 81% 182R-6 cells (Fig. 5D) were dead. This is comparable with the results from Fig. 2 (29% and 78%, respectively) showing that expression of the nonfunctional Bcl-2 does not alter the response to cisplatin.

Expression of full-length Bcl-2 partially rescued antiestrogen-resistant cells from cisplatin-induced cell death. In the case of TAMR-1 cells, the ectopic expression of Bcl-2 protein reduced cisplatin-induced cell death by \( \sim 45\% \) (Fig. 5C), and for the 182R-6 cells, Bcl-2 reduced cell death by \( \sim 35\% \) (Fig. 5D) irrespectively of the cisplatin concentration. Statistically significant differences between transfection with nonfunctional and functional Bcl-2 were shown for all concentrations of cisplatin (20, 40, and 60 \( \mu \text{mol/L} \)) for both cell lines. As seen in Fig. 5D, a small but statistically significant difference was also found between 182R-6 cells transfected with BCL-nt and BCL-2 even without cisplatin treatment, whereas this was not the case for TAMR-1 cells (Fig. 5C). Because 182R-6 cells were
more sensitive to the transfection procedure than TAMR-1 cells; ~20% of the 182R-6 cells were dead even without cisplatin treatment (Fig. 5D); reexpression of Bcl-2 seemed to protect partly against this effect. These results clearly show that Bcl-2 is expressed at a reduced level in the antiestrogen-resistant MCF-7 sublines and that reexpression of Bcl-2 can protect against cisplatin-induced cell death in TAMR-1 and 182R-6 cells. This suggests that the low expression of Bcl-2 in the antiestrogen-resistant cells plays a significant role in determining the sensitivity towards cisplatin.

Discussion

The data presented in this work show that breast cancer cells with acquired resistance to antiestrogen treatment can be killed efficiently with the platinum compound cisplatin, in fact even more effective than the cells from which they were subcloned. This indicates that some of the genetic alterations associated with the development of antiestrogen resistance in these cells eventually make them more prone to cell death induced by cisplatin and possibly also other compounds. In support of this, previous investigations of the antiestrogen-resistant cell lines TAMR-1 and 182R-6 showed an increased sensitivity to treatment with the vitamin D analogue EB1089 (32). Furthermore, antiestrogen-resistant cells were shown to be more sensitive to the phosphatidylinositol 3-kinase inhibitor wortmannin and the Akt inhibitor SH-6, as compared with MCF-7 cells (11).

DNA damage–induced cell death is a complex process involving several signaling pathways and organelles. The data presented here indicate that cisplatin-induced cell death of MCF-7 cells and their antiestrogen-resistant derivatives is dependent on lysosomal cathepsin release as well as activation of caspases. The mitochondrial and lysosomal death pathways are not mutually exclusive and can positively regulate each other (23, 28, 34). Camptothecin, a DNA topoisomerase I inhibitor, induces both lysosomal and mitochondrial membrane rupture in lymphoma cells, and it was found that lysosomal rupture is dependent, at least in part, on mitochondrial permeabilization (35). Similarly, overexpression of Bcl-2 in immortalized fibroblasts effectively inhibits etoposide (a DNA topoisomerase II inhibitor)-induced lysosomal permeabilization (34). Conversely, lysosomal permeabilization induced by quinolone antibiotics induces cell death that is dependent on mitochondrial membrane permeabilization (23). In line with these previous observations, we show that the cysteine cathepsin inhibitor zFA-fmk completely inhibits cisplatin-induced cleavage of the effector caspase substrate PARP, indicating that cysteine cathepsin activity is needed for the activation of caspases. Conversely, the decreased level of Bcl-2 in the antiestrogen-resistant cell lines compared with MCF-7 cells correlated with a marked increase in the lysosomal permeabilization (and cell death) induced by cisplatin. This suggests that Bcl-2, through its inhibition of the mitochondrial membrane permeabilization, also prevents lysosomal permeabilization. Alternatively, Bcl-2 might protect against lysosomal rupture in a more direct manner. It has been shown that oxidative stress–induced phospholipase...
A2 activation, which is associated with destabilization of both mitochondrial and lysosomal membranes, is inhibited by Bcl-2 overexpression in murine lymphoma J774 cells (36, 37). Due to its ability to inhibit both death pathways, Bcl-2 would be expected to play an important role in determining the outcome of cisplatin treatment of antiestrogen-resistant breast cancer cells. Indeed, overexpression of wild-type Bcl-2, but not the nonfunctional truncated version, significantly reduced the cisplatin sensitivity of two different antiestrogen-resistant cell lines. The positive coregulation between two death-promoting signaling pathways, as described above, may help to ensure that once a cell has activated either pathway above a certain threshold (here due to DNA damage), the death signal is amplified to ensure that the cell is effectively eradicated. A similar positive feedback mechanism has recently been described for activated effector caspases that feedback to enhance mitochondrial membrane permeabilization and thereby the release of proapoptotic factors from the mitochondria (38).

Clinical trials with the platinum compound carboplatin in combination therapy for metastatic breast cancer are ongoing and thus far show promising results (39), indicating the potency of using platinum compounds for treating breast cancer. To our knowledge, studies of the effect of cisplatin in antiestrogen-resistant patients have not yet been conducted. However, our data imply that breast cancer patients who have relapsed on antiestrogen treatment might be good responders to cisplatin and that low Bcl-2 expression could be used as a predictive marker. This gives hope to the future treatment of breast cancer patients with acquired antiestrogen resistance.

Acknowledgments
We thank Birgit Reiter for skillful technical assistance and Marja Jäättelä (Danish Cancer Society) for kindly providing pCEP4-BCL-2, pCEP4-BCL-nt, and H2B-pEGFP-N1 plasmids, as well as reagents for Cathepsin and NAG measurements.

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Molecular Cancer Therapeutics

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