Constitutively Active ErbB2 Regulates Cisplatin-Induced Cell Death in Breast Cancer Cells via Pro- and Antiapoptotic Mechanisms

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Abstract

Despite the frequent expression of N-terminally truncated ErbB2 (ΔNErbB2/p95HER2) in breast cancer and its association with Herceptin resistance and poor prognosis, it remains poorly understood how ΔNErbB2 affects chemotherapy-induced cell death. Previously it was shown that ΔNErbB2 upregulates acid extrusion from MCF-7 breast cancer cells and that inhibition of the Na+/H+ exchanger (SLC9A1/NHE1) strongly sensitizes ΔNErbB2-expressing MCF-7 cells to cisplatin chemotherapy. The aim of this study was to identify the mechanism through which ΔNErbB2 regulates cisplatin-induced breast cancer cell death, and determine how NHE1 regulates this process. Cisplatin treatment elicited apoptosis, ATM phosphorylation, upregulation of p53, Noxa (PMAIP1), and PUMA (BBC3), and cleavage of caspase-9, -7, fodrin, and PARP-1 in MCF-7 cells. Inducible ΔNErbB2 expression strongly reduced cisplatin-induced ATM- and p53-phosphorylation, augmented Noxa upregulation and caspase-9 and -7 cleavage, doubled p21\textsuperscript{WAF1/Cip1} (CDKN1A) expression, and nearly abolished Bcl-2 expression. LC3-GFP analysis demonstrated that autophagic flux was reduced by cisplatin in a manner augmented by ΔNErbB2, yet did not contribute to cisplatin-induced death. Using knockdown approaches, it was shown that cisplatin-induced caspase-7 cleavage in ΔNErbB2-MCF-7 cells was Noxa- and caspase-9 dependent. This pathway was augmented by NHE1 inhibition, while the Na+/HCO\textsubscript{3}− cotransporter (SLC4A7/NBCn1) was internalized following cisplatin exposure.

Implications: This work reveals that ΔNErbB2 strongly affects several major pro- and antiapoptotic pathways and provides mechanistic insight into the role of NHE1 in chemotherapy resistance. These findings have relevance for defining therapy regimens in breast cancers with ΔNErbB2 and/or NHE1 overexpression. Mol Cancer Res; 13(1); 63–77. ©2014 AACR.

Introduction

The ErbB2 (HER2/Neu) receptor tyrosine kinase is upregulated in about one-third of human breast cancers, usually as a consequence of gene amplification (1). N-terminal truncation of the extracellular domain, rendering the receptor constitutively active, is common and correlates with increased malignancy and poor prognosis (2, 3). Herceptin resistance is also widespread in patients expressing full-length ErbB2 (4) and anti-ErbB2-treatments exhibit a range of potential side effects, including cardiotoxicity (5). Thus, despite the overall success of Herceptin, novel treatment regimens and combination therapies are needed.

Although cis-diaminedichloroplatinum(II; cisplatin) chemotherapy has not been in widespread use in breast cancer, it has been shown to exhibit clinical advantages in synergy with antiHER2 antibodies, especially in previously untreated metastatic breast cancer, by mechanisms possibly involving interference with DNA damage repair (6–8). Cisplatin is a neutral inorganic complex that after spontaneous aquation in the low Cl− environment of the cell interacts with nucleophilic N7-sites of purine bases in DNA to form DNA–protein and DNA–DNA intrastrand and intranuclear cross-links (9). The latter appear largely responsible for the cytotoxic action, through multiple signaling pathways (8–10). However, not all of the cellular effects of cisplatin can be ascribed to its interaction with DNA (11): cisplatin also forms stable bonds with cellular sulfur donors such as cysteines and methionines (10), and can interact with cellular membranes, forming coordination complexes with phosphatidylserine, increasing membrane fluidity, and altering the function of ion channels and transporters (11, 12). Cisplatin-induced DNA damage has been shown to activate ataxia-telangiectasia–mutated kinase/ataxia-telangiectasia–mutated kinase related (ATM/ATR), which mediates Ser\textsuperscript{15} phosphorylation and upregulation of the tumor suppressor p53 (10). In turn, p53 can upregulate the expression of the cyclin-dependent kinase inhibitor (CDKI) p21\textsuperscript{WAF1/Cip1} and caspase-9, -6, and -7 (13, 14). Also, several Bcl-2 family proteins are transcriptionally upregulated by p53, including the proapoptotic Bcl-2 family proteins, p53-upregulated Modulator of Apoptosis (PUMA) and Noxa (14).
Given the clinical need for novel treatment combinations, as well as the frequent development of cisplatin resistance (7), increased understanding of the signaling pathways activated by cisplatin in ErbB2- and ΔNErbB2-overexpressing cancers is of essential importance, as are possible mechanisms that may alleviate cisplatin resistance in these cancers. Nonetheless, knowledge on ErbB2-induced effects on apoptotic pathways is limited, and pro- and antiapoptotic effects (6, 15), as well as no effects (16), of ErbB2 signaling on various forms of programmed cell death are reported. Furthermore, these questions have, to our knowledge, not previously been addressed specifically for ΔNErbB2-expressing breast cancer. Notably, despite their increased motility and invasiveness, ΔNErbB2-expressing cells do not exhibit increased proliferation, but in fact can decreased, their increased proliferative capacities (16), and pro- and antiapoptotic effects, of ErbB2 signaling on various forms of pro- and antiapoptotic and –proliferative pathways are upregulated by ΔNErbB2. We recently showed that cisplatin induces caspase- and caspase-depen- dent pathways in ΔNErbB2-expressing MCF-7 breast cancer cells (18), yet upstream events induced by cisplatin, as well as possible roles of other death pathways, were not investigat- ed. Furthermore, inhibition or knockdown of the Na+/H+ exchanger NHE1 (SLC9A1) strongly sensitized ΔNErbB2-expressing cells to cisplatin-induced death (18). This would imply a possible effect of cellular pH (pH1) regulation; however, inhibition of the other major acid extruding transporter in these cells, the Na+/HCO3− cotransporter NBCn1 (SLC4A7), had no net effect on cell death, and in fact attenuated lysosomal membrane permeabilization (LMP) and cytokine caspase release (18). This led us to hypothesize that the functional availability of the two transporters is differentially regulated by cisplatin.

The aim of the present study was to delineate the molecular mechanisms involved in cisplatin-induced death in MCF-7 breast cancer cells and determine the effects of ΔNErbB2 signaling on these processes, with particular focus on the roles of dysregulation of NHE1 and NBCn1. We characterize the main signaling pathways activated during cisplatin-induced death of MCF-7 cells, determine the functional contributions of central death pathways to ΔNErbB2-dependent death, identify a mechanism for the differential roles of NHE1 and NBCn1 during cisplatin-induced apoptosis, and point to novel pro- and antiapoptotic roles of ΔNErbB2 with potentially important clinical implications.

Part of these findings have previously been reported in abstract form (19).

Materials and Methods

Antibodies and reagents

The polyclonal NBCn1 antibody (20) was a kind gift from Jeppe Prætorius, Aarhus University, Denmark. Antibodies against NHE1 (sc-136239), μ-calpain (sc-7531), p21 (sc-397), Mcl-1 (sc-819), fodrin (sc-48382), and phospho Ser15 p53 (sc-101762) were from Santa Cruz Biotechnology. Antibodies against caspase-9 (#9292), caspase-7 (#9492), poly-(ADP-ribose) polymerase (PARP, #9542), Bad (#9292), Bcl-2 (#2876), Bcl-xl (#2764), p53 (#2527 and #2524), STAT3 (#9139), and phospho Tyr\textsuperscript{1181}STAT3 (#9131) were from Cell Signaling Technology. PUMA (#ab9643) and pATM (ab36810) antibodies were from Abcam. Antibodies against cytochrome c (#556432), p150 Glued (#610473), and Noxa (#M349A) were from BD Pharmingen, Transduction Laboratories, and IMGENEX, respectively. Antibody against LC3 (0231-100/ LC3-5F10) was purchased from nanoTools Antikörpertechnik. Antibody against cathepsin B was a kind gift from Dr. E. Weber, University of Halle, Germany. Cariporide and S0859 (C(29)H (24)CIN(3)O(3)S) were kind gifts from Sanoﬁ-Aventis and botheq were dissolved at 10 mmol/L in ddH2O. Cisplatin (Sigma-Aldrich) was dissolved at 3.3 mmol/L in growth medium. Rapamycin (#R8781) and 3-Methyladenine (3MA, #M9281), both from Sigma-Aldrich, were dissolved at 1 and 200 mmol/L, respectively, in ddH2O. ATM inhibitor KI-53933 was purchased from Santa Cruz Biotechnology, and dissolved at 10 mmol/L in DMSO.

Cell lines and culture conditions

MCF-tTA-ΔNErbB2 cells (hereafter ΔNErbB2-expressing cells), which express the truncated ErbB2 receptor under a tetracycline-off promoter, and the corresponding vector controls (MCF-tTA-pTRE; hereafter vector control cells) were a kind gift from Dr. J. Nylandsted, Danish Cancer Research Center, Copenhagen, Denmark, and were cultured as in (ref. 18). Brieﬂy, cells were grown in RPMI-1640 supplemented with 6% heat-inactivated FBS, 1% penicillin/streptomycin, 1 μg/mL puromycin, and 200 μg/mL G418 (hereafter Complete Medium, CM). Cells were maintained at 37°C/95% humidity/5% CO2, passed every 3 to 4 days, and not used after passage 22. Tetracycline was removed by washing the cells six times in tetracycline-free medium with 1% FBS and repeating this procedure 1 hour later. Unless otherwise noted, tetracycline was removed 48 hours before start of the experiment. As previously described (18), the tetracycline off promoter used is somewhat leaky, hence, to avoid contamination of the baseline system by ΔNErbB2 expression, the corresponding vector cells, treated identically, are used as controls.

FACS analysis of annexin V and propidium iodide staining

Cell death was detected using Annexin V-FITC Apoptosis Detection Kit (Abcam). Forty-eight hours after tetracycline, removal cells were washed in PBS and trypsinized. 500,000 cells were collected and resuspended in 200 μL Annexin V Binding Buffer (Abcam). Cells were stained with propidium iodide (PI) and Annexin V-FITC 1:100 as per the manufacturer’s instructions and incubated 5 minutes at room temperature shielded from light. Samples were analyzed using a FACS Calibur (BD Bioscience) and data analysis was carried out in Cell Quest Pro (BD Bioscience).

SDS-PAGE and Western blotting

Western blotting was performed as previously described (21). Brieﬂy, cells were grown to 80% to 90% confluency, washed in ice-cold PBS, and lysed in lysis buffer (10% SDS, 0.1 mol/L TRIS, pH 7.5, 1 mMol/L NaVO3). Protein content was determined (DC assay, Bio-Rad), equalized, and lysates mixed 1:1 with NuPage LDS sample buffer (Invitrogen, #NP0007). SDS-PAGE gel electrophoresis was carried out in NOVEX chambers with NuPAGE precast 10% Bis-Tris gels under reducing and denaturing conditions, using BenchMark protein ladder (Invitrogen, #10747-012). For proteins smaller than 10 kDa, MES running buffer (Invitrogen, #NP0002) and 12% Bis-Tris gels (Invitrogen,
Immuno-fluorescence analysis

Immuno-fluorescence analysis was carried out essentially as in (ref. 22). Cells grown on glass coverslips were fixed in 2% paraformaldehyde (15 minutes room temperature, 30 minutes on ice), washed in TBST, permeabilized for 5 minutes in 0.2% Triton X-100 (Plusone, #17-1315-01) in TBST or 20 μg/mL Digitonin (Sigma-Aldrich, #D141) in dH2O, blocked for 30 minutes (5% BSA in TBST), incubated with primary antibody in TBST + 1% BSA overnight at 4°C, washed in TBST, and incubated with AlexaFluor®488-conjugated anti-mouse or AlexaFluor®568-conjugated anti-rabbit secondary antibodies (Invitrogen, 1:600 in TBST + 1% BSA, 1 hour), washed in TBST, and mounted in 2% N-propyl-galleate in PBS/glycerine. Cells were visualized on an Olympus BX63 microscope, fitted with a 60×/1.35 NA objective. Images were collected and frame-averaged using Olympus CellSens dimension software, and processed (brightness/contrast adjustment only) using Adobe Photoshop CS4. Essentially no labeling was detectable in the absence of primary antibody (not shown).

Determination of cell surface fractions of NHE1 and NBCn1

All experiments were carried out at 4°C. Forty-eight hours after tetracycline removal, cells were washed twice in PBS and incubated for 30 minutes with 0.5 mg/mL EZ-Link Sulfo-NHS-SS-biotin (Pierce, Thermo Scientific) diluted in PBS. To remove excess Sulfo-NHS-SS-Biotin, cells were washed three times in cold quenching buffer (100 mM/L Glycine in PBS) and lysed in cold RIPA buffer (50 mM/L Tris HCl, pH 7.5, 150 mM/L NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, Complete mini protease inhibitor 1 tablet/10 ml buffer, 1% Igepal CA630). Cell lysates were centrifuged for 15 minutes at 16,000 RCF and 4°C. The supernatant was adjusted to equal protein concentration (DC assay, Bio-Rad) and an aliquot was mixed with NuPage LDS sample buffer and saved as total lysate fraction (TLF). The remaining supernatant was incubated under gentle rolling with streptavidine agarose beads (Sigma-Aldrich) for 2 hours at 4°C. Beads were washed three times in RIPA buffer, NuPage LDS sample buffer was added, and bound protein was released from the beads by heating for 5 minutes at 95°C before samples were processed for Western blot analysis.

siRNA sequences and transfection

siRNA against caspase-9 (GGAAUCAGCCAGGCGACGCU[dT][dT][dT][dT]), (GCUGGAAUCGCGUUGGUGUG[dT][dT][dT]), μ-caspase (GGACAAACGUGGACCCCAA[dT][dT][dT][dT]), (UGUUCUGGUGGUGGUGCCUG[dT][dT][dT]), cathepsin B (GCGAAUGACGCUUCACGCU[dT][dT][dT]), (UGUUCGUGGUGGUGCCUCU[dT][dT][dT]), Noxa (CGUGUGUGUGUGUGUGUGUGA)[dT][dT][dT], and mock control (SIC001), were purchased from Sigma-Aldrich. Optimization experiments showed that knockdown of caspase-9, μ-caspase, and cathepsin B was most effective using a combination of rank 1 and -2 siRNA, hence this was done in all subsequent experiments. Cells were seeded in 6-well dishes to reach 50% to 60% confluency on the day of transfection. One hour before transfection, cells were washed in transfection medium (growth medium without serum and antibiotics), and 1.5 μL transfection medium was added. Cells were transfected with 200 nmol/L of siRNA using Lipofectamine 2000 (Invitrogen). Four hours after the transfection, 200 μL of heat-inactivated FBS was added. After an additional 20 hours, the medium was replaced with complete medium. Fifty-six hours after transfection, cells were treated with 25 μmol/L cisplatin for 18 hours, lysed, and processed for Western blotting.

Quantification of LC3-eGFP puncta

Cells grown on 25 mm round glass coverslips to 60% to 70% confluency were transfected with Microtubule-Associated Protein-1 Light Chain 3 (LC3)[II]-EGFP (a kind gift from Dr. J. Nylandsted, Danish Cancer Research Center, Copenhagen, Denmark) using Lipofectamine 2000 (Invitrogen). Inhibitors and reagents were added 18 hours before experimental start. Autophagy was induced by incubating with 1μmol/L rapamycin and inhibited by incubating with 10 mmol/L 3MA. The cells were fixed in 4% paraformaldehyde, washed several times with PBS, and mounted in N-propyl-galleate mounting medium (2% w/v in PBS/glycerine). Punctate dots in 100 random cells per sample were counted at ×100 magnification (Leica DM IRBE inverted fluorescence microscope).

Cell viability assays

Cell viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as in (ref. 23). Ten thousand cells were seeded per well in 96-well dishes. Forty-eight hours before the experiment, tetracycline was removed. The following day, the cells were treated with cisplatin (18 hours before MTT addition) as indicated. Where 3MA or rapamycin was used, these were applied 1 hour before cisplatin exposure. Cells were treated with MTT (2.4 mmol/L) for 2 hour at 37°C. Extraction buffer (4.4% SDS in 0.01 mol/L HCl) was added, and plates were incubated 5 to 18 hours at room temperature before measuring absorbance at 592 nm on a FLUOstar-OPTIMA microplate reader (BMG Labtechnologies GmbH). Data were background subtracted and normalized to control.

Data and statistical analysis

Data are shown as representative individual experiments or as mean with SEM error bars. Within one cell line, significance between two groups was tested using Student t test, and one-way ANOVA, with Bonferroni post-test, for comparisons of more than two groups. For comparisons of two cell lines and multiple groups, or two-way ANOVA was applied, with Bonferroni post-test, for comparisons of more than two groups. For comparisons of two cell lines and multiple groups, or two-way ANOVA was applied, with Bonferroni post-test.

Results

Cisplatin treatment elicits ANErbB2-sensitive caspase-9 and -7 activation and ANErbB2-insensitive PARP-1 and fodrin cleavage

We previously demonstrated that cisplatin treatment elicits caspase- and cathepsin-dependent cell death in MCF-7 cells, in the absence and presence of ANErbB2 (18). To further elucidate the mechanisms involved in cisplatin-induced cell death and the
impact of ΔNErbB2 on these processes, MCF-7 cells expressing either ΔNErbB2 or the corresponding empty vector were used, as previously described (ref. 18; Supplementary Fig. S1A). First, FACS analysis was used to quantify the effect of cisplatin on Annexin V and PI staining. On the basis of our previous dose–response analyses (18), the cells were exposed to 25 μmol/L cisplatin for 18 hours. Cisplatin treatment elicited the greatest increase in the fraction of cells showing Annexin V staining in the absence of PI staining, that is, early apoptosis. Also late apoptotic cells were observed, whereas necrosis was not induced to a detectable extent (Fig. 1A–D). As assessed by this method, apoptosis induction tended to be slightly greater in vector than in ΔNErbB2-expressing cells, although the difference was not statistically significant.

Figure 1. Effects of 18-hour cisplatin exposure on cell death induction, and caspase-9, caspase-7, PARP, and fodrin cleavage. MCF-7 vector (V) and ΔNErbB2 (ΔN) cells were washed free of tetracycline 48 hours before the experiment, and exposed to cisplatin (25 μmol/L) or control conditions for 18 hours. Cells were subsequently processed for FACS analysis (A–D) or immunoblotting (E–H). Top, representative immunoblot analyses; arrows, relevant cleavage product. Bottom, cleavage product level relative to that in vector cells under control conditions. A, dot plot of annexin V versus PI staining. B, Annexin V (%). C, Annexin V + PI (%). D, PI (%). E, caspase-9 cleavage. F, caspase-7 cleavage. G, PARP cleavage. H, fodrin cleavage and μ-calpain expression. Data are representative of three to seven independent experiments for each condition. FACS data and Western blot data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, respectively (B–D, Student’s t test; E–H, two-way ANOVA with Bonferroni post-test).
We next determined which major death pathways were activated by cisplatin in MCF-7 cells in the absence and presence of ΔNErbB2 expression (Fig. 1E–H and Supplementary Fig. S1B). Caspase-7 is the main effector caspase in MCF-7 cells, which lack caspase-3 (24). Exposure to cisplatin (25 μmol/L, 18 hours) elicited cytochrome C release (Supplementary Fig. S1B), and cleavage of caspase-9 (Fig. 1E), caspase-7 (Fig. 1F), and PARP-1 (Fig. 1G), in both vector- and ΔNErbB2-expressing cells, in accordance with our previous finding in ΔNErbB2-expressing cells (18). No caspase-8 cleavage was detectable (n = 5, data not shown), suggesting that only, or by far predominately, the intrinsic apoptotic pathway was activated. Confirming our previous report (18), cisplatin-induced cleavage of caspase-9, and to a lesser extent of caspase-7, was increased by ΔNErbB2-expression, whereas PARP-1 cleavage was numerically similar in both cell types (Fig. 1E–G). Cisplatin treatment furthermore induced the appearance of the 150 kDa calpain-specific fodrin cleavage product (25), suggesting that calpain was activated (Fig. 1H, bottom). Fodrin cleavage was not significantly affected by ΔNErbB2 expression (Fig. 1H, bottom), and μ-calpain was expressed at similar levels in vector- and ΔNErbB2-expressing MCF-7 cells (Fig. 1H, top).

Cisplatin treatment reduces autophagic flux in MCF-7 cells in a manner sensitive to ΔNErbB2

The role of autophagy in cisplatin-induced death of ErbB2-positive breast cancer has, to our knowledge, not previously been investigated. Autophagic vesicles were visualized as distinct cytoplasmic LC3(II)-eGFP puncta after expression of LC3 (II)-eGFP. Confirming the functionality of the system, the number of LC3(II) puncta in both ΔNErbB2- and vector-expressing cells was decreased by exposure to the autophagy inhibitor 3MA (10 mmol/L) and increased by exposure to rapamycin (1 μmol/L), which induces autophagy by triggering the starvation response (Fig. 2A). ΔNErbB2 expression significantly increased the number of LC3(II)-eGFP puncta in MCF-7 cells under basal conditions (Fig. 2A and B). Cisplatin treatment strongly reduced the number of LC3(II)-eGFP puncta in both cell types, yet the relative decrease was more than doubled by ΔNErbB2-expression (Fig. 2B). These findings were confirmed by Western blotting for endogenous LC3 in its soluble form (LC3-I) and the lipiddated, membrane-associated form (LC3–II) to which LC3-I is converted during autophagosome formation. In congruence with the LC3-eGFP data, the endogenous LC3-II/LC3-I ratio was greater in ΔNErbB2-expressing cells than in vector controls. Furthermore, in ΔNErbB2-expressing cells, the LC3-II/LC3-I ratio decreased markedly over time after cisplatin exposure (Fig. 2C). Also in accordance with the LC3-eGFP data, the decrease in LC3-II/LC3-I ratio after cisplatin treatment was most pronounced in ΔNErbB2-expressing cells.

p62, aka sequestosome-1, is a scaffold protein interacting with LC3 and transporting ubiquitinated proteins for degrada-
tion in the autophagosome, and its accumulation is indicative of reduced autophagic flux (26). The endogenous level of p62 in MCF-7 cells appeared slightly greater in ΔNErbB2-expressing cells compared with vector cells, and was not significantly increased by cisplatin treatment although a small numerical increase was seen in cisplatin-treated ΔNErbB2 cells (Fig. 2D). There was no difference in the Beclin1 protein level between vector- and ΔNErbB2-expressing MCF-7 cells (n = 2, data not shown).

Depending on the specific cell type and conditions, reduced autophagic flux can be either a pro-death or a pro-survival event in cancer cells (27). To assess whether the cisplatin-induced reduction in autophagic flux impacted on cisplatin-induced death, MTT viability assays were carried out after 18 hours of cisplatin treatment in the absence and presence of 3MA or rapamycin (Fig. 2E). Confirming previous findings (18), cisplatin induced a loss of viability as assessed by MTT assay in both vector- and ΔNErbB2-expressing cells. Neither 3MA nor rapamycin significantly affected the cisplatin-induced loss of viability, regardless of ΔNErbB2 status (Fig. 2E). It may be noted that ΔNErbB2 expression does not increase viability under control conditions, in congruence with the known propensity of long-term ΔNErbB2 expression to induce a shift toward a senescent phenotype (17), see also below.

Collectively, these results show that cisplatin treatment of MCF-7 cells elicits cytochrome c release, cleavage of caspase-9, -7, PARP-1, and fodrin, and reduces autophagic flux. ΔNErbB2 expression increases cisplatin-induced caspase-9 and -7 cleavage and exacerbates the cisplatin-induced reduction of autophagic flux, although basal autophagy is increased by ΔNErbB2. Finally, neither inhibition nor induction of autophagy affects cisplatin-induced death of MCF-7 cells.

p21WAF1/Cip1 expression and cisplatin-induced ATM- and p53 signaling are ΔNErbB2 sensitive

To identify the ΔNErbB2-sensitive step(s) in the death pathways characterized above, we next determined the effects of ΔNErbB2 on cisplatin-induced upstream events. DNA damage-induced activations of ATM and p53 are hallmarks of cisplatin-induced death (10, 13, 14, 28). Accordingly, cisplatin treatment increased ATM phosphorylation on Ser1981 by about 10-fold in vector-expressing MCF-7 cells. Notably, in ΔNErbB2-expressing cells, cisplatin-induced ATM phosphorylation was strongly reduced and not significantly different from that in untreated controls (Fig. 3A). Similarly, although cisplatin treatment increased the protein level of p53 in both cell types, Ser15 phosphorylation of p53 was increased more than 10-fold in vector-expressing cells, yet the increase did not quite reach statistical significance in ΔNErbB2-expressing cells (Fig. 3B). The p53 activation was confirmed by immunofluorescence analysis, which also showed the nearby exclusive nuclear localization of p53 in MCF-7 cells (Fig. 3C).

A major effector of p53-induced cell death and cell-cycle arrest is p21 (a.k.a. p21WAF1/Cip1), which is transcriptionally upregulated by p53 (13). Notably, the cellular p21 protein level was nearly doubled upon ΔNErbB2 expression, yet was unaffected by cisplatin treatment in both cell types, despite the robust increase in p53 level under these conditions (Fig. 3D). These data were confirmed by immunofluorescence analysis, which also showed that the increase in p21 protein level in ΔNErbB2-expressing cells reflected that a much greater fraction of cells exhibited high p21 levels, whereas other cells had nondetectable p21 levels (Fig. 3E). Regardless of the cell type and condition, p21 was only found at detectable levels in the nucleus. Notably, there was no clear correlation between p21 and p53 expression in individual cells, substantiating the finding that p53 is not a major regulator of p21 by neither ΔNErbB2 nor cisplatin in MCF-7 cells (Fig. 3E). Because STAT3 signaling has been assigned a role in resistance to chemotheraphy, including cisplatin treatment (29, 30) and...
in upregulation of p21 (31), and because we previously showed that STAT3 activity is greatly increased by ΔNErbB2 expression in MCF-7 cells (32), we next assessed the effect of cisplatin treatment on STAT3 level and activity. Confirming our previous report, STAT3 phosphorylation was strongly increased by ΔNErbB2. Cisplatin treatment had no effect on STAT3 phosphorylation in vector cells, yet reduced STAT3 phosphorylation in ΔNErbB2-expressing cells by about 50% (Fig. 3F).

Taken together, these data show that cisplatin treatment of MCF-7 cells activated ATM and increased the protein level and Ser15-phosphorylation of p53, in a ΔNErbB2-sensitive manner. In contrast, p21 expression was doubled by ΔNErbB2, yet insensitive to cisplatin in both cell types.

Basal Bcl-2 expression and effects of cisplatin on other Bcl-2 proteins are ΔNErbB2 sensitive

Modulation of Bcl-2 family proteins in p53-dependent and -independent manners has been widely implicated in cell death induced by cisplatin- and other DNA damage (28, 33). We therefore next asked whether ΔNErbB2 impacts on the effects of cisplatin on proapoptotic (PUMA, Noxa, and Bad) and anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) Bcl-2 family proteins, respectively (Fig. 4). Both PUMA and Noxa were induced by cisplatin, and rapamycin and 3MA significantly reduced this induction in ΔNErbB2 cells, with PUMA showing a stronger inhibitory effect than Noxa.

Cisplatin itself had no effect on Bcl-xL and Mcl-1 expression in either cell line, but ΔNErbB2 cells showed a greater reduction in Bcl-xL expression following cisplatin treatment compared to vector cells (Fig. 4). These findings suggest that ΔNErbB2 may affect the sensitivity of MCF-7 cells to cisplatin-induced apoptosis by modulating the expression of pro- and anti-apoptotic Bcl-2 family proteins.
cisplatin treatment, whereas Bad expression was unaffected (Fig. 4A–C). Notably, although PUMA induction by cisplatin was slightly reduced by ΔNErbB2 (Fig. 4A), both basal (not quite statistically significant) and cisplatin-induced Noxa expression were strongly increased by ΔNErbB2 (Fig. 4B). Conversely, Bcl-2 expression was almost abolished by ΔNErbB2 (Fig. 4D). Finally, Mcl-1 and Bcl-xL levels were largely similar between cell types, yet in ΔNErbB2-expressing cells, the Bcl-xL level was decreased by cisplatin treatment, causing the level in these cells after treatment to be reduced compared with that in vector cells (Fig. 4E and F). Bid cleavage did not differ significantly between vector- and ΔNErbB2-expressing cells, and was not significantly affected by cisplatin (n = 4, data not shown).

Thus, cisplatin treatment increased the protein levels of pro-apoptotic PUMA and Noxa, and tended to decrease that of ErbB2 Regulates Cisplatin-Induced Death in Breast Cancer Cells

Figure 3.
Effects of 18-hour cisplatin exposure on p-ATM, p53, p21, and STAT3. MCF-7 vector (V) and ΔNErbB2 (ΔN) cells were washed free of tetracycline 48 hours before the experiment, and exposed to cisplatin (25 μmol/L) or control conditions for 18 hours. Cells were subsequently processed for immunoblotting (A, B, D, F) or immunofluorescence analysis (C and E). A, p-ATM; top, representative immunoblot analyses. Bottom, p-ATM level relative to that in vector cells under control conditions. B, p53 and p-p53; top, representative immunoblot analyses. Bottom, p-p53 level relative to that in vector cells under control conditions. C, fluorescence images of p53 (green) and p-p53 (red). Nuclei are stained with DAPI. D, p21; top, representative immunoblot analyses. Bottom, p21 level relative to that in vector cells under control conditions. E, fluorescence images of p53 (green) and p21 (red). Nuclei are stained with DAPI. F, STAT3 and p-STAT3; top, representative immunoblot analyses. Bottom, p-STAT3/STAT3 ratio relative to that in vector cells under control conditions. Data are representative of three to six independent experiments for each condition. Western blot data are shown as mean with SEM error bars. * comparison of the value in cisplatin treated to that in the respective cell line control; # comparison of values in vector and ΔNErbB2 cell lines under the same treatment. **/###/#### indicate P < 0.05, P < 0.01, P < 0.001, and P < 0.0001, respectively (two-way ANOVA with Bonferroni post-test).
antiapoptotic Bcl-2 and Bcl-xL. Strikingly, upon ΔNErbB2 expression, Bcl-2 expression was almost abolished, and Noxa induction by cisplatin was increased.

Inhibition of NHE1 but not of NBCn1 increases caspase-9, -7, and PARP-1 cleavage

We previously showed that inhibition of NHE1 by 5-(N-ethyl-N isopropyl)-amiloride (EIPA) sensitized ΔNErbB2-expressing MCF-7 cells to cisplatin-induced death, whereas inhibition of Na⁺,HCO₃⁻/CO₂ cotransporters (NBC) by S0859 had no effect, despite the fact that proteins are important for pH regulation in these cells (18). Although most potent toward NHE1, EIPA inhibits several NHE isoforms. Furthermore, the upstream mechanisms involved in the sensitization were not previously investigated in detail (18). To substantiate these findings, we therefore next asked whether cariporide, a structurally different and more NHE1-specific inhibitor (see ref. 34), impacted on the cisplatin-induced caspase-9, -7, and PARP-1 cleavage. As seen, caspase-9 and -7 cleavage was substantially increased by concomitant treatment with cariporide in ΔNErbB2-expressing cells, but not in vector controls (Fig. 5A–C). PARP-1 cleavage also tended to be increased by cariporide, although this did not reach statistical significance (Fig. 5D). Substantiating our previous report (18), inhibition of NBCs by S0859 had no sensitizing effect, and even tended to attenuate PARP-1 cleavage (Fig. 5A–D).

NBCn1 but not NHE1 is internalized after cisplatin exposure

To elucidate why inhibition of NHE1, but not of NBCn1, sensitizes ΔNErbB2-expressing MCF-7 cells to cisplatin-induced death, we next asked whether NBCn1 might be rendered unfunctional by cisplatin treatment, for example, by reduced expression or plasma membrane localization. As previously shown (18), NBCn1 expression was strongly induced by ΔNErbB2, whereas NHE1 expression was unaltered. Cisplatin treatment had no effect on NHE1 expression, and did not significantly reduce NBCn1 expression, although a slight numerical decrease was seen (Fig. 6A and B). Notably, however, NBCn1 plasma membrane expression was markedly reduced after cisplatin, and essentially all detectable NBCn1 protein localized to a perinuclear compartment (Fig. 6D). In contrast,
NHE1 plasma membrane localization was not detectably altered by cisplatin exposure (Fig. 6C). To further substantiate this potentially important result, cell surface biotinylation experiments were performed to quantitatively determine the cell surface levels of the two transporters over time after cisplatin exposure. Experiments were done in vector- and ΔNErbB2-expressing MCF-7 cells, as well as in native MCF-7 cells (Fig. 6E). Consistent with the immunofluorescence data, these experiments showed that NBCn1 abundance in the plasma membrane was reduced after cisplatin treatment. In contrast, and again consistent with the immunofluorescence data, there was no detectable internalization of NHE1 in either cell type (Supplementary Fig. S1C and S1D).

Noxa, caspase-9, cathepsin B, and μ-calpain regulate cisplatin-induced death pathways in vector- and ΔNErbB2-expressing MCF-7 cells

To obtain mechanistic insight into the causal roles of individual death pathway in ΔNErbB2-expressing MCF-7 cells, we first determined the effect of pharmacologically inhibiting ATM, one of the upstream effectors in the cisplatin-induced pathways. Pretreatment with the ATM inhibitor KU-55933 strongly reduced the cisplatin-induced phosphorylation of ATM and of p53 (Fig. 7A and Supplementary Fig. S2A and S2B), and tended to reduce cisplatin-induced caspase-7 cleavage (Fig. 7A and B), whereas it did not reduce the cisplatin-induced PARP cleavage (Fig. 7A and C).

To get more direct information on the death pathways used in the ΔNErbB2-expressing cells, we next performed siRNA-mediated knockdown of Noxa, caspase-9, cathepsin B, and μ-calpain in these cells and quantified caspase- and PARP-1 cleavage as markers of apoptosis induction. The knockdown efficacy for Noxa and caspase-9 was about 80% and 75%, respectively, and that for cathepsin B and μ-calpain was about 50% (Supplementary Fig. S2). An overview of representative immunoblot analyses is shown in Fig. 7D, and data are quantified in Fig. 7E–H. As expected, knockdown of Noxa or caspase-9 strongly reduced the levels of cleaved caspase-9 and -7 (Fig. 7E and F) and also tended to reduce PARP-1 and...
fodrin cleavage (Fig. 7G and H). Knockdown of cathepsin B had no detectable effect on any of the pathways assessed (Fig. 7E–H), likely due to the limited knockdown efficacy (Supplementary Fig. S2) and/or compensatory upregulation of other pathways (see Discussion). In contrast, knockdown of μ-calpain resulted in a dramatic increase in the levels of cleaved PARP-1 and fodrin (Fig. 7G and H), with no detectable changes in the levels of cleaved caspase-9 and -7 (Fig. 7E and F). Supporting this finding, pretreatment with two different calpain inhibitors (calpain inhibitor I and PD 150606) also increased PARP-1 cleavage ($n = 2$, data not shown).

**Discussion**

The widespread clinical importance of breast cancers over-expressing ErbB2 and its truncated variants, and the Herceptin resistance induced by N-terminal ErbB2 truncations, highlight the need for alternative treatment strategies. The effects of ErbB2 on cell death mechanisms are incompletely understood, and both in- and decreased sensitivity to apoptotic stimuli has been reported in ErbB2-overexpressing breast cancers (6, 15). Furthermore, although it is well recognized that full-length
and truncated ErbB2 elicits different signaling responses (17), little is known about the specific molecular mechanisms through which ΔNErbB2 signaling modifies chemotherapy-induced cell death—a knowledge that could potentially lead to novel treatment strategies. Cisplatin chemotherapy, while not in widespread use in breast cancer, has recently been proposed to have clinical advantages as combination therapy in ErbB2-positive breast cancers (6–8). Yet, the biologic mechanisms of cisplatin resistance are complex and incompletely understood (35) and have not been widely studied in breast cancer, and never in the context of ΔNErbB2 overexpression.
The aim of this study was to delineate molecular mechanisms regulating cisplatin-induced death in MCF-7 breast cancer cells and determine the effects of ΔNErbB2 signaling on these processes. A working model summarizing the major findings is shown in Supplementary Fig. S3.

Cisplatin-induced caspase-9 and -7 activation is augmented by ΔNErbB2

Reduced or no expression of caspase-3 is common in breast cancer (36). MCF-7 cells are excellent models for such cancers, as they harbor a premature stop codon mutation in the caspase-3 gene and instead rely on caspase-7 (37, 38). We previously showed that cisplatin treatment elicits caspase- and LMP-and cysteine-cathepsin–dependent loss of viability in MCF-7 cells (18). Here, we demonstrate that exposure to 25 μmol/L cisplatin elicited Annexin V binding, cytochrome c release, and activation of caspase-9 and -7. This agrees well with previous reports of caspase-3–independent, cisplatin-induced death in ovarian cancer cells (39), and caspase-3–independent PARP-1 cleavage in MCF-7 cells (25). Furthermore, in renal epithelial cells, caspase-6 and -7 yet not caspase-3 were assigned important roles in cisplatin-induced death (40). We found no detectable activation of caspase-8, pointing to a predominant role for the intrinsic, rather than the extrinsic, apoptotic pathway in cisplatin-induced MCF-7 cell death. Caspase-9 and -7 activation was augmented by ΔNErbB2 expression, in congruence with the increased caspase sensivity of ΔNErbB2-expressing cells previously demonstrated by us and others (18, 41). This underscores the notion that ΔNErbB2-expressing breast cancer cells, while exhibiting increased metastatic potential, do not exhibit increased chemotherapy resistance (18, 41).

ΔNErbB2 expression regulates autophagic flux in MCF-7 cells, yet this does not contribute to cisplatin-induced cell death

Autophagy has been assigned both pro- and anticancer effects, and its roles in chemotherapeutic cell death are incompletely understood (27). We show here that compared with vector control cells, ΔNErbB2-expressing cells exhibit increased basal autophagy and a greater reduction of autophagic flux upon cisplatin treatment, demonstrating that ΔNErbB2 signaling regulates the autophagic machinery. In metastatic skin cancer, it has been proposed that autophagy is involved in cisplatin resistance (42). However, neither inhibition nor induction of autophagy affected net cell death induced by cisplatin treatment in MCF-7 cells. Therefore, the mechanisms underlying the ΔNErbB2-induced increase in basal autophagy were not addressed here. However, as Bcl-2 downregulation has been reported to stimulate autophagy (43), it seems likely that the effect can at least, in part, be ascribed to the strong downregulation of Bcl-2 by ΔNErbB2 (see below).

ΔNErbB2 expression regulates basal p21 and Bcl-2 expression and attenuates cisplatin-induced ATM- and p53 signaling

In congruence with the well-accepted upstream signaling induced by cisplatin (10), cisplatin treatment activated ATM and increased the protein level and Ser15-phosphorylation of p53 in MCF-7 cells. Notably, cisplatin-induced phosphorylation of ATM and p53 was reduced by ΔNErbB2 expression, as was the cisplatin-induced upregulation of PUMA, whereas Noxa upregulation by cisplatin was strongly augmented by ΔNErbB2. Although both PUMA and Noxa are known transcriptional targets of p53 (14), the strong Noxa induction despite p-p53 downregulation by ΔNErbB2 strongly suggests that Noxa induction is p53 independent (Supplementary Fig. S3). This agrees well with other reports of p53-independent Noxa induction after a variety of stimuli (44, 45). Noxa is important for the ensuing apoptosis, as shown by the marked inhibition of caspase-9 and -7 cleavage upon Noxa knockdown. In contrast, ATM inhibition effectively inhibited p53 phosphorylation yet had little if any effect on caspase cleavage and no detectable effect on PARP cleavage. In conjunction with the p53 independence of Noxa activation, this is most consistent with the notion that Noxa, yet not the ATM-p53 axis, is essential for net cisplatin-induced cell death (Supplementary Fig. S3). Most likely, this at least in part reflects that p53 has both protective and proapoptotic roles in the cisplatin-treated cells, in good agreement with its known cellular functions (14).

In the present work, Bcl-2 was strongly downregulated by ΔNErbB2, and the cisplatin-induced downregulation of another antiapoptotic Bcl-2 protein, Bcl-xL, was augmented. Recent microarray analyses reported upregulation of the Bcl-2 family member BCL2A1, and downregulation of BCL2L11 after expression of another truncated ErbB2 variant in MCF-7 breast cancer cells (Supplementary Table S1 in ref. 17). Collectively, this shows that multiple Bcl-2 family proteins are regulated by ErbB2 signaling, making the net effect on cell death/survival balance difficult to predict. Interestingly, a search of Bcl-2 expression levels in breast cancer patient samples in the Oncomine database (www.oncomine.org), filtering for ErbB2 status, indicates that ErbB2-positive breast cancers generally exhibit lower Bcl-2 expression than ErbB2-negative breast cancers. Also in agreement with our findings, the ErbB3 and -4 ligand heregulin, which activates ErbB2 by heterodimerization, strongly reduced Bcl-2 expression in SkBr3 breast cancer cells (46). On the other hand, a study addressing the effect of full-length ErbB2 on apoptotic pathways in MCF-7 cells by Western blotting concluded that Bcl-2 was upregulated (47). Hence, the effects of ErbB2 signaling on Bcl-2 may be context dependent, and differences between full-length and truncated ErbB2 effects on Bcl-2 are possible.

Also notably, the basal p21 level in MCF-7 cells was more than doubled upon ΔNErbB2 expression yet was not significantly increased by cisplatin, suggesting that under the conditions tested, p53 did not regulate p21. In congruence our findings, both expression of a truncated ErbB2 (17) and overexpression of heregulin (48) have been shown to increase p21 expression, and p53-independent regulation of p21 is well documented (13). Notably, elevated p21 expression is characteristic of senescent cells, and long-term ΔNErbB2 expression was demonstrated to shift MCF-7 cells toward a senescent phenotype suggested to sustain metastasis by secreting factors regulating neighboring non-senescent cells (17). The conditions in our study (48 hours ΔNErbB2 expression) represent an intermediate phenotype, in which cell division is largely unaffected, and the cells therefore remain chemotherapy sensitive.

Both STAT3 and KLF4 have been assigned roles in activation of CDKN1A (the gene coding for p21) promoter (13), and we have previously shown both to be activated by ΔNErbB2 expression (32). That cisplatin treatment strongly attenuated STAT3 phosphorylation in ΔNErbB2-expressing cells while having no effect on p21 argues against a role for STAT3 in this context, leaving
viability, inhibition of NBCn1 failed to affect any of the death
bition had no signi
tributing to cell death [Supplementary Fig. S3 (18)]. The
for normal apoptosis, including that induced by chemothera-
cleavage. In accordance with this view, PARP is dispensable
suggested (54).

NHE1 and NBCn1 are differentially regulated by cisplatin and
play different roles in cisplatin-induced death

Numerous studies by us and others have implicated the
Na+/H+ exchanger protein NHE1 in regulation of death/survival balance after various stimuli including chemotherapeu-
ctic treatment (18, 23, 49–51). This has mainly been
ascribed to pH dependence of cell death pathways, yet, sur-
prisingly, we found that although acid extrusion capacity in
unstimulated ΔNErbB2-expressing MCF-7 cells involves NHE1 and the Na+/HCO3− cotransporter NBCn1 to an approximate-

ly equal extent, inhibition of NHE1, yet not of NBCn1, sen-
sitized these cells to cisplatin-induced death (18). Substanti-
ing this, we show here, first, that the highly specific NHE1
inhibitor cariporide further augmented caspase-9 and -7 activa-
tion in ΔNErbB2-expressing cells, yet not in vector controls.
In contrast, PARP-1 cleavage was only very modestly increased
by NHE1 inhibition, thus, the EIPA-induced augmentation of
cell death (18) cannot be explained by increased PARP-1

cleavage. In accordance with this view, PARP is dispensable
for normal apoptosis, including that induced by chemotherapy
agents (52, 53), and we previously showed that NHE1
inhibition augments LMP and cysteine cathepsin release, con-
tributing to cell death [Supplementary Fig. S3 (18)]. The
precise mechanisms through which NHE1 inhibition enhances
caspase-9 cleavage remain to be determined. It is interesting
to note, however, that NHE1-sensitive regulation of Bcl-xl dea-
midation and hence function after DNA damage has been
suggested (54).

Second, supporting our previous finding that NBCn1 inhibi-
tion had no significant effect on cisplatin-induced loss of
viability, inhibition of NBCn1 failed to affect any of the death
pathways assessed, despite its importance for steady state
global pHi regulation of MCF-7 cells (18). In congruence with
this, we here provide the novel finding that NBCn1, yet not
NHE1, is strongly internalized upon cisplatin exposure. The
molecular mechanisms of NBCn1 trafficking and degradation
are yet unelucidated, hence, understanding the mechanisms
of internalization by cisplatin requires further studies. However,
it seems reasonable to suggest that this marked reduction in
the fraction of NBCn1 protein available for acid extrusion at least
partly accounts for the lack of effect of NBCn1 inhibition on
cisplatin-induced cell death and on pHi in MCF-7 cells after
cisplatin treatment (18).

Clinical implications

Cisplatin is currently receiving renewed interest for combi-
nation treatment of ErbB2-positive breast cancer (6–8), yet the
understanding of its mechanisms of action in this context is
limited. Importantly, we show that several central apoptotic
pathways important for cisplatin-induced cell death are strongly
affected by ΔNErbB2, including, ATM, p53, Noxa, Bcl-2, and,
in agreement with previous reports, p21. The work also sheds
new light on the sensitization of ΔNErbB2-expressing MCF-7
cells to cisplatin chemotherapy by inhibition of the Na+/H+
exchanger NHE1. These findings have potential implications
for the choice of therapy regimen in breast cancer subtypes with
ΔNErbB2 and/or NHE1 overexpression. Specifically, they
indicate that the specific coexpression pattern of ΔNErbB2 with
ATM, p53, p21, Noxa, Bcl-2, and NHE1 in a given patient may
affect sensitivity to cisplatin chemotherapy. Furthermore, they
substantiate our previous proposal that combination treatment
with NHE1 inhibitors may be particularly beneficial in at least
some ErbB2-positive breast cancers.

In conclusion, in MCF-7 cells, ΔNErbB2 expression increases
the basal expression of p21WAF1/Cip1 and nearly abolishes that
of Bcl-2. After cisplatin treatment, ΔNErbB2-expressing cells
exhibit reduced ATM- and p53 phosphorylation, augmented
Noxa upregulation, and increased caspase-9- and -7 cleavage,
compared with vector control cells. Cisplatin-induced caspase-
7 cleavage is strongly dependent on Noxa and caspase-9. Both
caspase-9 and -7 cleavage is augmented by specific inhibition
of NHE1, whereas NBCn1 is lost from the plasma membrane
upon cisplatin treatment and NBCn1 inhibition has no effect
on death signaling. These findings identify novel pro- and
antiapoptotic roles of ΔNErbB2 with potentially important
clinical implications and explain the differential roles of NHE1
and NBCn1 during cisplatin-induced apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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