Epidermal Growth Factor Receptor Pathway Analysis Identifies Amphiregulin as a Key Factor for Cisplatin Resistance of Human Breast Cancer Cells

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The use of platinum complexes for the therapy of breast cancer is an emerging new treatment modality. To gain insight into the mechanisms underlying cisplatin resistance in breast cancer, we used estrogen receptor-positive MCF-7 cells as a model system. We generated cisplatin-resistant MCF-7 cells and determined the functional status of epidermal growth factor receptor (EGFR), MAPK, and AKT signaling pathways by phosphoropeptor tyrosine kinase and phospho-MAPK arrays. The cisplatin-resistant MCF-7 cells are characterized by increased EGFR phosphorylation, high levels of AKT1 kinase activity, and ERK1 phosphorylation. In contrast, the JNK and p38 MAPK modules of the MAPK signaling pathway were inactive. These conditions were associated with inactivation of the p53 pathway and increased BCL-2 expression. We investigated the expression of genes encoding the ligands for the ERBB signaling cascade and found a selective up-regulation of amphiregulin expression, which occurred at later stages of cisplatin resistance development. Amphiregulin is a specific ligand of the EGFR (ERBB1) and a potent mitogen for epithelial cells. After exposure to cisplatin, the resistant MCF-7 cells secreted amphiregulin protein over extended periods of time, and knockdown of amphiregulin expression by specific short interfering RNA resulted in a nearly complete reversion of the resistant phenotype. To demonstrate the generality and importance of our findings, we examined amphiregulin expression and cisplatin resistance in a variety of human breast cancer cell lines and found a highly significant correlation. In contrast, amphiregulin levels did not significantly correlate with cisplatin resistance in a panel of lung cancer cell lines. We have thus identified a novel function of amphiregulin for cisplatin resistance in human breast cancer cells.

The use of platinum complexes for the therapy of breast carcinomas is an emerging new treatment modality that has recently been introduced into the clinical setting (reviewed in Ref. 1). Breast cancer is a family of diseases that consists of major categories, including HER-2-positive breast cancer; “triple-negative” tumors that are ER3-negative, progesterone receptor-negative, and HER-2-negative; and hormonally sensitive breast cancers. The estrogen receptor-expressing (ER-positive) breast cancers are the most prevalent (2). For the therapy of HER2-overexpressing metastatic breast cancer, platinum complexes have been used in combination with paclitaxel and trastuzumab, a humanized monoclonal IgG1 that binds the extracellular domain of the ERBB2 (HER-2/neu) receptor (3). For the treatment of HER-2-positive locally advanced breast cancer, a combination of docetaxel, cisplatin, and trastuzumab has been used as primary systemic therapy (4). Several ongoing phase II studies explore the use of platinum salts for the therapy of breast cancer, including “double-negative” (ER-, progesterone receptor-, and HER-2-negative) breast carcinomas.

Cisplatin enters the cells predominantly by passive diffusion, where it undergoes aquation to form [Pt(NH3)2Cl(OH2)]+ and [Pt(NH3)2(OH2)]2+. Cisplatin functions as a bivalent electrophile predominantly inducing formation of 1,2-intrastrand d(GpG) DNA cross-links (6). Although many cellular components interact with cisplatin, DNA is thought to be the primary biological target of the drug (5). Recently, it was demonstrated that the epidermal growth factor receptor (EGFR) becomes phosphorylated at Thr-669 by p38 MAPK when nonresistant MCF-7 breast cancer cells were exposed to cisplatin (7). Thus, the EGFR signaling pathway is involved in cellular defense against the toxic effects of cisplatin compounds. The ERBB receptor-ligand network comprises a total of four receptors, including EGFR, HER-2, HER-3, and HER-4.
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EGFR (ERBB1), ERBB2 (HER-2), ERBB3, and ERBB4, and multiple ligands reviewed in Ref. 8. Ligands that bind to the ERBB receptor family include EGF, transforming growth factor-α, heparin-binding EGF-like ligand, amphiregulin, betacellulin, epiregulin, epigen, and neuregulin (NRG) family members (9). It is known that an extraordinary variety of different isoforms are produced from the NRG1 gene by alternative splicing. These isoforms include heregulins (HRGs), glial growth factors, and sensory motor neuron-derived factor. The NRG1 gene is located on chromosome 8 and additional neuregulin genes were identified on chromosome 5 (NRG2), 10 (NRG3), and 15 (NRG4) (10). It is well established that the ERBB receptor ligands activate distinct subsets of ERBB receptors and differ in their biological activities (11). The EGFR signaling system is connected to a variety of other related pathways, and a comprehensive pathway map has been constructed based on published scientific papers (12).

The development of cellular resistance to anticancer drugs is a dynamic biological process of high complexity. To better understand this clinically important issue, novel approaches like systems biology are needed. To study cellular mechanisms of resistance to cisplatin, we utilized ER-positive MCF-7 breast cancer cells as a model system. We selected cisplatin-resistant MCF-7 breast cancer cells by exposure to sequential cycles of cisplatin that mimic the way the drug is used in the clinic. We systematically investigated the EGFR signaling system and related pathways, and we identified autocrine amphiregulin as a novel molecular mechanism that confers resistance to cisplatin. Examination of a panel of human breast cancer cells revealed that high levels of amphiregulin are associated with resistance to cisplatin thus demonstrating the generality of our findings.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Cell Lysates—HCC1419 breast cancer cells were purchased from a vendor affiliated with the ATCC (LGC Promochem GmbH, Wesel, Germany). Cells were grown at 37 °C under humidified air, supplemented with 5% CO2 in phenol red-free DMEM (Biochrom AG, Berlin, Germany) containing 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml glucose. Cells were grown to 80% confluency in T-75 cell culture flasks (Corning Inc. San Diego). Incubation was terminated by aspirating the media, and 300 μl of MTT solution (5 mg/ml in PBS) were added to each well. Formazane formation was terminated after 15 min by removing the MTT solution. Subsequently 700 μl of Me2SO were added to each well to solubilize formazane, and the formazane-containing samples were transferred to a new 24-well plate and measured at 590 nm in a microplate reader. To measure cell survival after exposure to cisplatin, MTT assays were performed exactly as described above. The cisplatin concentrations and experimental details are described in the text and the legends of the corresponding figures. To measure cell survival after inhibition of amphiregulin by a neutralizing antibody (AF 262, R & D Systems, Wiesbaden, Germany), we added 1 μg/ml of the polyclonal antibody to the tissue culture 1 h before the addition of cisplatin, and MTT assays were performed as described above.

Matrigel Invasion Assay—To determine invasive potential of MCF-7 CisR cells, we utilized the CytoSelect™ 24-well cell migration and invasion assay, colorimetric format (Cell Biolabs Inc. San Diego). The assay was done exactly as described in the invasion assay protocol. In brief, cells were serum-starved for 24 h and subsequently they were seeded into the upper chamber onto a rehydrated basement membrane covering a Matrigel preparation with a diameter of 8 μm. Cells were allowed to invade toward 10% fetal calf serum for 24 h. Invaded cells on the bottom of the membrane were stained and quantified as described in the assay protocol. The assay was repeated several times (n = 4).

Signaling Pathway Analysis—To investigate signaling pathways we used the Proteome Profiler™ arrays (R & D Systems, Wiesbaden, Germany). For the analysis of ERBB phosphorylation, a human phospho-RTK antibody array was used. The human phospho-RTK antibody array is a nitrocellulose membrane where 42 different anti-RTK Abs have been spotted, including four positive controls and five negative controls, which are spotted in duplicate. Positive controls are phosphorylated tyrosine kinase receptors, which are recognized by the anti-RTK Abs. For the analysis of serine/threonine kinases, a human phospho-MAPK antibody array was used. The human phospho-MAPK array is a nitrocellulose membrane where 21 different anti-kinase Abs have been spotted, including three positive controls and six negative controls, which are spotted in duplicate. Positive controls are phosphorylated proteins, which are recognized by the anti-kinase Abs.

To conduct a Proteome Profiler™ array experiment, the appropriate cells were rinsed twice with PBS, and Nonidet P-40 lysis buffer was added at density of 1 × 107 cells/ml. Cell lysates were gently rocked for 30 min at 4 °C and then centrifuged at 14,000 × g for 5 min (4 °C), and the supernatants were frozen at −80 °C. A total of 250 μg of protein was used for each array. To prevent unspecific protein binding, arrays were blocked using 2% bovine serum albumin in PBS for 1 h at room temperature. Subsequently cell lysates were diluted with PBS containing 2% bovine serum albumin, and the arrays were incubated with the diluted cell lysates overnight at 4 °C. The arrays were then washed three times for 10 min with a wash buffer as specified by the manufacturer. Processing of the arrays differs slightly for phospho-RTK and phospho-MAPK antibody arrays. To proc-
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ess phospho-RTK antibody arrays, they were incubated with horseradish peroxidase-conjugated mouse anti-phosphotyrosine Ab for 2 h at room temperature. To process phospho-MAPK antibody arrays, they were incubated with a biotinylated detection antibody mixture for 2 h at room temperature, followed by a washing step and incubation with a streptavidin-horseradish peroxidase conjugate (30 min, room temperature). After another washing step both types of arrays were processed using a luminol-based reagent, which is used in combination with horseradish peroxidase-conjugated secondary antibodies (WesternGlo®, R & D Systems, Wiesbaden, Germany). Subsequently, arrays were exposed to x-ray films (5–20 min) and developed under standard conditions. Please note that all array experiments were carried out in triplicate.

**Immunoblot Analysis**—Cell lysates (30 μg of protein) were loaded per lane and size-fractionated on a 12% SDS-PAGE. Fractionated proteins were transferred to a polyvinylidene difluoride membrane using established protocols. To control that equal amounts of protein were loaded in each lane, the membranes were stained with Ponceau S (Sigma). For immunoblotting we used a polyclonal affinity-purified goat Ab specific for p53 at a concentration of 1 μg/ml (AF1355, R & D Systems, Wiesbaden, Germany) and a polyclonal affinity-purified goat Ab specific for p21 at a concentration of 1 μg/ml (AF1047, R & D Systems, Wiesbaden, Germany). As secondary antibody we used a horseradish peroxidase-conjugated antibody that detects total goat IgG (HAF109, R & D Systems, Wiesbaden, Germany). The secondary antibody was used at a 1:2500-fold dilution. Western blots were processed with the enhanced chemiluminescence system (Amersham Biosciences) and exposed to x-ray films. X-ray films were developed using standard conditions.

**Enzyme-linked Immunoassays, AKT Kinase Activity, and BrdUrd Cell Proliferation Assay**—To determine the levels of p53 and p21 in cell lysates, we have used a human total p21 and a human total p53 ELISA kit, and for the detection of amphiregulin in cell culture supernatants we have used a human amphiregulin ELISA kit (R & D Systems, Wiesbaden, Germany). BCL-2 levels in cell lysates were measured using a human BCL-2 ELISA kit (Calbiochem). ELISAs were performed as specified by the manufacturer. The activity of AKT kinase in breast cancer cells was determined by an AKT kinase activity assay based on a solid phase ELISA, which utilizes a specific synthetic peptide as a substrate and a polyclonal Ab that recognizes the phosphorylated form of the substrate (StressGen Bioreagents, Victoria, Canada). The kit was used in accordance with the manufacturer’s recommendations. Cell proliferation was quantified using a cell proliferation ELISA, which is based on the measurement of BrdUrd incorporation during DNA synthesis (Roche Applied Science). The assay was performed as recommended by the manufacturer.

**Microarray Analysis with the Agilent System**—For gene expression analysis, Agilent 44k whole genome microarray slides were used. RNA samples (500 ng each) were amplified and labeled with CY-3-CTP and CY-5-CTP, respectively (PerkinElmer Life Sciences), to gain labeled cRNA following a protocol published by the manufacturer. Dye incorporation ratio (≥10 pmol of dye per μg of cRNA) was measured using the Nanodrop photometer (Kisker, Steinfurt, Germany). For hybridization, 1 μg of CY-3-labeled control and 1 μg of CY-5-labeled treated samples were mixed and incubated according to the manufacturer’s instructions (Agilent Technologies, Böblingen, Germany). Washing steps were performed for 1 min in 2× SSPE, 0.01% N-lauryl sulfate, and 1 min in 0.01× SSPE, 0.01% N-lauryl sulfate. Finally, the slides were incubated 1 min in acetonitrile. Completely dried slides were scanned using Agilent microarray scanner. Data analysis and interpretation were performed using Rosetta Luminator software (Rosetta Biosoftware, Seattle).

**Affymetrix Microarray GeneChip Expression Analysis**—Total RNA (5 μg) was extracted with RNeasy mini kit (Qiagen) protocol. The quality of the total RNA was checked with denaturing formamide gel electrophoresis, which showed two sharp and distinct bands of 18 S and 28 S. Quality check was also done by the Agilent Bioanalyzer with graphic analysis showing two distinct peaks of 18 S and 28 S without additional peaks of degradation. The total RNA was then hybridized onto Affymetrix GeneChip HG-U133 A and B sets according to standard protocols (Affymetrix Microarray Suite User Guide 5.0).

**Quantification of Amphiregulin mRNA by Real-time RT-PCR**—Total cellular RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The concentration of purified RNA was determined by the Agilent 2100 bioanalyzer using RNA 6000 NanoChips (Agilent, Waldbronn, Germany). As an internal standard, the β-actin gene was chosen. RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) on DNA Engine Opticon (Bio-Rad). All reactions were performed with 500 ng of total RNA in a volume of 25 μl. Thermal cycling conditions were 30 min at 50 °C, 15 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60°C, and 30 s at 72°C and finished with a melting curve. Expression levels of amphiregulin and β-actin were quantified by the ΔΔCt method. The primers used were amphiregulin forward (5’-GCTCTTTGATACTCGGCTCAG-3’) and amphiregulin reverse (5’-CCCCAGACGTTCTACACTAC-3’); β-actin forward (5’-AGAAAATCTGGCACCACACC-3’) and β-actin reverse (5’-CAGAGGCGTAGGCAGATAGC-3’). The amphiregulin mRNA concentration was normalized to β-actin mRNA.

**Amphiregulin Knockdown by siRNA**—Transfection of 21-nucleotide siRNA duplexes (Qiagen, Hilden, Germany) for targetting endogenous amphiregulin was carried out using Lipofectamine 2000 (Invitrogen). Transfection was performed in suspension, and cells were plated at 1 × 10⁴ per well into 24-well plates. The final volume of 0.5 ml contained 33 nm of both siRNA 1 and siRNA 2 (siRNA 1, CCACAAAUAACCG-GCUAuAdTdT; siRNA 2, AUAUCAUGUAUAGCAAGA-dTdT) and 1 μl of Lipofectamine 2000. As described elsewhere, the highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest (13). As control we used a nonsilencing siRNA (AllStars RNAi control, Qiagen, Hilden, Germany). In general, siRNA-treated cells were analyzed 72 h after transfection.
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Fitting of Concentration/Effect Curves—Indicated are the mean values ± S.E. Concentration/effect curves were fit to the data by nonlinear regression analysis using the following four-parameter logistic Equation 1,

\[ y = \min + \frac{\text{max} - \min}{1 + \left(\frac{\text{IC}_{50}}{10^x}\right)^{n_x}} \]  

(Eq. 1)

where \( x \) refers to the drug concentration; \( y \) refers to the response. The parameters “Max” and “Min” refer to the upper and lower plateau of the sigmoidal curve, respectively. \( \text{IC}_{50} \) denotes the \( x \) value at the inflection point of the sigmoidal curve, and \( n_x \) is the slope factor of the curve.

Statistical Methods—Assays were performed in triplicate unless otherwise indicated. The data are reported as means ± S.E. Statistical significance was assessed by two-tailed Student’s \( t \) test for single comparison and analysis of variance for multiple comparisons, respectively.

Results

Generation and Characterization of Cisplatin-resistant MCF-7 Breast Cancer Cells—Hormone receptor-positive breast cancer is the most prevalent form of the disease (2). In this study, we used ER-positive MCF-7 mammary carcinoma cells as a representative model system to investigate cellular mechanisms of resistance to cisplatin. MCF-7 cells were originally derived from a malignant pleural effusion from a postmenopausal woman with metastatic infiltrating ductal carcinoma of the breast (14). After transplantation into nude mice, MCF-7 cells form tumors with a very low potential of visceral metastasis (15). To generate cisplatin-resistant MCF-7 breast cancer cells, we exposed the cells to sequential cycles of cisplatin using a similar dose that is used for the treatment of women with breast cancer. At a confluency of 80% (~1 \times 10^7 cells), the tissue culture medium was removed, and new medium containing 3 \( \mu \)M cisplatin was added. This concentration of cisplatin represents an \( \text{IC}_{50} \) concentration that was determined empirically using MCF-7 cells. After 8 h of cisplatin exposure the cells were washed three times with DMEM and cultivated as described. During the first 2 months, MCF-7 cells were treated with 8 cycles of cisplatin at weekly intervals. After that, the cells were treated with cisplatin in monthly intervals. After 6 months the extent of cisplatin resistance was quantified by an MTT cell survival assay, which is shown in Fig. 1A. In the MTT cell survival assay, nonresistant MCF-7 cells were used as control that had also been cultivated for 6 months. Nonlinear regression curves were fit to the data points following the four-parameter logistic equation as outlined under “Experimental Procedures.” The resistance factor was calculated as 3.3 from cell survival curves (\( p < 0.05 \)). Cisplatin-resistant MCF-7 cells that represent an end point of our cisplatin treatment regimen were denoted MCF-7 CisR in this work.

To test whether cellular resistance to cisplatin is associated with cross-resistance, we analyzed the anthracycline doxorubicin, which is a prominent chemotherapeutic drug for the treatment of breast cancer (16). We treated MCF-7 and the cisplatin-resistant MCF-7 CisR cells with increasing concentrations of doxorubicin and determined cell survival by the MTT assay (Fig. 1B). The figure shows that MCF-7 CisR cells developed a partial cross-resistance to doxorubicin.

Next, we determined the proliferation rates of MCF-7 CisR cells in comparison with the nonresistant MCF-7 cells. To this end, \( 6.5 \times 10^4 \) cells were seeded into individual wells of 6-well plates and cultivated for 72 h, and cell numbers were determined using a cell counter. The population doubling time of MCF-7 cells was 37 h. In contrast, it was 19 h in the resistant cells. This result is highly significant (\( p < 0.001 \)). Similar results were obtained by a proliferation assay that measures BrdUrd incorporation into DNA in the S-phase of the cell cycle (Table 1). Thus, the cisplatin-resistant state in MCF-7 breast cancer cells is characterized by increased proliferation rates.

To test whether cisplatin resistance would affect tumor cell behavior, we examined the metastatic potential of MCF-7 CisR cells by a Matrigel invasion assay that monitors whether the cells have an increased ability to invade the matrix of a reconstituted basement membrane (Fig. 1C). In comparison to MCF-7 cells, the cisplatin-resistant MCF-7 CisR cells have a significantly increased invasive ability. The assay was done several times (\( n = 4 \)). These results demonstrate that the development of a cisplatin-resistant phenotype is associated with increased tumor cell aggressiveness.

Analysis of the EGFR Signaling Pathway in MCF-7 CisR Cells—It was reported previously that the EGFR is activated in response to cisplatin in normal and in tumor cells as part of a cellular survival response (7, 17). This response can be classified as a cellular defense mechanism that is activated within several hours after exposure to cisplatin. In contrast, it is well established that the development of drug resistance is a long term, time-dependent process. To gain insight into the mechanisms of cisplatin resistance, we investigated the epidermal growth factor receptor (ERBB) signaling cascade in MCF-7 CisR cells. To investigate the phosphorylation status of the ERBB receptor family, we used a phosphoreceptor tyrosine kinase (phospho-RTK) array. In this assay, monoclonal capture antibodies, specific for a variety of RTKs, have been spotted in an array format. Phosphorylation of ERBB subunits is subsequently detected by a pan anti-phosphotyrosine Ab conjugated to horseradish peroxidase. In nonresistant cells the EGFR was phosphorylated at a low level. In contrast, in resistant MCF-7 CisR cells both the EGFR and ERBB2 receptors were strongly phosphorylated (Fig. 2A). The phospho-RTK array detected very low (if any) ERBB3 and ERBB4 phosphorylation in both MCF-7 and MCF-7 CisR cells. Thus, these receptor subtypes are not activated in cisplatin-resistant breast cancer cells.

The ERBB signaling pathway is connected to three major MAPK pathways and the PI3K/AKT survival pathway (12). The MAPK pathways consist of the ERK1/2 module, the p38 MAPK module, and the JNK module (18). To gain insight into the activities of these MAPK modules in MCF-7 CisR cells, we investigated the phosphorylation status of these modules by a human phospho-MAPK array. The principle of this assay is that capture antibodies specific for MAPKs have been spotted on nitrocellulose membranes. After incubation with cell lysates, a mixture of phospho-site-specific biotinylated antibodies was used to detect phosphorylated MAPKs. The phospho-MAPK

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FIGURE 1. Characterization of cisplatin-resistant MCF-7 breast cancer cells. A, degree of cisplatin resistance measured by the MTT cell survival assay. $1 \times 10^4$ cells were seeded into individual wells of 24-well plates and exposed to increasing concentrations of cisplatin for 72 h. The cisplatin-containing tissue culture medium was exchanged with MTT solution and incubated for 15 min. Formazane dye was solubilized by Me$_2$SO, and formazane formation was quantified by absorbance at 590 nm. MCF-7 cells (filled circles) and MCF-7 CisR cells (open circles) are shown. Curves were fitted to data points as specified under “Experimental Procedures,” and the factor of cross-resistance was calculated as 2.0. Arrows indicate IC$_{50}$ concentrations of cisplatin for MCF-7 cells (left arrow) and IC$_{50}$ concentrations of cisplatin for MCF-7 CisR cells (right arrow) ($n = 3$, $p < 0.05$). B, partial cross-resistance of MCF-7 CisR cells to doxorubicin. The degree of cross-resistance was measured by the MTT cell survival assay. $1 \times 10^4$ cells were seeded into individual wells of 24-well plates and exposed to increasing concentrations of doxorubicin for 72 h. The assay was done as described in A. MCF-7 cells (filled circles) and MCF-7 CisR cells (open circles) are shown. Curves were fitted to data points as specified under “Experimental Procedures,” and the factor of cross-resistance was calculated as 3.3. Arrows indicate IC$_{50}$ concentrations of doxorubicin for MCF-7 cells (left arrow) and IC$_{50}$ concentrations of doxorubicin for MCF-7 CisR cells (right arrow) ($n = 3$). C, determination of invasive ability of MCF-7 CisR cells. The Matrigel invasion assay was done following a protocol of the producer. Invaded cells were detected by a fluorometric procedure 24 h after seeding into rehydrated Matrigel invasion chambers. ($n = 4$, Student’s t test, *, $p < 0.05$).

TABLE 1
Increased proliferation rates of cisplatin-resistant MCF-7 breast cancer cells

| Relative BrdUrd incorporation | No. of cells ($\times 10^6$) | Population doubling time |
|-----------------------------|-----------------------------|-------------------------|
| Mean | S.E. | n | Mean | S.E. | n |
| MCF-7 | 100 | 12.7 | 3 | 27.5 | 2.8 | 6 | 37 |
| MCF-7 CisR | 192 | 26 | 3 | 119.6 | 10.2 | 5 | 19 |

array shows that ERK1 (MAPK3) phosphorylation was notably increased in the resistant MCF-7 CisR cells (Fig. 2B). The phospho-MAPK array detects phosphorylation of ERK1 at the Thr-185/Tyr-187 phosphorylation site.

Next, we investigated the p38 MAPK module. p38 MAPK consists of four isoforms as follows: p38-α (MAPK14), p38-β (MAPK11), p38-γ (MAPK12), and p38-δ (MAPK13). In mammalian cells, the p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines but not appreciably by mitogenic stimuli (18). The phosphorylation of the p38 MAPK isoforms is mediated by a complex cascade of protein kinases that is illustrated in detail by PhosphoSite®. The human phospho-MAPK array detects phosphorylation at Thr-180/Tyr-182 (p38-α), Thr-180/Tyr-182 (p38-β), Thr-183/Tyr-185 (p38-γ), and Thr-180/Tyr-182 (p38-δ). It is evident that the phosphorylation levels of all four isoforms of p38 MAPKs are very similar in MCF-7 and MCF-7 CisR cells (Fig. 2C). Thus, the p38 MAPK module is not activated in cisplatin-resistant cells.

Next, we investigated the JNK module using the phospho-MAPK array. The JNK family consists of JNK1 (MAPK8), JNK2 (MAPK9), and JNK3 (MAPK10). The JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, and DNA-damaging agents (19). JNK activation requires dual phosphorylation on tyrosine and threonine residues within a conserved TPY motif (18). Like p38 MAPKs, the JNKs are also activated by a complex cascade of kinases (19). The phospho-MAPK array detects phosphorylation of the phosphorylation site Thr-183/Tyr-185 (JNK1), Thr-183/Tyr-185 (JNK2), and Thr-221/Tyr-223 (JNK3). The phospho-MAPK array shows equal although very low levels of JNK1, JNK2, and JNK3 phosphorylation in MCF-7 and MCF-7 CisR cells (Fig. 2D). Thus, the JNK module is not activated in MCF-7 CisR cells.

The PI3K/AKT cell survival pathway is linked to the EGFR pathway by the docking protein GAB1 that recruits PI3K in response to EGF stimulation of the EGFR (20). PI3K converts phosphatidylinositol 4,5-bisphosphonate (PI(4,5)P$_2$) to PI(3,4,5)P$_3$, and in consequence AKT1 kinase translocates to the cell membrane and interacts with PI(3,4,5)P$_3$ via its pleckstrin homology domain, being phosphorylated at Thr-308 in the activation loop by phosphoinositide-dependent kinase (PDK) 1 and most likely by the rictor-mTOR complex at Ser-473 (21). Three isoforms of AKT kinases (AKT1, AKT2, and
AKT3) have been identified so far. Activation of AKT2 is associated with phosphorylation of Thr-309 and Ser-474, whereas activation of AKT3 is associated with Thr-305 and Ser-472 phosphorylation. The human phospho-MAPK array detects Ser-473 phosphorylation (AKT1) and Ser-474 and Ser-472 phosphorylation on AKT2 and AKT3, respectively. Fig. 2E shows that the levels of AKT phosphorylation are very low in nonresistant MCF-7 cells confirming data from the literature (22). In contrast, we find pronounced AKT1 phosphorylation on Ser-473 in MCF-7 CisR cells. However, we did not detect increased phosphorylation of AKT2 (Ser-474) and AKT3 (Ser-472) in cisplatin-resistant cells. We conclude that selective phosphorylation of AKT1 is a feature of cisplatin-resistant MCF-7 breast cancer cells.

Inactivation of the p53 Pathway in Cisplatin-resistant MCF-7 Breast Cancer Cells—It has been shown recently that AKT induces nuclear localization of MDM2 and, in consequence, degradation of p53 (23). To quantify the activity level of AKT1 kinase in MCF-7 CisR cells, we used an AKT kinase activity assay (Fig. 3A). It is evident that the level of AKT kinase activity is strongly increased in cisplatin-resistant MCF-7 CisR cells.

To analyze p53 protein by immuno blotting, we used a mouse monoclonal Ab specific for human p53 (Fig. 3B). The immunoblot shows that p53 protein is strongly down-regulated in MCF-7 CisR cells to a level below detectability (Fig. 3B, lane 2). To quantify p53 in MCF-7 and MCF-7 CisR cells, we utilized a sandwich ELISA that measures human total p53 in cell lysates and found a 90% lower p53 protein level in MCF-7 CisR cells when compared with nonresistant MCF-7 cells (Fig. 3C). Thus, cisplatin-resistant cells are characterized by a p53 pseudonull phenotype as a result of markedly decreased p53 protein expression. Degradation of p53 will ultimately inactivate the p53 pathway (24), which can be monitored by
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determining p21 expression. We thus investigated p21 expression in MCF-7 and MCF-7 CisR cells by immunoblotting and a sandwich ELISA that measures total p21 in cell lysates (Fig. 3, D and E). It can be seen that p21 expression in cisplatin-resistant breast cancer cells is drastically reduced. These data indicate that the p53 pathway is not active in resistant MCF-7 CisR cells.

It is known that wild-type p53 can bind to BCL-2 and neutralize the death-protective function of BCL-2 (25). Moreover, p53 is a negative regulator of BCL-2 expression (26), suggesting that a lack of p53 in MCF-7 CisR cells might be associated with altered levels of BCL-2 protein. To determine the levels of BCL-2 in resistant MCF-7 CisR and nonresistant MCF-7 cells, we used a sandwich ELISA that measures human total BCL-2 in cell lysates. Although MCF-7 cells express a low level of BCL-2 protein, the cisplatin-resistant MCF-7 CisR cells showed highly elevated BCL-2 levels (Fig. 3F). We conclude that both the functional inactivation of p53 and the high levels of BCL-2 in MCF-7 CisR cells are an important facet of acquired cisplatin resistance in these cells.

Up-regulation of Amphiregulin Gene Expression during the Development of Cisplatin Resistance in MCF-7 Breast Cancer Cells—Next, we wished to investigate activities of genes encoding the known EGFR/ERBB ligands during development of cisplatin resistance. For this analysis, a new batch of nonresistant MCF-7 cells was treated by cycles of cisplatin in weekly intervals for a total of 6 months, and mRNA was isolated 1 week after each treatment cycle. For the isolation of temporally matched control RNAs, untreated MCF-7 cells were cultivated in parallel for a total of 6 months. For gene expression analysis, Agilent 44k whole genome microarray slides were used. Gene expression data were analyzed using the Rosetta Luminator software. We analyzed amphiregulin, betacellulin, EGF, epiregulin, epi-
gen, heparin-binding EGF-like ligand, NRG1, NRG2, NRG3, NRG4, and transforming growth factor-α gene expression. We detected a transient induction of amphiregulin gene expression in response to cisplatin exposure in the 1- and 3-week time points, but nearly control levels in the 6-week and 8-week time points. We found that the levels of amphiregulin gene expression began to rise again after 3 months and steadily increased in MCF-7 CisR cells until the end point (6 months) of our cisplatin treatment regime (supplemental Fig. S1). In contrast to amphiregulin, the transcription of epigen, betacellulin, epi-
gen, EGF, HBEGF, transforming growth factor-α, NRG1 (variant glial growth factor 2), NRG1 (variant sensory motor neuron-derived factor), NRG1 (variant HRG-β1), NRG1 (variant HRG-γ), NRG2 (variant 5), NRG2 (variant 3), NRG3, and NRG4 did not change significantly after exposure to cisplatin at any time (data not shown). In fact, only amphiregulin was detectably expressed in MCF-7 cells, and the expression levels for all other ERBB ligands were below background. The amphiregulin microarray expression data were verified by RT-PCR, and this analysis yielded identical results (Fig. 4A). We conclude that ER-positive MCF-7 breast cancer cells express the amphiregulin gene at a low level with strongly increased expression in MCF-7 CisR cells at later stages of cisplatin resistance development.

Sustained Secretion of the Epidermal Growth Factor Receptor Ligand Amphiregulin by MCF-7 CisR Cells in Response to Cisplatin Exposure—We then analyzed whether the up-regulation of amphiregulin gene expression in MCF-7 CisR cells translates into increased amphiregulin protein levels. The transmembrane amphiregulin precursor protein consists of 252 amino acids, and the biologically active 84-amino acid-long amphiregulin protein is released from the membrane by proteolytic activity of the metalloproteinase ADAM17 (also known as tumor necrosis factor α-converting enzyme) (13). To detect secreted (shedded) amphiregulin, we used an ELISA. MCF-7 and MCF-7 CisR cells were exposed to 3 μM cisplatin for 8 h, and after removal of the drug, the tissue culture supernatants were analyzed with the amphiregulin-specific ELISA in 24-h intervals. Amphiregulin secretion was first detected 24 h after cisplatin exposure. This result shows that amphiregulin secretion occurs as a response to cisplatin treatment. Moreover, the amphiregulin-specific ELISA detected a strong increase in the concentration of secreted amphiregulin over an extended period of time in supernatants of cisplatin-treated MCF-7 CisR cells (Fig. 4B, open circles). In this experiment, the highest levels of secreted amphiregulin were found 72 h after exposure to cisplatin. In contrast, nonresistant MCF-7 cells did not secrete amphiregulin after exposure to cisplatin. The levels of amphiregulin in supernatants of cisplatin-treated nonresistant MCF-7 cells were very low and did not significantly change over a period of 72 h (Fig. 4B, filled circles). Thus, sustained amphiregulin secretion in response to cisplatin treatment is a unique feature of cisplatin-resistant MCF-7 breast cancer cells.

Impact of Amphiregulin and AKT Kinase on Cisplatin Resistance—Our data suggested that amphiregulin is directly linked to cisplatin resistance. We thus wished to determine the impact of amphiregulin for the cisplatin-resistant state of MCF-7 CisR cells by an amphiregulin knockdown experiment. MCF-7 CisR cells were treated with Lipofectamine 2000® and siRNA was specifically targeted against the amphiregulin mRNA transcript. As control, we used a commercially available nonsilencing siRNA. Knockdown efficiency was controlled by
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FIGURE 3. Analysis of AKT kinase, downstream signaling reveals inactivation of the p53 pathway in MCF-7 CisR cells. A, quantification of AKT kinase activity. To measure AKT kinase activity, a solid phase ELISA, which utilizes a specific synthetic peptide as a substrate and a polyclonal antibody that recognizes the phosphorylated form of the substrate, was used. (n = 3, *** p < 0.001). B, detection of p53 protein by immunoblotting (IB) using a polyclonal affinity-purified goat Ab specific for p53. MCF-7 cells (lane 1) and MCF-7 CisR cells (lane 2). Lane M, molecular weight marker. p53 is indicated by an arrow. C, quantification of p53 by a sandwich ELISA that measures human total p53 in cell lysates (n = 3, *** p < 0.001). D, p21 expression indicates p53 pathway activity. Detection of p21 in whole cell lysates of MCF-7 (lane 1) and MCF-7 CisR cells (lane 2) by immunoblotting using a polyclonal affinity-purified goat Ab specific for p21. Lane M, molecular weight marker. p21 indicated by an arrow. E, to quantify the levels of p21 protein a sandwich ELISA that measures p21 in cell lysates was used. (n = 3, ***, p < 0.001). F, to quantify BCL-2 expression an ELISA that detects human BCL-2 in cell lysates was used (n = 3, ***, p < 0.001).

As amphiregulin activates the ERBB signaling cascade and this pathway is linked to the PI3K/AKT pathway through GAB1, we wished to investigate the impact of PI3K/AKT signaling on cisplatin resistance of MCF-7 CisR cells. To inhibit the PI3K/AKT kinase pathway we used wortmannin, which irreversibly targets PI3K and inhibits its activity (27). MCF-7 CisR cells were cultivated in the presence of 25 nM wortmannin and exposed to increasing amounts of cisplatin. Subsequently, cell viability was determined by the MTT cell survival assay. As a control, MCF-7 CisR cells cultivated without the addition of wortmannin were exposed to increasing amounts of cisplatin and then analyzed by the MTT cell survival assay (Fig. 4E). Inhibition of PI3K caused reversal of cisplatin resistance. This is illustrated by comparing Fig. 4E with Fig. 1, where MTT cell survival assays of nonresistant and MCF-7 CisR cells after exposure to cisplatin are shown. We conclude that activation of the PI3K/AKT signaling pathway is an important event downstream of amphiregulin for the development of cisplatin resistance in MCF-7 breast cancer cells.

Significant Correlation of Amphiregulin Expression with Cisplatin Resistance in Diverse Human Breast Carcinoma Cell Lines—MCF-7 breast cancer cells served as a model system to investigate molecular mechanisms of cisplatin resistance. To test whether our results are of more general importance, we analyzed amphiregulin expression in a panel of 12 human breast carcinoma cell lines. In a second step, the sensitivities of these cell lines to cisplatin exposure were measured by MTS cell survival assays. The summary of these data is shown (Fig. 5A). In a final step, we correlated the amphiregulin expression levels with the IC50 values from MTS cell survival assays (Fig. 5B). This analysis revealed a correlation coefficient of 0.674, which is highly significant (**, p value 0.002). Thus, elevated levels of amphiregulin expression indicate a cisplatin-resistant phenotype in diverse human breast cancer cells. To verify this experimentally, we selected HCC1419 breast cancer cells as a representative example for amphiregulin knockdown experiments. The HCC1419 cell line expresses high levels of amphiregulin and was originally established from a nodal positive ductal carcinoma that was treated by prior chemotherapy (28). The MTT cell survival assay shows that the inhibition of amphiregulin by a specific siRNA was associated with a significant reversal of cisplatin resistance in HCC1419 breast cancer cells (Fig. 5C).
FIGURE 4. Identification of amphiregulin as key factor for cisplatin resistance in MCF-7 CisR cells. A, amphiregulin mRNA expression in the course of cisplatin resistance development. Amphiregulin mRNA levels were measured by real time quantitative RT-PCR using gene-specific primers for amphiregulin and β-actin. The amphiregulin PCR products (Ct value) were normalized to the level of β-actin (Ct value). (n = 2, *p < 0.05; ***, p < 0.001). B, MCF-7 CisR cells respond to cisplatin exposure with sustained secretion of amphiregulin. MCF-7 CisR cells (open circles) were exposed to 3 μM cisplatin for 8 h. After replacement of the cisplatin-containing medium with DMEM, an amphiregulin ELISA was performed on cell culture supernatants (100 μl) at the indicated time points. (n = 3, *p < 0.05; ***, p < 0.001; n = 3). Nonresistant MCF-7 cells (filled circles) were exposed to an IC50 dose of cisplatin (3 μM) for 8 h. After replacement of the cisplatin-containing medium with DMEM, an amphiregulin ELISA was performed on cell culture supernatants (100 μl) at the indicated time points (n = 3). C, regulation of cisplatin resistance by amphiregulin. To inhibit amphiregulin expression, MCF-7 CisR cells were transfected using Lipofectamine 2000® and an amphiregulin-specific siRNA. To determine the impact of amphiregulin inhibition on cisplatin resistance, 1 × 10⁴ siRNA transfected MCF-7 CisR cells were seeded into individual wells of a 24-well plate and exposed to increasing concentrations of cisplatin. After 72 h MTT cell survival assays were used to measure the degree of cisplatin resistance. siRNA transfected MCF-7 CisR cells (asterisks), nonsilencing siRNA transfected MCF-7 CisR cells (filled triangles), and untreated MCF-7 CisR cells (open circles) are shown. Curves were fitted to data points as specified under "Experimental Procedures," and the shift factor caused by amphiregulin silencing was calculated as 3.8. Arrows indicate the IC₅₀ concentration of cisplatin for MCF-7 CisR cells transfected with the nonsilencing siRNA (right arrow) and the IC₅₀ concentration of cisplatin for siRNA transfected MCF-7 CisR cells (left arrow). (n = 2, *p < 0.05). D, functional inhibition of secreted amphiregulin by a neutralizing antibody (neutr. Ab). MTT cell survival assay of MCF-7 CisR cells after exposure to cisplatin in the presence of a neutralizing antibody specific for amphiregulin. The antibody (1 μg/ml) was added to the tissue culture 1 h before the addition of cisplatin. MCF-7 CisR cells (open circles), MCF-7 CisR cells in the presence of the neutralizing antibody (asterisks), (n = 2, *p < 0.05. E, impact of the PI3K/AKT kinase pathway for the cisplatin-resistant phenotype of human breast cancer cells. MCF-7 CisR cells were cultivated in the presence of 25 nm wortmannin to inhibit the PI3K pathway. Wortmannin was added 30 min prior to addition of cisplatin (filled triangles), MCF-7 CisR cells without the addition of wortmannin (open circles). The cells were exposed to increasing concentrations of cisplatin (1–100 μM), and the degree of cisplatin resistance was determined after 72 h by the MTT cell survival assay. Curves were fitted to data points as specified under “Experimental Procedures,” and the wortmannin-dependent shift factor was calculated as 4.4. Arrows indicate the IC₅₀ concentration of cisplatin for MCF-7 CisR cells (right arrow) and the IC₅₀ concentration of cisplatin for MCF-7 CisR cells in the presence of 25 nm Wortmannin (left arrow). n = 3, *p < 0.05.
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A

| Breast cancer Cell Line | IC\textsubscript{50} from MTS assay 
|-------------------------|-------------------------|
| Cell Line               | Cisplatin [\mu M]       | Amphiregulin gene expression log\textsubscript{2} \n|-------------------------|-------------------------|
| HCC1143                 | 4.8                     | 7.986                    |
| HCC1419                 | 12.2                    | 11.872                   |
| HCC1569                 | 3.4                     | 6.150                    |
| HCC1954                 | 8.5                     | 9.289                    |
| HCC2218                 | 14.7                    | 8.928                    |
| HCC2688                 | 1.4                     | 5.792                    |
| HCC3153                 | 1.4                     | 6.458                    |
| HTB122                  | 1.2                     | 5.333                    |
| HTB126                  | 6.9                     | 5.873                    |
| HTB131                  | 2.0                     | 9.005                    |
| HTB24                   | 2.6                     | 6.687                    |
| MDA-MB 231              | 4.1                     | 6.932                    |

We conclude that amphiregulin is a key factor for cisplatin resistance in human breast cancer cells. To test whether increased amphiregulin expression has an impact on cisplatin resistance in another tumor entity, we correlated the levels of amphiregulin expression with cisplatin resistance in a cohort of lung cancer-derived cell lines (n = 43). We found high levels of amphiregulin expression in a large fraction of lung cancer cells (n = 43), some of which were highly resistant to cisplatin (supplemental Fig. S2A). However, a statistical analysis of these data did not reveal a significant correlation of amphiregulin expression with resistance to cisplatin. The correlation coefficient was calculated as −0.02027 with a nonsignificant p value (p = 0.895). Future work is needed to clarify whether amphiregulin is linked to anticancer drug resistance in other malignant diseases besides breast cancer.

DISCUSSION

ER-positive breast cancers are the most prevalent form of the disease (2). Eventually, in most women, metastatic breast cancer becomes refractory to hormonal treatment and chemotherapy (16). These clinical findings demonstrate that the development of resistance to therapy is a time-consuming biological process. Here we have generated cisplatin-resistant ER-positive breast cancer cells (MCF-7 CisR) by sequential cycles of cisplatin exposure over a period of 6 months. During the first 2 months the cells received weekly cycles of cisplatin followed by monthly cycles of cisplatin exposure. It is a goal of our work to use systems biology approaches to unveil basic principles of cisplatin resistance. The MCF-7 CisR cells represent the end point of our cisplatin treatment regimen, and we used these cells to investigate systematically the activities of ERBB and MAPK signaling pathways using phospho-RTK and phospho-MAPK arrays.

In MCF-7 CisR cells, the EGFR is activated (phosphorylated). It has been reported that the adaptor protein GAB1 (Grb2-associated binder 1) recruits PI3K to the activated EGFR, which lacks canonical PI3K-binding sites (20). A systems biology approach demonstrated that the essential function of GAB1 is to enhance PI3K/AKT activation and to extend the duration of Ras/MAPK signaling (29). In keeping with this, we have detected selective phosphorylation of ERK1 at the Thr-202/Tyr-204 phosphorylation site and selective phosphorylation of AKT1 at Ser-473. It is important to figure out how these phosphorylation events could be linked to the cisplatin-resistant state of MCF-7 CisR cells. Several reports from the literature show that ERK1 and ERK2 have different functions (30). Although ERK1 is linked to cell proliferation and the survival of tumor cells (30), ERK2 has been linked to the regulation of cell motility (31). Thus, the activation of ERK1 in MCF-7 CisR cells can contribute to increased cell proliferation and cell survival. It has also been shown that the three AKT isoforms have different functions (32). For example, only AKT1 is required for proliferation, whereas AKT2 promotes cell cycle exit through p21 binding (32). Likewise, knock-out mice have shown that loss of Akt1 leads to growth defects, whereas loss of Akt2 primarily affects glucose homeostasis (33). Most notably, however, it was found that AKT1 amplification in lung cancer tissues was associated with resistance to cisplatin (34). In line with this, inhibition of PI3K/AKT by wortmannin in MCF-7 CisR cells was associated with an almost complete reversal of the cisplatin-resistant phenotype.

MCF-7 CisR cells are characterized by elevated levels of AKT kinase activity. It is a goal of our work to unveil mechanisms of cisplatin resistance by a systematic analysis of selected pathways, and we thus focused on signaling downstream of AKT. It
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is well established that AKT phosphorylates a number of substrates at specific serine and threonine residues, including transcription factors, protein kinases, apoptosis regulators, and components of the proteasome (35). For example, AKT-dependent phosphorylation of MDM2 at Ser-166 and Ser-186 promotes translocation to the nucleus where it negatively regulates p53 function (23, 36). MCF-7 cells are wild type for p53, and MCF-7 CisR cells are characterized by strongly reduced p53 protein levels that are associated with an inactivation of the p53 pathway. It has to be debated whether the reduction of p53 is because of increased cell proliferation or whether the loss of p53 is causing this increase in proliferation. It has been shown that silencing of p53 by siRNA promotes accelerated DNA synthesis (37). MCF-7 CisR cells show accelerated DNA synthesis as determined by BrdUrd incorporation. In addition, chemotherapeutic drugs, including anthracycline, induce p53 expression and p53-dependent apoptosis (37). If the lower levels of p53 and p21 in MCF-7 CisR cells are because of accelerated proliferation, one might expect that they are sensitive to other chemotherapeutic drugs. We chose doxorubicin to address this issue and found that MCF-7 CisR cells are partially cross-resistant to this drug. This result supports the notion that loss of p53 and p21 in MCF-7 cells is responsible for accelerated proliferation and drug resistance.

It is evident that inactivation of p53 is an important step for the development of cisplatin resistance as p53 antagonizes BCL-2 function at several levels (25). p53 is also controlling the expression of Bcl-2 at the level of transcription as antisense inhibition of p53 is associated with overexpression of Bcl-2 (38), and transient transfection analysis revealed that wild-type p53 repressed the Bcl-2 full-length promoter (26). In MCF-7 CisR cells, the down-regulation of wild-type p53 protein expression is associated with increased levels of BCL-2 suggesting that both events are interconnected in the resistant cells. In addition, AKT kinase up-regulates Bcl-2 expression through phosphorylation of a cAMP-response element-binding protein. It is well established that BCL-2 prevents apoptosis induced by most chemotherapeutic drugs (39).

The JNK and p38 MAPK modules of the MAPK signaling pathway were not activated in MCF-7 CisR cells. It is established that these two stress-activated modules are directly linked to apoptosis, and it is known that apoptosis-signal regulating kinase 1 (ASK-1) acts upstream of these MAPK modules (40). ASK-1 is a substrate of AKT kinase, and phosphorylation inhibits ASK-1 function (35). It is conceivable that ASK-1 phosphorylation by AKT1 in MCF-7 CisR cells prevents activation of the JNK and p38 MAPK modules. It is, however, part of future work to elaborate this issue. So far we have discussed the current status of ERBB and MAPK signaling pathways in MCF-7 CisR cells. In the long run, however, we are interested in analyzing the process of cisplatin resistance development in a time-resolved fashion. To address this issue, we used Agilent 44k whole genome microarrays and analyzed gene expression profiles during the process of cisplatin resistance development. For microarray analysis, the MCF-7 cells were exposed to cisplatin in weekly intervals over a total period of 6 months.

The ERBB pathway is activated by a family of diverse ligands that bind to the ERBB receptor subunits (9, 10). These ligands can be defined as the input level of the ERBB signaling pathway. Gene expression profiling revealed that amphiregulin is the only EGFR ligand that was expressed in nonresistant MCF-7 cells. When we analyzed expression of the ERBB ligands in a time-resolved fashion, we found that amphiregulin gene expression was transiently up-regulated during the first 3 weeks of cisplatin treatment and returned to a level similar to the nonresistant MCF-7 cells in the fourth week. Thereafter the levels of amphiregulin expression were unchanged for the next 8 weeks. However, after 12 weeks of weekly cisplatin treatment amphiregulin expression increased again reaching the highest levels after 6 months. Amphiregulin is an exclusive ligand of the EGFR that induces tyrosine phosphorylation and receptor activation (41). Amphiregulin was originally purified from the conditioned media of MCF-7 breast cancer epithelial cells treated with the tumor promoter phorbol 12-myristate 13-acetate (42). A comparison between the biological effects of EGF and amphiregulin reveals distinct differences (43). Amphiregulin increases invasion capabilities of MCF-7 breast cancer cells, and transcriptional profiling experiments revealed that amphiregulin and EGF promote dramatically distinct patterns of gene expression (43, 44). Several genes involved in cell motility and invasion were up-regulated when nontumorigenic breast epithelial cells were cultivated in the presence of amphiregulin (43). The cytoplasmic tail of the EGFR plays a critical role in amphiregulin mitogenic signaling but is dispensable for EGF signaling (45). Breast cancer cells that were derived from an aggressive inflammatory breast carcinoma overexpress amphiregulin, which renders them EGF-independent (43). Escape of dependence on extrinsic proliferative signals is a key event in the evolution of malignant tumors. Clinical investigations revealed that the levels of amphiregulin protein are generally higher in invasive breast carcinomas than in ductal carcinoma in situ or in normal mammary epithelium (46–48). We have used Matrigel invasion assays to characterize tumor cell behavior of MCF-7 CisR cells and found a significantly increased ability to invade and penetrate the basement membrane that is the essential component of the Matrigel invasion assay. These results are in line with published data, and they show that drug resistance and tumor aggressiveness are interconnected biological processes.

Here we have identified a novel function of amphiregulin for the cisplatin-resistant state of MCF-7 breast cancer cells. The initial transient up-regulation of amphiregulin expression suggests that this is part of a cellular defense mechanism. The continuous up-regulation of amphiregulin expression during the last 3 months of cisplatin treatment cycles shows that this is part of a cellular resistance mechanism. Our results suggested that amphiregulin could play an essential role for the development of cisplatin resistance. This consideration was tested by amphiregulin knockdown experiments. It was possible to reverse the cisplatin-resistant state of MCF-7 CisR cells to a large part by siRNA-mediated inhibition of amphiregulin expression. Amphiregulin protein is anchored to the cell membrane as a 50-kDa proamphiregulin form and is preferentially cleaved by ADAM17 at distal site within the ectodomain to release a major 43-kDa amphiregulin form into the medium (49). We conclude that MCF-7 CisR cells show persistent alter-
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Amphiregulin (Amph) is a member of the epidermal growth factor (EGF) family. Its expression has been correlated with tumor progression and resistance to chemotherapy in various malignancies. In breast cancer, Amphiregulin expression correlates with outcomes and can be induced by cisplatin, suggesting a role in cisplatin resistance.

Here we have used MCF-7 cells as a model to study the mechanism of cisplatin resistance. Once a molecular mechanism is unveiled, it is mandatory to explore whether this finding is of greater importance for the disease. To address this issue we correlated amphiregulin expression levels with the cisplatin-resistant state of a collection of human breast cancer cells. We found a highly significant correlation that demonstrates that human breast cancer cells use amphiregulin as a survival signal to resist exposure to cisplatin. We also analyzed a collection of lung cancer cells that tend to express elevated levels of amphiregulin. However, we did not find a significant correlation between cisplatin resistance and amphiregulin expression. From these data we conclude that it is necessary to systematically investigate different tumor types to determine the role of amphiregulin for cisplatin resistance in other malignant tumors besides breast cancer. Future clinical studies will determine the full impact of amphiregulin expression for therapy response and outcome in women with breast cancer.

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