Involvement of the HER2 pathway in repair of DNA damage produced by chemotherapeutic agents

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Abstract
HER2 (ErbB2) is overexpressed in up to 30% of human breast cancers. Preclinical and clinical studies suggest synergy between some chemotherapeutic agents and the humanized anti-HER2 antibody trastuzumab (Herceptin). This study investigated the effects of etoposide and cisplatin on the repair of DNA damage in breast cancer cell lines. We examined the potential significance of HER2 nuclear expression in DNA repair. MCF-7, SK-BR-3, and MDA-MB-453 cells were treated with cisplatin and etoposide. Repair of DNA interstrand crosslinks (ICL) and strand breaks, following incubation with cisplatin and etoposide, respectively, were quantitated by the single-cell gel electrophoresis (comet) assay. Intrastrand crosslinks produced by cisplatin were assessed by ELISA. The effects of trastuzumab were measured in combination with these drugs. Similar experiments were done using HER2-negative MDA-MB-468 cells transfected with HER2 and a construct lacking the nuclear localization sequence. Incubation of breast cancer cell lines with trastuzumab delayed the repair of ICL produced by cisplatin. There were no effects on the repair of interstrand crosslinks produced by cisplatin, or repair of DNA strand breaks following etoposide treatment. Transfection of HER2 into MDA-MB-468 cells inhibited the repair of cisplatin-induced ICL, whereas transfection of a HER2 construct lacking the nuclear localization sequence did not affect DNA repair. These results indicate that HER2 expression modulates the repair of specific DNA lesions produced by chemotherapy. The effect on ICL repair requires nuclear expression of HER2. Understanding the mechanisms of interaction between DNA-interacting agents and HER2 inhibitors will inform the design of clinical trials and optimize the therapeutic effects of these combinations. [Mol Cancer Ther 2009;8(11):3015–23]

Introduction
Overexpression of the HER2 oncogene occurs in 25% to 30% of human breast cancers and is a marker of poor prognosis (1, 2). Additionally, increased expression of HER2 has also been found in other malignancies including gastric and prostate cancers (3). Activation of HER2, primarily through heterodimerization with other members of the epidermal growth factor receptor (EGFR) family, results in downstream signaling through the Ras and phosphoinositide-3-kinase pathways (4–6). This results in the promotion of tumorigenic effects such as increased proliferation, metastasis, and angiogenesis. Inhibition of HER2 by targeted antibodies causes the regression of breast cancers in a variety of models (7).

Although the defined intracellular signaling pathways downstream of HER2 are based on the membrane localization of HER2, there is increasing evidence that nuclear expression of HER2 may be significant in mediating some effects (8, 9). Detection of nuclear HER2 by immunohistochemistry in breast cancer is associated with a worse prognosis (10). There is evidence that nuclear translocation of HER2 is associated with increased transcription of several genes, notably COX-2 (11).

In view of the importance of the HER2 pathway in the pathogenesis of breast cancer, there has been major interest on the inhibition of this pathway as a therapeutic strategy. The humanized monoclonal antibody, trastuzumab (Herceptin), has been shown to improve survival in both adjuvant and advanced settings of breast cancer (12). Several studies in vitro and in vivo have shown that both additive and synergistic effects occur when trastuzumab is combined with chemotherapeutic agents (13–17). The synergistic effects of combining trastuzumab with cisplatin, carboplatin, and paclitaxel have been shown in several breast cancer cell lines (18, 19). Additive effects occur with combinations of trastuzumab and doxorubicin (18). Clinically, the combinations of trastuzumab with vinorelbine and taxanes have been shown to be most efficacious (20). However, there is extensive preclinical and some clinical evidence for benefit in combining trastuzumab with cisplatin and doxorubicin (13, 18).

It is therefore critical to understand how new agents targeting the HER pathway should be optimally combined with conventional chemotherapeutic agents. Inhibition of EGFR by antibodies (cetuximab) or small molecules (gefitinib) results in increased sensitivity to radiation and chemotherapy.
HER2 Inhibition and Chemotherapy

in a variety of cellular models (21–23). Clinically, combining EGFR inhibitors with radiation and chemotherapy has shown synergy (24). Thus far, the design of clinical studies combining trastuzumab and chemotherapeutic agents has been empiric, with trastuzumab added to established therapeutic regimens. However, the mechanism of interaction between trastuzumab and specific chemotherapeutic agents has not been extensively investigated. An important study showed that trastuzumab had effects on DNA repair following cisplatin administration to xenografts, with inhibition of unscheduled DNA synthesis following trastuzumab treatment (25).

The aim of this study was to determine the effects of trastuzumab on the repair of DNA damage produced by cisplatin and etoposide. Although doxorubicin has been most widely used, there is evidence of the efficacy of these agents in the treatment of breast cancer (26, 27). Additionally, with recent reports of the expression of HER2 in other malignancies including gastrointestinal cancer, it is important to understand the interaction of trastuzumab with a variety of chemotherapeutic agents. To determine the effects of HER2 inhibition on DNA repair, we examined three breast cancer cell lines with differing levels of HER2, and transfected HER2 into a HER2-negative background in the MDA-MB-468 cell line. We used the single cell electrophoresis (comet) assay to quantitate the formation and repair of DNA interstrand crosslinks (ICL) and DNA strand breaks following treatment of cells with cisplatin, melphalan, and etoposide. To examine the effects of trastuzumab on the formation and repair of cisplatin-induced DNA interstran crosslinks, which account for >80% of the DNA damage produced by this agent, we used a competitive ELISA assay. Additionally, we investigated the significance of nuclear HER2 expression in the modulation of DNA repair using cells transfected with HER2 constructs lacking a nuclear localization sequence (NLS). These experiments show the effect of HER2 on the repair of specific DNA lesions. Understanding the mechanism of these interactions will inform the design of clinical studies, which will optimize combinations of HER inhibition and chemotherapeutic agents.

Materials and Methods

Materials

Cisplatin (DBL) and trastuzumab (Herceptin, Roche). Wheat germ agglutinin (WGA; lectin from Triticum vulgaris) was purchased from Merck Biosciences. Melphalan and etoposide were purchased from Sigma-Aldrich.

Cell Lines and Culture Conditions

MCF-7 cells (obtained from CR-UK London Research Institute) were grown in RPMI 1640 (Autogen Bioclear). SK-BR-3 cells (obtained from CR-UK London Research Institute) were cultured in McCoy’s 5A modified medium (Sigma-Aldrich). MDA-MB-453 cells (obtained from CR-UK London Research Institute) were grown in DMEM (Autogen Bioclear). Human breast cancer cell line, MDA-MB-468, was grown in DMEM (Autogen Bioclear). Transfected MDA-MB-468 cells were grown in the same complete medium containing G418-selective agent (Sigma-Aldrich) at a concentration of 750 μg/mL. All cell culture media were supplemented with 10% FCS and 1% glutamine and incubated at 37°C in 5% CO2.

Plasmid Transfection

cDNA3 vector control and pcDNA3-ErbB2 plasmid were obtained from Dr. Segatto (Laboratory of Immunology, Regina Elena Cancer Institute, Rome, Italy). pEGFP-N1 vector control, pEGFP-N1 ErbB2, and pEGFP ErbB2 ΔNLS were kindly provided by Prof. Hung (Department of Molecular and Cellular Biology, University of Texas, M.D. Anderson Cancer Center, Houston, TX). The ErbB2 ΔNLS construct contains a deletion of the NLS (amino acids 676-KRRQQRKYTMRR-689; ref. 8). Transfections were achieved using Genejuice transfection reagent (Novagen EMD Biosciences). Cells were pooled transfected using G418-selective agent (Sigma-Aldrich).

Growth Inhibition Assay

The cytotoxicity of drugs was assessed using the colorimetric assay Sulforhodamine B (28) in 96-well microtiter plates. Cells were incubated into 96-well microtiter plates (Nunclon, VWR) and incubated for 24 h at 37°C with 5% CO2 prior to drug treatment. Drugs were diluted in complete growth medium and 200 μL of the appropriate concentration was added to the appropriate well. Cells were treated with a range of concentrations to wells in triplicate and left in solution continuously for 1 h followed by 3 d in drug-free medium in a humid box at 37°C in 5% CO2. Control wells were treated in the same way with drug-free medium. At the end of the incubation period, cells were fixed with ice-cold 10% w/v trichloroacetic acid and stained with 0.4% SRB in 1% v/v acetic acid. The absorbance (OD) of each well was read at 540 nm using a Spectrafluor Plus plate reader (Tecan). The mean absorbance of each drug concentration was expressed as a percentage of the untreated control well’s absorbance.

Western Blotting

Western blotting was done as described (23). Proteins were probed using anti-EGFR, anti-HER2, anti-HER3, anti-HER4, anti-calnexin, anti-lamin A/C (Cell Signaling), or anti-α-tubulin (clone B-5-1-2; Sigma-Aldrich). Finally, the primary antibody was probed with horseradish peroxidase–conjugated polyclonal antibodies for chemiluminescence detection (ECL System, Amershams Biosciences).

Immunofluorescence

Cells were cultured in Lab-Tek II chamber slides (Nalge Nunc International, VWR) overnight before drug exposure. Exponentially growing cells were cultured in the presence of complete medium alone, 40 μg/mL of cisplatin alone or in combination with trastuzumab, or 100 μmol/L of cisplatin alone or in combination with trastuzumab for 60 min followed by replacement with drug-free complete medium or 40 μg/mL of trastuzumab for 23 h. Cells were washed twice with PBS followed by fixing with 50:50 (v/v) acetone/methanol for 8 min at 4°C. Cells were permeabilized with a buffer containing 0.5% Triton X-100 in PBS, followed by washing with PBS. Slides were mounted using Vectashield hard set mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories). Cells were visualized.
using a Perkin-Elmer Ultraview confocal microscope using excitation wavelengths of 405 nm for 4′,6′-diamidino-2-phenylindole and 488 nm for green fluorescent protein with appropriate emission discrimination. Final images were prepared with Image J to show subcellular localization of HER2.

**Alkaline Single-Cell Gel Electrophoresis (Comet)**

Measurement of DNA ICLs was achieved using a modification of the comet assay as previously described (29, 30). Immediately before analysis, cells were irradiated (12.5 Gy) to deliver a fixed number of random DNA strand breaks. Results were expressed as a percentage decrease in tail moment, based on the definition of Olive (31), compared with untreated controls calculated by the following formula:

\[
\% \text{ decrease in tail moment} = \left[1 - \frac{(\text{TMDi} - \text{TMcu})}{(\text{TMcu} - \text{TMci})}\right] \times 100
\]

where TMdi, tail moment of drug-treated irradiated sample; TMcu, tail moment of untreated, unirradiated control; and TMci, tail moment of untreated, irradiated control.

For strand breaks agents, tail moment data were analyzed as a function of time postincubation or drug concentration.

**Cisplatin Intrastrand Crosslink Assay by ELISA**

Exponentially growing cells were treated with cisplatin for 1 h and incubated in drug-free medium for various periods of time. Following incubation, genomic DNA was extracted using a DNeasy blood and tissue extraction kit (Qiagen). The competitive ELISA assay used was developed by Tilby et al. (32, 33). Data obtained were fitted to the logistic equation \([FV = MxCs / (Cs + Ks)]\) as described by Tilby et al. (32), and the 50% inhibition (K value) was determined using GraphPad Prism 4 software.

**Student’s t Test**

This was used for calculating the significance of differences in unhooking crosslinks caused by single agent and combination treatments. Each cell line was considered individually and statistical values of \(P < 0.05\) were considered significant.

**Results**

### Effect of HER2 Inhibition on DNA Repair

The synergy between trastuzumab and DNA-interactive chemotherapeutic agents has been well described, and an effect on DNA repair documented (18). However, the DNA lesions for which repair is modulated by inhibition of HER2 have not been defined. To investigate the effects of HER2 inhibition on DNA repair, we investigated SK-BR-3, MDA-MB-453, and MCF-7 breast cancer cell lines with different HER expression profiles. The level of expression of EGFR, HER2, HER3, and HER4 was determined by Western blotting from total protein lysates of exponentially growing cells. Figure 1 shows that HER2 is expressed at high levels in SK-BR-3 and MDA-MB-453 cell lines, and is detectable in MCF-7 cells. Additionally, EGFR and HER3 are expressed in all cell lines.

Cells were either treated with cisplatin alone or a combination of trastuzumab and cisplatin for 1 hour, incubated in drug-free medium or medium containing trastuzumab, and repair of DNA damage was measured for 72 hours. The comet assay, with modifications to allow the quantitation of ICL formation and repair (29), was used. Results are presented in Fig. 2 for the SK-BR-3 cell line. Similar results were seen with MCF-7 and MDA-MB-453 cell lines (data not shown). In all three cell lines, treatment with trastuzumab did not affect the formation of cisplatin-induced ICLs which peak at 9 hours, as shown previously (23). However, when compared with cells treated with cisplatin alone, trastuzumab caused a clear reduction in efficiency of ICL repair (unhooking) when combined with cisplatin in all cell lines. Maximal inhibition of DNA repair at 24 hours was in SK-BR-3 cells expressing the most HER2 (Fig. 2A), with 52.9 ± 0.6% of peak ICLs present in cells treated with cisplatin alone and 91.1 ± 2.2% in cells treated with a combination of cisplatin and trastuzumab. All three cell lines showed complete repair of ICL after 72 hours. Therefore, reduction in repair of cisplatin-induced ICL was due to the effect of trastuzumab.

Using the t test, results obtained with cisplatin were compared with the results of the drug combination. At a confidence interval of 95%, results were significantly different at 24 and 48 hours (t values of 2.52 and 5.13, respectively). In addition, with a confidence interval of 99%, ICL formation with cisplatin alone was significantly different from the drug combination at 48 hours with a t value of 5.13.

The effect of trastuzumab on the repair of ICL produced by melphalan was also examined in the SK-BR-3 line (Fig. 2B). As with cisplatin-induced ICL, the peak of ICL for melphalan was not modified by trastuzumab. However, in contrast to cisplatin, melphalan-induced ICL were not affected by the presence of trastuzumab. After 24 hours, 67.7 ± 4.2% of peak ICLs remained in cells treated with melphalan alone and 71.2 ± 8.7% in cells cotreated with trastuzumab. Similar results were seen with MCF-7 and MDA-MB-453 cell lines (data not shown).

To examine the effects of trastuzumab on repair of DNA strand breaks secondary to agents targeting topoisomerase II, cells were treated with etoposide alone or in combination...
with trastuzumab. Cells were incubated for 2 hours and medium was replaced by drug-free medium or medium containing trastuzumab only. Repair of strand breaks was observed over 72 hours postincubation. Figure 2C shows the formation and repair of DNA strand breaks. Trastuzumab alone did not alter the level of strand breaks produced by etoposide. DNA strand break repair was detectable within 30 minutes following drug removal and almost complete repair was achieved after 6 hours in all three cell lines. Combining trastuzumab with etoposide did not affect the repair of DNA strand breaks. In SK-BR-3 cells, after 1 hour postincubation, the tail moment of cells treated with etoposide alone was 4.7 ± 0.1 and the combination treatment gave a tail moment of 4.4 ± 0.1. Similar results were seen with MCF-7 and MDA-MB-453 cell lines (data not shown). Therefore, repair of ICL secondary to cisplatin damage is modulated by trastuzumab in these cell lines.

HER2 Expression is Required for Modulation of DNA Repair by Trastuzumab

To examine the effects of HER2 on repair of ICL, the HER2-negative cell line, MDA-MB-468, was stably transfected with either pcDNA3 vector or pcDNA3-ErbB2 to create a cell line expressing HER2. Pooled transfectants were used to minimize interclonal variation. Expression of HER2 was measured by Western blotting and immunofluorescence. Figure 3 shows that HER2 was not expressed in MDA-MB-468 and was only expressed in MDA-MB-468 cells transfected with pcDNA3-ErbB2. Furthermore, transfection did not affect the expression level of the other receptors of the HER family (Fig. 3).

Trastuzumab did not alter the kinetics of repair of cisplatin-induced DNA damage in MDA-MB-468 (Fig. 3B) and MDA-MB-468 pcDNA3 (Fig. 3C) cells not expressing HER2. For example, in the wild-type cell line, after 48 hours, 58.5 ± 3.4% of peak ICLs remained in cells treated with cisplatin alone, and 48.9 ± 6.6% in cells treated with the combined drugs. In contrast, inhibition of HER2 by trastuzumab, in MDA-MB-468 pcDNA3-ErbB2 (Fig. 3D), caused a delay in the repair of cisplatin-induced DNA ICL. After 48 hours, 35.9 ± 11.6% of peak ICLs remained in cells treated with cisplatin alone, whereas 79.2 ± 2.1% was unrepaired in cells treated with cisplatin in combination with trastuzumab. Transfection of HER2 did not affect the level of formation of crosslinks, as 61.7 ± 3.6% of crosslinks were formed after 9 hours posttreatment in MDA-MB-468 wild-type cells and 66.8 ± 2.1% in HER2-overexpressing cells. Cells transfected with the vector showed that 70.4 ± 1.8% of crosslinks formed after 9 hours (data not shown).

Repair of Intrastrand Crosslinks Are Not Affected by the Inhibition of HER2

Although ICLs are critical lesions produced by cisplatin, intrastrand crosslinks are more numerous and likely to contribute to the biological action of the drug. MDA-MB-468 ErbB2-transfected cells were treated with cisplatin alone or with a combination of cisplatin and trastuzumab as shown previously. As shown in Fig. 4, formation of cisplatin-induced intrastrand crosslinks was not affected by HER2 overexpression. In these cell lines, the peak of intrastrand adduct formation was observed immediately after 1 hour of treatment, and was not modified by HER2 overexpression. Furthermore, adducts were rapidly repaired within the first 5 hours posttreatment. After 24 hours, 162.0 ± 8.4 nmol of intrastrand adducts/g of DNA remained in MDA-MB-468...
cells (Fig. 4A) and 160.5 ± 9.7 nmol of intrastrand adducts/g of DNA was present in cells overexpressing HER2 (Fig. 4B). Furthermore, inhibition of HER2 by trastuzumab did not affect the kinetics of DNA intrastrand crosslink formation or repair. In cells overexpressing HER2, after 9 hours, 222.3 ± 9.1 nmol of intrastrand adducts/g of DNA was present in cells treated with cisplatin alone and 238.5 ± 9.0 nmol of intrastrand adducts/g of DNA in cells treated with a combination of cisplatin and trastuzumab. Therefore, repair of intrastrand crosslinks was not altered by modulation (overexpression or inhibition) of HER2.

**HER2 Nuclear Expression and Modulation of DNA Repair**

There is evidence that HER2 translocates to the nucleus and can activate gene expression (11). To investigate the effects of nuclear expression of HER2, the HER2-negative cell line, MDA-MB-468, was stably transfected with three plasmids (MDA-MB-468 vector control, MDA-MB-468 ErbB2, and MDA-MB-468 ErbB2 ΔNLS). The MDA-MB-468 ErbB2 ΔNLS construct contains a deletion of the putative NLS (8). The functionality of this construct has been shown by its ability to transduce the HER2 mitogen-activated protein kinase pathway and to activate the Elk1 target promoter (8). There was transient phosphorylation of both the wild-type and mutant HER2 following trastuzumab treatment, also indicating functionality (data not shown). Figure 5A shows that HER2 is overexpressed in MDA-MB-468 ErbB2 cells and in MDA-MB-468 ErbB2 ΔNLS cells. Expression of other EGF receptors is not affected by transfection. HER2 is not present in the nucleus

![Figure 3](image1.png)

*Figure 3.* Effects of HER2 transfection on the repair of cisplatin-induced ICL. **A,** Western blotting of MDA-MB-468, MDA-MB-468 pcDNA3 (vector control), and MDA-MB-468 pcDNA3-ErbB2 lysates probed with anti-EGFR, anti-HER2, anti-HER3, and anti-HER4 antibodies. Measurement of cisplatin-induced DNA ICL in MDA-MB-468 (B), MDA-MB-468 pcDNA3 vector control cells (C), and MDA-MB-468 pcDNA3-ErbB2 cells (D) treated with 40 μg/mL of trastuzumab alone (■), 100 μmol/L of cisplatin alone (◊), or 40 μg/mL of trastuzumab in combination with 100 μmol/L of cisplatin (▲).

![Figure 4](image2.png)

*Figure 4.* Measurement of cisplatin-induced DNA intrastrand crosslinks in MDA-MB-468 wild-type cells (A) treated with 100 μmol/L of cisplatin alone (◊) or 100 μmol/L of cisplatin with 40 μg/mL of trastuzumab (■), and MDA-MB-468 pcDNA3-ErbB2 cells (B) treated with 100 μmol/L of cisplatin alone (◊) or 100 μmol/L of cisplatin with 40 μg/mL of trastuzumab (▲).
of MDA-MB-468 ErbB2 ΔNLS cells (Fig. 5B) but is present in both the nucleus and the cytoplasm of MDA-MB-468 ErbB2 cells (Fig. 5B). As previously found (8), the nuclear HER2 does not account for the majority of expressed HER2.

To investigate the significance of nuclear translocation on modulation of DNA repair, MDA-MB-468 wild-type, MDA-MB-468 pEGFP-N1 vector control, MDA-MB-468 ErbB2, and MDA-MB-468 ErbB2 ΔNLS were treated with cisplatin for 1 hour followed by incubation in drug-free medium. To investigate the effects of inhibiting nuclear import on DNA repair, the lectin WGA was used. Cells were incubated with WGA and trastuzumab in combination with cisplatin. Figure 6A shows that transfection of the empty vector did not affect the kinetics of cisplatin-induced DNA ICL formation and repair. As HER2 is not present in the wild-type and vector control cell line, trastuzumab did not alter the kinetics of DNA repair. Additionally, generalized inhibition of nuclear translocation by WGA did not affect the kinetics of DNA repair.

However, HER2 overexpression caused an increase in ICL repair efficiency (Fig. 6B), with 61.3 ± 0.7% of peak ICLs still present after 24 hours compared with the parental cells which had 91.9 ± 0.6% of peak ICLs remaining after 24 hours. Inhibition of HER2 by trastuzumab delayed repair of cisplatin-induced ICL with 100.6 ± 0.1% of peak ICLs

**Figure 5.** A, Western blotting of MDA-MB-468 wild-type (1), pEGFP-N1 vector control (2), pEGFP-N1 ErbB2 (3), and pEGFP-N1 ErbB2 ΔNLS (4) untreated cell lysates probed with anti-EGFR, anti-HER2, anti-HER3, and anti-HER4. B, Western blotting of MDA-MB-468 pEGFP-N1 ErbB2 and pEGFP-N1 ErbB2 ΔNLS nuclear (N) and cytoplasmic (C) cell lysates probed with anti-HER2. Calnexin was used as a loading control for the nuclear fraction and lamin as a loading control for the cytoplasmic fraction.

**Figure 6.** Effects of cisplatin on ErbB2 and ErbB2 ΔNLS-transfected cells. Measurement of cisplatin-induced DNA ICL in MDA-MB-468 pEGFP-N1 vector control (A), MDA-MB-468 pEGFP-N1 ErbB2 (B), and MDA-MB-468 pEGFP-N1 ErbB2 ΔNLS (C) cells treated with 100 μmol/L of cisplatin alone (○), 100 μmol/L of cisplatin with 40 μg/mL of trastuzumab (+), 50 μg/mL of WGA and 100 μmol/L of cisplatin (×), or 50 μg/mL of WGA with 40 μg/mL of trastuzumab and 100 μmol/L cisplatin (•). ICL formation is represented as a percentage of decrease in tail moment. D, confocal microscopy of vector, ErbB2, and ErbB2 ΔNLS-transfected cells following treatment with trastuzumab and cisplatin.
remaining after 24 hours. The effect of trastuzumab is comparable to that following treatment with WGA, which resulted in 100.8 ± 4.1% of peak of ICLs remaining after 24 hours.

Mutation of the ErbB2 NLS sequence caused a delay in ICL repair (Fig. 6C) because after 24 hours 97.9 ± 6.0% of peak ICLs is unrepaired, comparable to 91.9 ± 0.6% in the parental cells and 61.3 ± 0.7% in cells overexpressing HER2. Trastuzumab and WGA did not change the kinetics of ICL formation and repair in MDA-MB-468 ErbB2 ΔNLS cells. After 24 hours, 97.9 ± 6.0% of the peak of ICLs remained in cells treated with cisplatin alone and 99.8 ± 0.2% in cells treated with a combination of cisplatin and trastuzumab. Using small interfering RNA to HER2 in the stable HER2 MDA-MB-468 transfectants, we were able to obtain almost complete knockdown of HER2. This resulted in complete inhibition of ICL unhooking at 48 hours (as previously shown following trastuzumab treatment) in cells expressing HER2, as compared with 43% of ICL unhooking using a scrambled small interfering RNA (data not shown).

To determine the effects of cisplatin and trastuzumab treatment on nuclear expression of HER2, confocal microscopy was done following drug treatment. Treatment with cisplatin increased the expression of HER2 in cells transfected with HER2 but not in cells transfected with the ErbB2 ΔNLS plasmid (Fig. 6D).

Discussion
There has been extensive interest in the development of inhibitors of receptor tyrosine kinases as a strategy for cancer therapy. Inhibition of HER2/ErbB2 by trastuzumab has been one of the most effective interventions, with single-agent trastuzumab significantly reducing the risk of recurrence as adjuvant therapy in HER positive breast cancer (34). Additionally, trastuzumab has shown activity in combination with chemotherapeutic agents. In patients with metastatic breast cancer, the addition of trastuzumab with chemotherapy (epirubicin and cyclophosphamide) resulted in a significant improvement in response rates and overall survival (35). Although doxorubicin is most frequently used in combination with trastuzumab, there is preclinical evidence that platinum salts are synergistic with trastuzumab (14). However, the mechanism by which combinations of trastuzumab and chemotherapy synergize has not been fully investigated. An important study indicated that unscheduled DNA synthesis was inhibited by treatment of cells with trastuzumab in combination with cisplatin and doxorubicin (36). The removal of specific platinum adducts from DNA was reduced by trastuzumab treatment. It is important to understand the mechanisms by which HER2 inhibition modulates the effects of chemotherapeutic agents. This will allow for the design of clinical studies optimizing these combinations in breast cancer, in which trastuzumab is well-validated therapeutically, and in other cancers, in which HER2 inhibition is promising.

Experiments with EGFR inhibitors have also shown that modulation with both antibodies and small molecules modulate the repair of DNA damage produced by chemotherapy and radiation. Gefitinib, a quinazoline inhibitor of EGFR, reduced the repair of DNA ICLs and DNA strand breaks following cisplatin and etoposide treatment (23). Inhibition of DNA-dependent protein kinase has been shown following treatment with gefitinib (37). Other studies have implicated the inhibition of rad51 as a factor in modulation of DNA damage (38).

The modification of the single-cell gel electrophoresis (comet) assay has been fully validated for the detection of ICLs, including those produced by platinum-based agents (29, 39). It is now increasingly used to monitor ICL formation and unhooking in clinical samples (30, 40) as a pharmacodynamic end point in clinical trials (41, 42), and following ex vivo treatment (39, 43).

In this study, we measured the effects of HER2 inhibition on three types of DNA lesions produced by treatment with chemotherapeutic agents—ICLs, intrastrand crosslinks, and DNA strand breaks. Our results show that the major effect on DNA repair following trastuzumab treatment was found with the inhibition of ICLs following cisplatin treatment. No effect was found on the repair of intrastrand crosslinks. It is well established that ICLs are a highly cytotoxic lesion and contribute to cisplatin cytotoxicity, and that repair (unhooking) of ICL may be an important determinant of clinical sensitivity (39). Our data clearly shows that the processing of these lesions is altered by trastuzumab treatment. Recent evidence suggests that different mechanisms exist for the unhooking of melphalan and cisplatin-induced ICL, both in vitro and in tumor samples in which resistance is acquired (44). For example, ovarian cancer cells from patients resistant to cisplatin are able to unhook cisplatin-induced ICL but not those produced by melphalan. Conversely, plasma cells from myeloma cells resistant to melphalan are able to unhook melphalan-induced ICL, but not those produced by cisplatin. The differing response to cisplatin and melphalan-induced ICL shown in the current study was also found with the inhibition of EGFR by gefitinib, which inhibited the repair of cisplatin, but not melphalan-induced ICL (23). Results following trastuzumab treatment with etoposide differ from those found with the inhibition of EGFR, in which a marked delay in unhooking occurred following treatment with gefitinib (23).

The reasons for the synergy of platinum drugs and trastuzumab may include effects on DNA damage and repair, as shown here, but other important mechanisms including glutathione transferase expression and apoptotic markers such as bcl2 might also be involved. We are not postulating that the sole reason for synergy is ICL repair inhibition by trastuzumab, only that a significant effect on the repair of ICL occurs with trastuzumab. To determine the precise contribution for ICL in the synergy would require using agents whose mode of action is through the production of ICL. Novel agents currently under development, such as the pyrrolobenzodiazepine S1G136, which acts predominantly through the formation of covalent ICL, will be candidates for further study (45).

The importance of HER2 in the modulation of response to DNA damage was confirmed by experiments in cells...
transfected with HER2, whereby the inhibition of ICL repair by trastuzumab was only shown in cells expressing HER2. Similarly, a study on MCF-7 breast cancer cells engineered to overexpress heregulin with resultant HER2 activation showed resistance to cisplatin, which was reversed with trastuzumab treatment (46).

The mechanism of repair of ICLs in metazoans is unclear and processes including nucleotide excision repair, translesion synthesis, and homologous recombination have been implicated. Although nucleotide excision repair has been implicated in the repair of cisplatin, and increased ERCC1 expression is found in cisplatin-resistant tumors, there was no alteration of intrastrand crosslink repair following trastuzumab treatment in this study. Additionally, the absence of any alteration in the kinetics of repair of strand breaks following etoposide treatment, which requires homologous recombination, suggests that this pathway is not involved following trastuzumab treatment. There is evidence that HER2 may interact with the promoter of the FANCC gene (11) and this might modulate responses to DNA lesions as found in our study. Interestingly, expression of FANCC has been shown to correlate with cellular response to cisplatin treatment but not to etoposide (47). However, we did not find that there was any alteration in expression of FANCC mRNA or protein in cells transfected with vector as compared with cells transfected with ErbB2 wild-type or ErbB2 ΔNLS (data not shown). We are currently carrying out a chromatin immunoprecipitation sequencing approach to identify which genes are implicated in nuclear HER2 binding.

The recent identification of HER2 expression in the nucleus and its role in transcriptional regulation of COX-2 expression suggests additional roles apart from those identified as a result of activation at the cell membrane. There is evidence that full-length HER2 is expressed in the nucleus, although some studies have suggested that COOH-terminal fragments rather than full-length transcripts are translocated (48). We have found that inhibition of nuclear import using wheat germ agglutinin abrogated the effect of EGFR inhibitors on DNA repair. Therefore, we investigated the effects of blocking HER2 nuclear expression by using a construct lacking the putative NLS. In these experiments, the HER2-expressing cells showed no effect of trastuzumab treatment and indeed were less efficient at repairing ICL than the vector-expressing controls. This requires further investigation but could be due to a possible dominant-negative effect by dimerization of the ErbB2 NLS mutant with EGFR which also plays a role in ICL repair. However, the key point is that nuclear localization of HER2 is required for the effects on DNA repair.

Understanding the mechanisms of interaction of HER2 inhibition and DNA repair is critical in the design of novel strategies to combine chemotherapy with HER2 inhibition. There have been extensive investigations of the effects of HER2 inhibition by trastuzumab in increasing apoptosis by a variety of mechanisms including activation of antibody responses and reduced expression of antiapoptotic genes (49, 50). Our results suggest that in addition to these effects, the inhibition of HER2 by trastuzumab markedly affects DNA ICL repair and this suggests strategies for future combinatorial regimens. These strategies will be important in assessing novel therapies targeting these pathways such as lapatinib and pertuzumab.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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Involvement of the HER2 pathway in repair of DNA damage produced by chemotherapeutic agents

Julien J.M. Boone, Jaishree Bhosle, Mike J. Tilby, et al.


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