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Prelinical report

Evidence for epidermal growth factor receptor-enhanced chemosensitivity in combinations of cisplatin and the new irreversible tyrosine kinase inhibitor CI-1033

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Irreversible inhibitors of the epidermal growth factor receptor (EGFR) are showing promise in clinical trials. This report is the first to show that inhibition of the EGFR tyrosine kinase by an irreversible binder synergizes with cisplatin, at least in EGFR-overexpressing tissue culture cell lines *in vitro*. Unlike previous synergies demonstrated between ErbB2 blockade and DNA-damaging drugs, the synergy between the irreversible EGFR inhibitor and cisplatin does not appear to involve the repair of DNA–cisplatin adducts. Given the current clinical data, this combination may be of more than theoretical interest. [© 2001 Lippincott Williams & Wilkins.]

Key words: CI-1033, cisplatin, DNA repair, epidermal growth factor receptor inhibitor, synergy.

Introduction

Overexpression of members of the epidermal growth factor receptor (EGFR) family of trans-membrane growth factor receptor tyrosine kinases has been identified as an important contributor to tumor malignancy and progression.[1–3] These enzymes (EGFR, ErbB2, ErbB3 and ErbB4) have therefore become the focus of a large drug discovery effort. Both monoclonal antibodies to the extracellular domains,[4] and small-molecule inhibitors to the tyrosine kinase domains[5] have been developed, to block the intracellular signaling cascade resulting from the receptor and inhibit cell growth.

The combination of both anti-EGFR (e.g. C225) and anti-ErbB2 (e.g. trastuzumab) antibodies with cytotoxic anticancer drugs shows promise both in animal models and in an increasing range of clinical trials against solid tumors that overexpress the target receptor. Combinations of C225 and cisplatin or topotecan showed superior results as compared with either drug alone in mouse xenograft models.[6] Phase I trials of C225 either as a single agent or in combination with cisplatin (the latter in head and neck or non-small cell lung cancer) resulted in disease stabilization and (in the combination study) two out of 13 partial responses.[7] The anti-ErbB2 antibody trastuzumab is also clinically active in breast cancer as a single agent,[8] but shows higher response rates (24%) in combination with cisplatin than the single agents alone (7% for cisplatin and 11% for trastuzamab).[9] More extensive studies with trastuzumab have shown its activity is also potentiated by a range of other cytotoxic agents, including carboplatin, doxorubicin and paclitaxel.[8,10,11]

An increase in cell killing resulting from the addition of a cytotoxic drug in combination with receptor blockade is described as receptor-enhanced chemosensitivity.[12] Several reports have demonstrated a possible mechanism for this observed synergy between ErbB2 blockade with monoclonal antibodies and treatment with DNA-damaging agents.[12–15] Arteaga *et al.*[13] used the ErbB2 antibody Tab 250 in combination with cisplatin to show the repair of cisplatin–DNA adducts was inhibited. Cisplatin–DNA adducts were detected with a polyclonal antiserum raised against DNA modified with cisplatin. Pietras *et al.*[12] used unscheduled DNA synthesis and atomic absorption spectrometry to show that a different ErbB2 antibody, 4D5, would inhibit the repair of cisplatin–DNA adducts. Other approaches have further confirmed this result.[14,15]

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It has also been shown that the inhibition of cisplatin–DNA repair is dependent on the ErbB2 antibody being administered within 24 h of the cisplatin adducts.[15]

Small-molecule inhibitors of the intracellular kinase site of EGFR have similar effects as monoclonal inhibitors directed towards members of the EGFR family. The anilinoquinazoline Iressa, a reversible inhibitor at the ATP site of EGFR, shows activity as a single agent in early clinical trials.[16] This activity is also potentiated both *in vitro* and *in vivo* by low doses of a variety of cytotoxic drugs, including cisplatin,[17] possibly by activating the receptor.[18,19] The related irreversible inhibitor CI-1033 is also an anilinoquinazoline that binds at the ATP site of EGFR,[20] but carries an additional acrylamide unit that alkylates a cysteine 773 at the mouth of the ATP binding pocket, thus irreversibly and specifically inactivating the EGFR.[21] It prevents MAP kinase activation, inhibits c-*fos* expression and produces growth suppression *in vivo* of advanced A431 tumor xenografts,[22] and is showing encouraging results in phase I clinical trials.[23]

The purpose of the present study was to investigate the involvement of CI-1033 in receptor-enhanced chemosensitivity with the DNA alkylator cisplatin. We hypothesized that the combination of cisplatin and CI-1033 would give synergistic growth inhibition of the EGFR-over-expressing human squamous carcinoma cell line A431 *in vitro*, and that CI-1033 would inhibit the repair of cisplatin–DNA adducts. The synergy of drug combinations was analyzed by the median effect principle and the combination index of Chou and Talalay.[24] To determine whether CI-1033 inhibited the repair of cisplatin–DNA adducts, the host cell reactivation (HCR) assay was employed. This measures the *in vivo* restoration of biological activity to *in vitro*-damaged DNA.[25] In addition to A431, two further cell lines were investigated; the mouse fibroblast NIH-3T3 which does not overexpress EGFR and the NIH-EGFR line derived by transfection of NIH-3T3 with an overexpression vector containing EGFR.[26]

Materials and methods

Drugs

CI-1033 was available in this laboratory from previous work[20] and was employed as the free base.

Cell culture

The cell lines used in this study were the mouse fibroblast lines NIH3T3 and NIH-EGFR (provided by Pfizer Global Research and Development, Ann Arbor, MI) and the human squamous cell line A431. Cells were grown in alpha minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (Life Technologies/Gibco, Gaithersburg, MD) and 100 U/ml of penicillin and 100 μg/ml of streptomycin. They were passaged in 25-cm^2 tissue culture flasks in an atmosphere of 5% CO_2 in air and were subcultured weekly, using 0.1% trypsin (Difco, Franklin Lakes, NJ) in citrate saline (trisodium citrate dihydrate, 4.4 g/l, KCl, 10 g/l; pH 7.3).

Inhibition of cell growth

Sulfurhodamine B (SRB) assay[27] was used to measure inhibition of cell proliferation following drug addition. Briefly, cells were plated into 96-well plates (Nunclon, Rochester, NY) at 400 cells/well in 150 μl medium. Cells were allowed to adhere for 24 h before drug addition. Drugs were serial diluted across the plate in quadruplicate and cells were incubated with the drugs for 4 days. The cells were fixed with 75 μl of prechilled 30% (v/v) trichloroacetic acid and incubated for 30 min at 4°C. Plates were vigorously washed in two changes of deionized water, air dried and stained with 100 μl of 0.4% (w/v) SRB in 1% acetic acid for 30 min at room temperature. The unbound dye was flicked off and plates washed 4 times in a 1% acetic acid solution. After air drying, the bound SRB was solubilized with 10 mM Tris. Absorbance was measured at 570 nm using a Dynatech (Chantilly, VA) dual-wavelength spectrophotometer with a reference wavelength of 410 nm.

For drug combinations, drugs were added together to cells at the same concentration as used in single-agent experiments, either at the same time or 8 h after the first drug. The multiple drug effect analysis of Chou and Talalay,[24] which is based on the median-effect principle, was used to analyze the significance of combined drug effects. The computer program Calcusyn (Biosoft, Ferguson, MO) was used to calculate drug synergy from the drug combinations.

Host cell reactivation assay

The plasmids pGL2 (Promega, Madison, WI) and pCMVSPORT-βgal (Life Technologies) were isolated from *Escherichia coli* strain JM109 using Qiagen (Valencia, CA) plasmid purification kits. Plasmid DNA was damaged according to the protocol of Chou *et al.*[28] pGL2 was treated in the dark with 0.25 μM cisplatin in 3 mM NaCl and 1 mM sodium phosphate (pH 7.4) at 37°C for 18 h. The DNA concentration was 100 μg/ml (3×10^{-4} nucleotide phosphate). After drug treatment, the DNA was ethanol precipitated by

adding 3 M sodium acetate (pH 5.2) (0.1 v/v) and ethanol (2.5 v/v), and centrifuging at 15 000 *g* for 30 min. The DNA was washed once with 70% ethanol, centrifuged at 15 000 *g* for 15 min and then resuspended in 10 mM Tris–HCl, pH 8/1 mM EDTA. The DNA concentration was determined by fluorescence using 1 μg/ml Hoescht 33258 (Sigma, St Louis, MO) dissolved in TNE buffer (10 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4) and a Hitachi F-2000 fluorescence spectrophotometer.

Cells were plated out 1 day prior to transfection at 1.5×10^5 for NIH3T3 and NIH3T3-EGFR, and 2×10^5 for A431 in 3-cm diameter plates. Cells were transfected with 1 μg of each plasmid per plate using LipofectAMINE PLUS reagent (Life Techologies) as per the manufacturer's instructions. After 5 h, the transfection medium was replaced and the cells were incubated in fresh media for 24 h. Following this, the medium was aspirated and the cells washed twice with 1 ml PBS. Cells were lysed by the addition of 400 μl of 1 × reporter lysis buffer (Promega) and the cells scraped off using an inverted pipette tip. The cell lysate was collected and underwent one freeze/thaw cycle and centrifuged at 12 000 *g* for 5 min at 4°C. The pellet was discarded and the supernatant retained for luciferase assay as per manufacturer's instructions (Promega) using a Wallac (Turku, Finland) Trilux luminometer. β-galactosidase activity was determined by addition of *o*-nitrophenyl-β-D-galactopyranoside (Sigma) at 1 mg/ml in phosphate buffered saline and 0.36% (v/v) 2-mercaptoethanol, incubated at 37°C for 15 min and the abosrbance at 415 nm measured (model 3550 microplate reader; BioRad, Hercules, CA).[29] The protein concentration was assayed using the BCA protein assay. Cell extracts (50 μl) were incubated with 100 μl of BCA reagent (Sigma) for 30 min in a 96-well plate. Absorbance was measured on a dual wavelength spectrophotometer at 570 nm. Protein concentration was determined from a BSA standard curve. Relative luciferase activity was determined as: [luciferase activity/50 μg protein]/[β-galactosidase activity/50 μg protein].

Results

Inhibition of cell growth

To investigate whether cisplatin and CI-1033 produced synergistic growth inhibition, the EGFR-overexpressing cell line A431 was grown with cisplatin, CI-1033 or both for 4 days, and the inhibition of cell growth measured using the SRB assay (an example is presented in Figure 1). The median-effect principle was employed as a rigorous method of identifying drug synergy. The growth inhibition values were analyzed with the median effect plot to determine potency (*Dm*), shape (*m*) and conformity (*r*) of the dose–response curves (an example is presented in Figure 2). The pooled results for all experiments are shown in Table 1. Both compounds were found to be potent cytotoxic agents with IC_{50} values of approximately 600 nM for cisplatin and 80 nM for CI-1033.

The *Dm* and *m* values for single drugs and for their combination mixtures were used for calculating synergism or antagonism from the combination index (CI) equation. From this equation synergy is defined as $CI < 1$, additivity as $CI = 1$ and antagonism as $CI > 1$. The CI values were calculated at drug doses that gave equivalent growth inhibition of 50, 75 and 90% (ED_{50}, ED_{75} and ED_{90}, respectively).

When both drugs were added to A431 cells at the same time, synergy was observed at the ED_{90} and ED_{75}

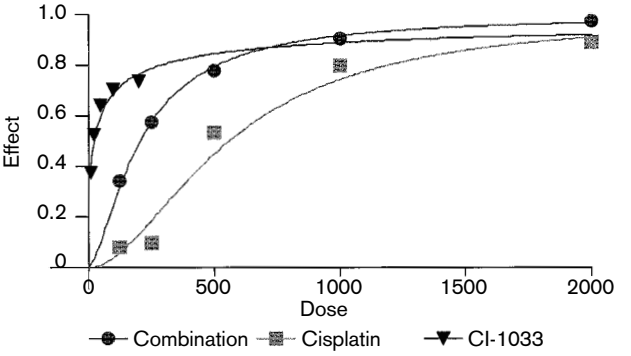

Figure 1. An example of the dose–response curves for the cell line A431 when treated with cisplatin, CI-1033 or both. Effect (*y*-axis) is defined as the percentage of cells inhibited (f_a)/percentage of cell not inhibited (f_u).

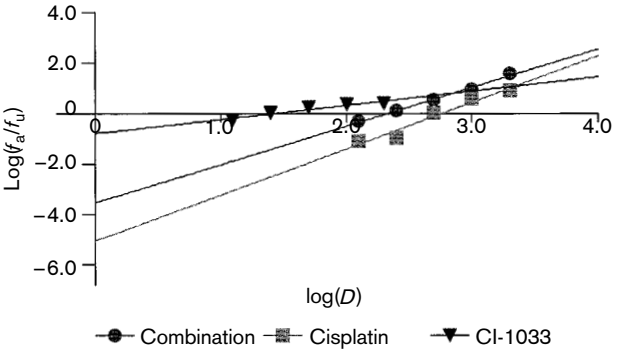

Figure 2. An example of the median-effect plot for the cell line A431 when treated with cisplatin, CI-1033 or both. The parameters *Dm*, *m* and *r* (the slope, antilog of the *x*-intercept and the linear coefficient of the median-effect plot, respectively) are calculated from the lines shown. d=Dose.

Table 1. Dose–effect relationship parameters of cisplatin and CI-1033 on the growth of A431 cells *in vitro*

	Dm	*m*	*r*	CI		
				ED_{50}	ED_{75}	ED_{90}
Simultaneous drug administration						
cisplatin	663±69	1.696±0.217	0.984±0.006	–	–	–
CI-1033	83.7±6.9	0.887±0.078	0.969±0.009	–	–	–
combination	395±26	1.519±0.101	0.995±0.002	1.083±0.085	0.862±0.075	0.759±0.082
Drug administration 8 h apart						
cisplatin (0 h)	553±45	1.696±0.085	0.996±0.001	–	–	–
CI-1033 (8 h)	93.2±11	0.961±0.144	0.980±0.006	–	–	–
combination	359±54	1.803±0.209	0.994±0.001	1.027±0.099	0.841±0.069	0.741±0.051
cisplatin (8 h)	615±104	1.616±0.184	0.995±0.001	–	–	–
CI-1033 (0 h)	80.5±13	1.036±0.087	0.976±0.010	–	–	–
combination	419±65	1.673±0.017	0.996±0.001	1.209±0.036	1.008±0.027	0.878±0.056

The parameters *Dm*, *m* and *r* are the slope, antilog of *x*-intercept and the linear coefficient of the median-effect plot, respectively. These signify the shape of the dose–effect curve, the potency (IC_{50}) and the conformity of the data to the mass-action law, respectively. Values are means±SEM calculated from seven independent experiments. CI>1=antagonism, CI<1=synergy, CI=1=additivity.

doses), while additivity was observed at the ED_{50} (Table 1). The timing of the drug additions influenced the degree of synergy (Table 2). When the addition of CI-1033 was delayed until 8 h after the addition of cisplatin, the observed degree of synergy was unchanged at all doses compared with the drugs being added together. However, if cisplatin addition was delayed by 8 h there was a decrease in the observed synergy, which was only observed at the ED_{90} dose, while ED_{75} and ED_{50} doses became additive and antagonistic, respectively.

Repair of cisplatin adducts

The HCR assay was employed to investigate whether the repair of DNA–cisplatin adducts was inhibited by CI-1033. The HCR assay measures the *in vivo* restoration of biological activity to *in vitro*-damaged DNA. By exposing naked DNA (as plasmid) to the DNA-damaging agent, the physiology of the cell is not itself perturbed by the agent and transfection becomes solely dependent upon the host cell's capacity to process damaged DNA.[25] Three different cell lines were investigated: the human squamous cell line A431 that overexpresses EGFR, the mouse fibroblast NIH-3T3 which does not overexpress EGFR and NIH-EGFR which was derived from transfection of NIH-3T3 with an overexpression vector containing *EGFR*.[26] These cell lines were chosen to investigate whether there was any difference between lines that required EGFR overexpression for growth and division (A431), and those that had artificial overexpression (NIH-EGFR).

A plasmid (pGL2) containing the *luciferase* reporter gene driven by the *SV40* promoter was used along with a second plasmid containing the *β-galactosidase* gene. In the experimental system the luciferase plasmid (pGL2) was damaged with 0.25 μM cisplatin before transfection of the cell line and subsequent reporter gene expression assays. A second plasmid containing the *β-galactosidase* reporter gene was not damaged and was co-transfected with the luciferase plasmid to act as a control for transfection efficiency. A concentration of 0.25 μM cisplatin was found to inhibit luciferase activity to around 20–40% of the control value 24 h after cell transfection (data not shown). The cisplatin–DNA adducts inhibited transcription of the luciferase gene and reduced luciferase protein expression, and hence luciferase activity. The repair of the cisplatin–DNA adducts can be measured as an increase in luciferase activity over time compared to undamaged luciferase control. Factors that affect the repair and removal of the cisplatin–DNA adducts will change the luciferase activity due to changes in the transcription of the damaged reporter gene. Following cell line transfection, CI-1033 was added at different concentrations depending on the cell line used.

When the A431 was transfected with the cisplatin-damaged plasmid the luciferase activity dropped to 40% of the undamaged control (Figure 3A). The addition of the inhibitor CI-1033 reduced luciferase activity of both the undamaged control and the cisplatin-damaged plasmid at all concentrations tested. However, it did not considerably change the luciferase activity of the cisplatin-damaged plasmid when normalized to the control, which remained constant at around 40% (Figure 3B).

When the mouse fibroblast cell line NIH-3T3 was transfected with cisplatin-damaged luciferase plasmid the luciferase activity dropped to approximately 25% of the undamaged luciferase control (Figure 4A). The addition of the EGFR inhibitor CI-1033 did not

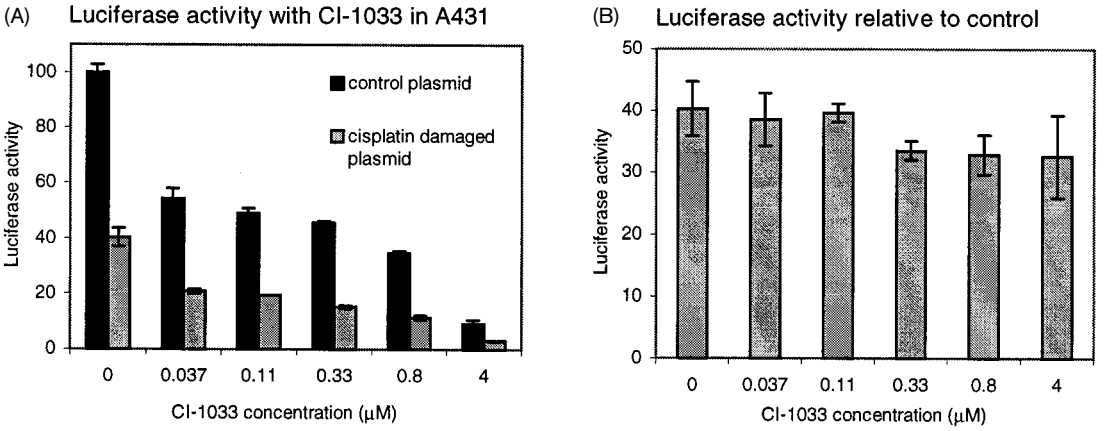

Figure 3. Host cell reactivation assay results from the cell line A431. Luciferase activity was measured 24 h after cell lines were transfected with either an undamaged control plasmid or a cisplatin-damaged plasmid and treated with increasing amounts of CI-1033. (A) Luciferase activity from both the control plasmid and the cisplatin-damaged plasmid. (B) Relative luciferase activity calculated by dividing the luciferase activity of the damaged plasmid by the luciferase activity of the control plasmid for each CI-1033 concentration.

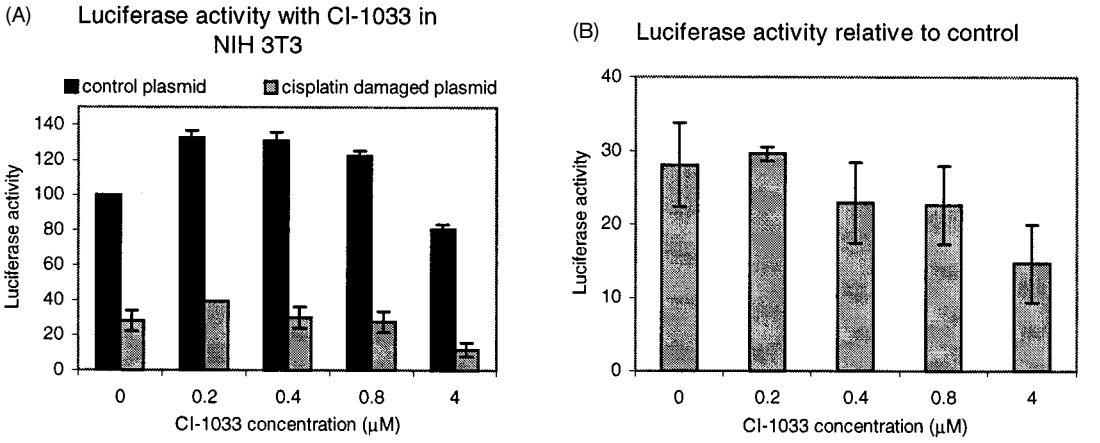

Figure 4. Host cell reactivation results for the cell line NIH-3T3. See legend to Figure 3 for details.

substantially reduce the luciferase activity of the damaged plasmid until the concentration reached 4 μM (Figure 4B). Surprisingly at CI-1033 concentrations of 0.2–0.8 μM there was a consistent elevation of control luciferase activity by approximately 30% which was unrelated to DNA repair (Figure 4A). It is unknown how CI-1033 induced this increase in luciferase activity.

Transfection of the mouse fibroblast cell line NIH-EGFR, which overexpressed the EGF receptor, gave a similar result (Figure 5A). The luciferase activity dropped to 20% of the undamaged control and there was no substantial change in activity from the addition of CI-1033 until the drug concentration reached 4 μM (Figure 5B). However, no increase in the control luciferase activity with lower concentrations of CI-1033 was observed.

Discussion

The results presented in this study showed a synergistic response when the EGFR-overexpressing cell line A431 was treated with CI-1033 and cisplatin in combination. The degree of synergy observed is affected by the order of drug exposure. Addition of the cytotoxin before or at the same time as receptor inhibition results in greater synergy compared to receptor inhibition followed by the addition of the cytotoxin. This result is in contrast to that observed

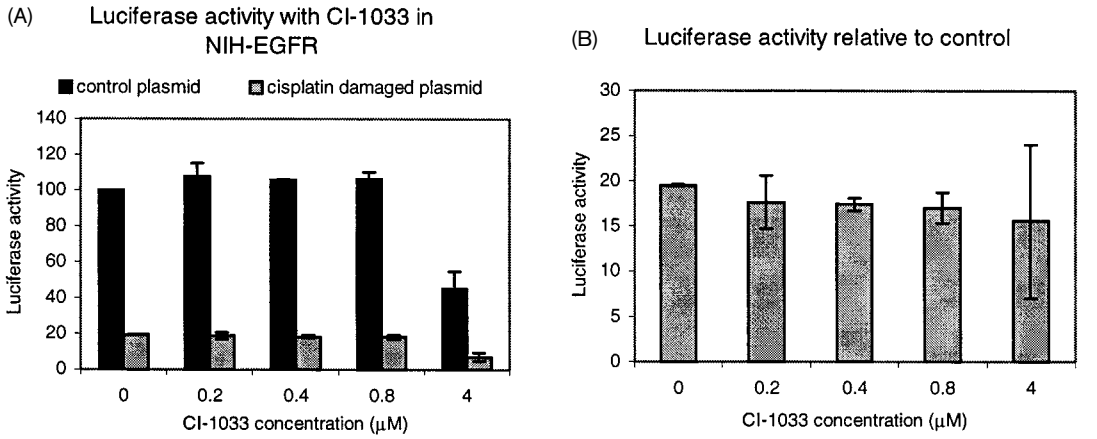

Figure 5. Host cell reactivation results for the cell line NIH-EGFR. See legend to Figure 3 for details.

with ErbB2 inhibitors and cytotoxic drugs. Optimal *in vivo* antitumor effect against MCF-7 mouse xenografts was found to occur when the ErbB2 inhibitor rhuMAb HER-2 was given shortly before or simultaneously with cisplatin.[15]

Such receptor-enhanced chemosensitivity has been previously described with combinations of cytotoxic drugs and inhibitors of function of the EGFR family. Thus the monoclonal antibody Mab 225, which is an antagonist of EGF, also shows additive cytotoxicity in combination with cisplatin in A431 cells and the combination also had enhanced activity against A431 xenografts in nude mice.[30] Combinations of the anti-ErbB2 antibodies traztuzumab[9] and rhuMAb HER2[31] also show synergistic cytoxicity in cell lines. Preliminary reports[17–19] have appeared of a similar effect with combinations of Iressa (a small molecule reversible ATP site inhibitor of EGFR) and cisplatin.

The question of whether CI-1033 would inhibit the repair of cisplatin–DNA adducts in a variety of EGFR-overexpressing cell lines was studied using the HCR assay. In all the lines tested, EGFR inhibition by CI-1033 appeared to have no effect on the repair of cisplatin–DNA adducts. Furthermore, there was no difference in DNA repair, regardless of whether the EGFR overexpression was artificially induced (NIH-EGFR cells) or was required for growth (A431 cells). This indicates that the synergistic mechanism operating between CI-1033 and cisplatin cannot be the inhibition of DNA repair of cisplatin–DNA adducts. This result is in contrast to work performed on ErbB2, where receptor blockade has been shown to inhibit DNA repair,[12–15] and suggests that EGFR and ErbB2 have significantly different signal pathways, that may contribute to tumor growth in different ways.

However, it should be noted that CI-1033 did modify the luciferase activity in a manner that was unrelated to DNA repair. Both increases (NIH 3T3) and decreases (A431 cell line) in luciferase control activity were observed. It is unknown how CI-1033 induced these changes or why these changes were so different depending on the cell line investigated.

The substantial homology between EGFR and ErbB2, and the almost simultaneous development of inhibitors to both, has encouraged comparisons to be made between the responses resulting from blockade of the two receptors. However, the results presented here suggest that such comparisons and the inferences that can be made from them may not necessarily be valid. If blockade of the EGFR does not inhibit DNA repair, what other mechanisms could explain the observed drug synergy between CI-1033 and cisplatin? There is now considerable evidence that DNA alkylating agents modulate gene expression both directly by inhibiting transcription, and indirectly by modifying signaling pathways and inducing the cytotoxic stress response. Cisplatin alkylates DNA primarily at G-N^7, forming both intra- and inter-DNA crosslinks[32] and has been shown to modulate gene expression in a number of different ways. Cisplatin adducts strongly sequester both TBP/TFIID[33] and hUBF,[34] and in reconstituted systems these effects were shown to inhibit transcription.

Cisplatin adducts also inhibit chromatin remodeling of the mouse mammary tumor virus promoter and block transcription factor NF1 from binding, resulting in transcription inhibition.[35] Furthermore, Evans and Gralla[36] found differential effects on gene expression by cisplatin; cell lines transfected with eight different promoters upstream of the CAT reporter gene showed different sensitivities to cisplatin with a correlation

between a strong promoter and greater sensitivity. Cisplatin treatment is also known to affect cell signaling, with increased expression of c-*jun* demonstrated, along with an increase in protein kinase C activity.[37] Furthermore, Sánchez-Pérez and Perona[38] have shown that cisplatin treatment of cell lines leads to activation of the JNK signaling pathway and activation of the AP-1 transcription factor complex.

Conclusions

These data clearly suggest that cisplatin can significantly modulate gene expression. We therefore hypothesize that cisplatin leads to the inhibition of key genes required for cell survival if the EGFR signaling pathway has been blocked. The result is a synergistic growth inhibition when CI-1033 and cisplatin are combined. Further evidence for this hypothesis is provided by the work of Koumenis and Giaccia.[39] They showed that the selective inhibition of RNA polymerase II resulted in substantially increased apoptosis, which was dependent on oncogene expression. Their work implied the need for the constitutive expression of an anti-apoptotic gene to maintain cellular homeostasis.

Acknowledgments

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