

Radiosensitization by Pan ErbB Inhibitor CI-1033 *in Vitro* and *in Vivo*

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ABSTRACT

Purpose: Overexpression of the ErbB family of receptor tyrosine kinases has been associated with uncontrolled growth of many tumor types and, therefore, presents a promising molecular target for cancer therapy. CI-1033 is a small molecule tyrosine kinase inhibitor that differs from other 4-anilinoquinazolines by being a pan ErbB (instead of epidermal growth factor receptor-specific) irreversible (instead of reversible) inhibitor. Therefore, we investigated the antitumor effect of CI-1033 alone and in combination with ionizing radiation *in vitro* and *in vivo*.

Experimental Design: We selected three human colon carcinoma cell-lines (LoVo, Caco-2, which express activated epidermal growth factor receptor and ErbB-2 family members, and SW620, which does not), and analyzed the effects of CI-1033 both *in vitro* and *in vivo*. For *in vivo* studies LoVo and Caco-2 cells were implanted s.c. in the flank of nude mice. After the tumor reached ~100 mm³, treatment was initiated with 20 mg/kg of CI-1033 (orally once daily × 5 for 3 successive weeks), radiation treatment (a total of 30 Gy given in 2 Gy once daily × 5 for 3 successive weeks), or a combination of both CI-1033 and radiation treatment.

Results: We found that exposure of LoVo and Caco-2, but not SW620 cells, to CI-1033 in the range of 1–3 μM could inhibit constitutive signaling by tyrosine kinases, arrest cell growth, inhibit cells in G₁, stimulate expression of p53, and induce apoptosis. The inhibition of cell growth by CI-1033 seemed to produce only minimal radiosensitization in LoVo and Caco-2 cells. In contrast, the combination of CI-1033 and radiation produced significant ($P < 0.0005$ and $P =$

0.0002, respectively) and prolonged suppression of tumor growth in both the tumor types when compared with either treatment alone.

Conclusions: These findings suggest that CI-1033 can increase the effectiveness of radiation therapy. The extent of suppression of tyrosine kinase activity by CI-1033, rather than the amount of activity in untreated cells, seemed to be more closely associated with the efficacy of combination treatment.

INTRODUCTION

Strong evidence has implicated the aberrant expression of the ErbB family of receptors, including epidermal growth factor receptor (EGFR; ErbB-1), ErbB-2 (HER-2), and ErbB-3 (HER-3; Ref. 1) in the development and progression of a variety of human tumors (2–5). Overexpression of the receptor tyrosine kinases is also associated with poor prognosis in a significant proportion of human tumors (6–9). For instance, in the case of colorectal cancer, 25–77% of tumors overexpress EGFR and 50% overexpress ErbB-2. In addition, the majority (72%) of colorectal cancer patients refractory to Irinotecan show EGFR expression, which may underlie the refractory nature of their disease (10).

Because of the apparent role of aberrant expression of receptor tyrosine kinases in the uncontrolled growth that occurs in many cancers, there have been intensive efforts to develop agents that inhibit EGFR autophosphorylation and concomitantly epidermal growth factor-stimulated signal transduction (11–14). One strategy for inhibition in combination with radiation therapy has involved the use of monoclonal antibodies, which bind to the extracellular domain of the EGFR and of ErbB-2 (15, 16). A second approach has been to target the tyrosine kinase itself. The 4-anilinoquinazolines, which bind to the ATP binding domain of the ErbB family of tyrosine kinases can inhibit kinase activity in the nanomolar range (12). One example of this class of compounds is CI-1033 (14), a pan ErbB tyrosine kinase inhibitor that irreversibly binds to the receptor tyrosine kinase (17–20). Thus, it differs in two potentially important ways from the compounds in this category that have been most intensively investigated in the clinic (ZD1839 and OSI-774), which reversibly bind only the EGFR tyrosine kinase with high affinity (21–23).

These observations led us to evaluate the combination of CI-1033 and radiation in cultured human colorectal cancer cells and in tumors. Thousands of patients receive radiation therapy each year for either primary locally advanced rectal cancer, recurrent rectal cancer, or metastatic colorectal cancer, and the potential role of growth factor receptor inhibition in combination therapy has been reviewed (24). For example in one recent Phase II clinical trial, treatment with a combination of Cetuximab (C225) and Irinotecan produced a 67% response rate in colorectal carcinoma patients (25). We have shown, using cul-

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tured breast cancer and normal breast epithelial cells, that radiation and CI-1033 can interact synergistically both through cytostatic and radiosensitizing effects (26). In the present report, we have carried out additional studies to determine the mechanism of interaction of CI-1033 and radiation both in cultured colorectal cancer cells and in animals bearing these tumors as xenografts.

MATERIALS AND METHODS

Reagents. CI-1033 was provided by Pfizer Global Research Inc (Ann Arbor, MI). Phospho-EGFR (pY845), phospho-ErbB-2 (pY1248), p44/42 extracellular signal-regulated kinase (ERK), and phospho-p44/42 ERK (Thr202/Tyr204) antibodies were purchased from Cell Signaling Inc. (Beverly, MA). Anti-phosphotyrosine antibody that binds to tyrosine phosphorylated proteins (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY), anti- β -actin antibody (clone AC-15) from Sigma (St. Louis, MO), p53 (Ab6) from Oncogene Research (San Diego, CA), and CD31 (sc-1506) from Santa Cruz Biotechnology (Santa Cruz, CA). For immunoprecipitation assays, EGFR (ab13) and ErbB-2 (ab11) antibodies were obtained from Lab Vision (Fremont, CA).

Cell Culture and Irradiation. Human colon cancer cell lines Caco-2, LoVo, and SW620, and breast cancer cell lines MCF-7 and MDA-MB-468 (used in control experiments) were obtained from the American Type Culture Collection (Manassas, VA). The LoVo cell line was maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. The remaining cell lines were grown in RPMI 1640 supplemented with 10% bovine serum. In this study cells were neither serum starved nor stimulated by epidermal growth factor. Cultures were tested routinely for *Mycoplasma* infection. For all of the experiments log phase cells were produced by releasing cells from the flasks with PBS containing 0.01% trypsin and 0.20 mM EDTA and plating them into 100-mm culture dishes 2 days before drug or radiation treatments. Irradiations were performed at a dose rate of 1–2 Gy/min at room temperature using an AECL Theratron 80 (^{60}Co) calibrated with an ionization chamber directly traceable to the National Institute of Standards and Technology standard.

Cell Growth and Apoptosis. Cells were treated with 0, 1, or 3 μM CI-1033 for up to 96 h, and floating (apoptotic) and adherent cells were counted every 24 h using a Coulter Counter (Beckman Coulter, Miami, FL) after the beginning of the treatment. The apoptotic fraction was calculated by taking the ratio of floating cells:total cells (floating plus adherent cells). The values from the control groups were subtracted from the treated group to obtain the final values. To confirm that the floating cells were apoptotic, cells were washed in PBS, fixed in 70% ethanol, stained with 1% propidium iodide, and viewed for nuclear fragmentation using a fluorescent microscope. Greater than 90% of the floating cells and <5% of the adherent cells were apoptotic, as has been confirmed by gel electrophoresis and reported previously (27). Approximately 200 cells were counted from random fields.

Clonogenic Cell Survival Assay. Clonogenic assays were performed using standard techniques as described previously (28). Radiation survival data from drug-treated cells were

corrected for plating efficiency using an unirradiated plate treated with CI-1033 under the same conditions. Cell survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose was calculated according to the method of Fertil *et al.* (29). The cell survival enhancement ratio was calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose after drug exposure. A value significantly greater than one indicates radiosensitization.

Flow Cytometry. Cells were exposed to bromodeoxyuridine (30 μM) for 15 min just before the termination of the experiment. They were then trypsinized, washed, and resuspended in PBS, fixed by drop-wise addition of 2.5 volumes of ice-cold 70% ethanol, and stored at 4°C until the day of analysis. For two-parameter flow cytometry, cells were processed by treatment with detergent (Triton X-100 in 0.1 N HCl) followed by boiling to permeabilize the cells. They were then incubated with antibromodeoxyuridine antibody (BD Pharmingen, San Diego, CA) followed by FITC-goat-antimouse IgG (Sigma Chemical Co.). Cells were then suspended in propidium iodide solution (180 $\mu\text{g}/\text{ml}$) and analyzed in the Flow Cytometry Core of the University of Michigan Cancer Center at Ann Arbor.

Immunoblotting. Cells were scraped into PBS containing sodium orthovanadate and protease inhibitor mixture (Roche Diagnostic Co., Indianapolis, IN). Detergent-soluble proteins were extracted by incubating cells at 4°C for 15 min in extraction buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{ml}$ aprotinin]. After particulate material was removed by centrifugation at 13,000 rpm for 15 min at 4°C, protein concentrations in the supernatant were estimated by Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Aliquots containing 40 μg of protein were diluted with an equal volume of loading buffer [63 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromophenol blue], heated to 95°C for 5 min, and applied to SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred to polyvinylidene fluoride membrane. Membranes were incubated for 1 h at room temperature in blocking buffer consisting of 3% BSA and 1% normal goat serum in Tris-buffered saline [137 mM NaCl, 20 mM Tris-HCl (pH 7.6), and 0.1% (v/v) Tween 20]. They were subsequently incubated overnight at 4°C with 1 $\mu\text{g}/\text{ml}$ primary antibody in blocking buffer, washed, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology Inc., Birmingham, AL). After three additional washes in Tris-buffered saline, bound antibody was detected by enhanced chemiluminescence plus reagent (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation. Cells were scraped as described for immunoblotting. Five $\times 10^6$ cells were suspended in radio-immunoprecipitation assay buffer [Tris-HCl 50 mM (pH 7.4), NP40 1%, Na-deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, phenylmethylsulfonyl fluoride 1 mM, aprotinin, leupeptin, pepstatin 1 $\mu\text{g}/\text{ml}$ each, Na_3VO_4 1 mM, and NaF 1 mM], incubated on ice for 15 min, sonicated (two to four bursts), and incubated again on ice for 15 min. Supernatant was collected by spinning at 13,000 rpm at 4°C. Cell-lysates were cleared by addition of 100 μg normal mouse/rabbit (depending on the host of primary

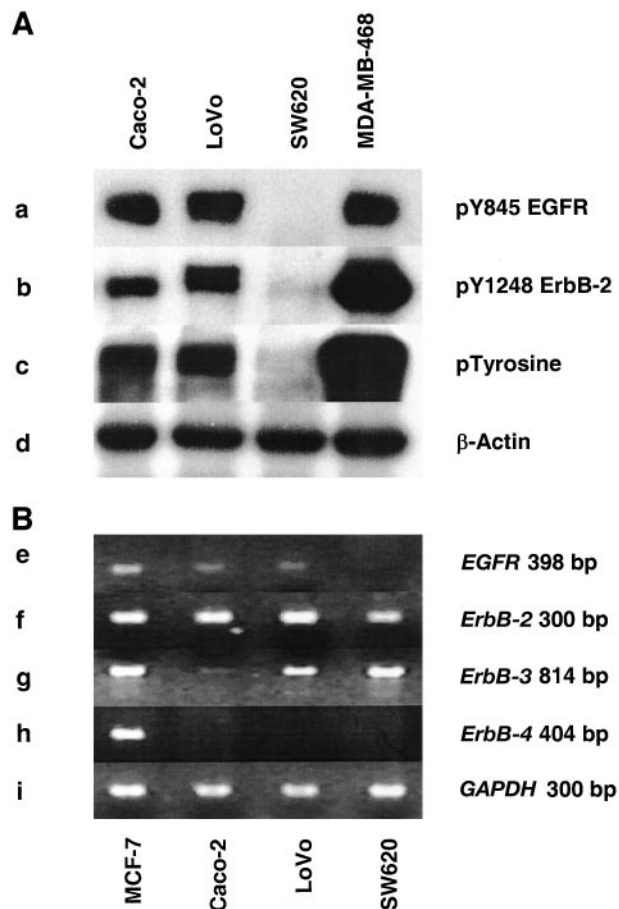


Fig. 1 Analysis of epidermal growth factor receptor (*EGFR*) and ErbB-2 phospho-protein and transcript levels of EGFR family in different cell lines. **A**, Caco-2, LoVo, SW620, and MDA-MB-468 (as a positive control) cells were assessed using immunoblotting for (a) pY845 EGFR, (b) pY1248 ErbB-2, (c) antiphosphotyrosine, and (d) β -actin (as a loading control). **B**, Caco-2, LoVo, SW620, and MCF-7 (as a positive control) cells were assessed for RNA transcripts of (e) *EGFR*, (f) *ErbB-2*, (g) *ErbB-3*, (h) *ErbB-4*, and (i) glyceraldehyde-3-phosphate dehydrogenase (as a loading control).

antibody IgG/5 $\times 10^6$ cells and addition of protein-G Sepharose beads. Primary antibody (10 μ g) was used to immunoprecipitate EGFR or ErbB-2, using protein-G Sepharose. Beads were washed five times in ice-cold radioimmunoprecipitation assay buffer, boiled in 1 \times SDS loading buffer, and subjected to immunoblot analysis.

Tissue Microarray Construction. Tissue microarrays were constructed at the University of Michigan Comprehensive Cancer Center Tissue Core by standard methods. For this purpose Caco-2 tumor xenografts and mouse skin tissue were used. Tissue cores from the circled areas of interest were targeted for transfer to the recipient array blocks. The 0.6-mm-diameter tissue microarray cores were spaced at 0.8 mm from core center to core center.

Immunohistochemistry. After slides were deparaffinized and rehydrated, antigen retrieval was performed using Proteinase K (DAKO Cytomation, Carpinteria CA), before antibody detection. Slides were incubated with primary antibody

overnight at 4°C (from 1:75 to 1:200). The Envision+ Peroxidase detection kit (DAKO Cytomation) was used to visualize the signal. Slides were counterstained with Hematoxylin, then dehydrated to Xylene before the coverslip was placed.

RNA Isolation and Reverse Transcription-PCR. Cells were harvested in TRIzol (Invitrogen Co., Carlsbad, CA) and passed through a 28-gauge needle fitted syringe to shear the DNA. RNA was extracted and purified by phenol-chloroform, precipitated with isopropanol, dissolved in RNase free water, and stored at -20°C until used. RNA (1 μ g) was reverse transcribed to cDNA under standard conditions. The cDNA product was then used for amplification of *EGFR*, *ErbB-2*, *ErbB-3*, and *ErbB-4* genes by PCR reactions. The primers used in this study were: *EGFR*: 5'-ACT AGC CAG GAA GTA CTT CC-3' and 5' GGC CTT CTT GGA TCT TTA GT 3' (product size: 398 bp); *ErbB-2*: 5'-CGG GAG ATC CCT GAC CTG CTG GAA 3' and 5' CTG CTG GGG TAC CAG ATA CTC CTC-3' (product size: 300 bp); *ErbB-3*: 5'-GAG GCT GAG CTC CAG GAG AA-3' and 5'-CTG GGA CCT GGG AGA GAG AG-3' (product size: 814 bp); and *ErbB-4*: 5' -CCT CTC CTT CCT GCG GTC TGT-3' and 5'-AAG TCT GGC AAT GAT TTT CTG TGG G-3' (product size: 404 bp).

After PCR amplification, the products were separated on a 2% (w/v) agarose gel in Tris-acetate-EDTA buffer, stained with 0.5 μ g/ml ethidium bromide, and photographed under UV light. Glyceraldehyde-3-phosphate dehydrogenase transcripts were used as a control for the RNA used to amplify the genes by reverse transcription-PCR.

Tumor Growth Studies. LoVo or Caco-2 cells (5×10^6) were transplanted in the flank of NU/NU CD-1 nude mice. When tumors had reached an average volume of 100 mm^3 , mice were randomized into four groups (6 mice/group), and the treatment was initiated. CI-1033 was dissolved in water and was given orally in the morning. The tumors in the combination or radiotherapy only group were irradiated 4 h later based on the observation that receptor tyrosine kinase activity was reduced

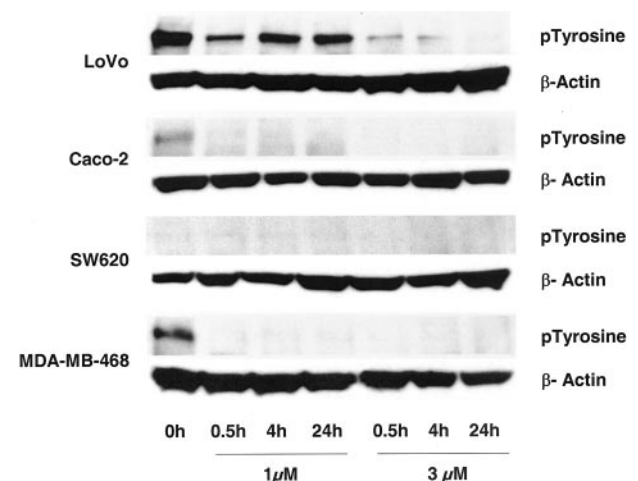


Fig. 2 Effect of CI-1033 concentration and duration of exposure on receptor tyrosine kinase phosphorylation in LoVo, Caco-2, SW620, and MDA-MB-468 cells. Cells were exposed to either 1 or 3 μ M CI-1033 for 0.5 to 24 h and assessed for tyrosine phosphorylation.

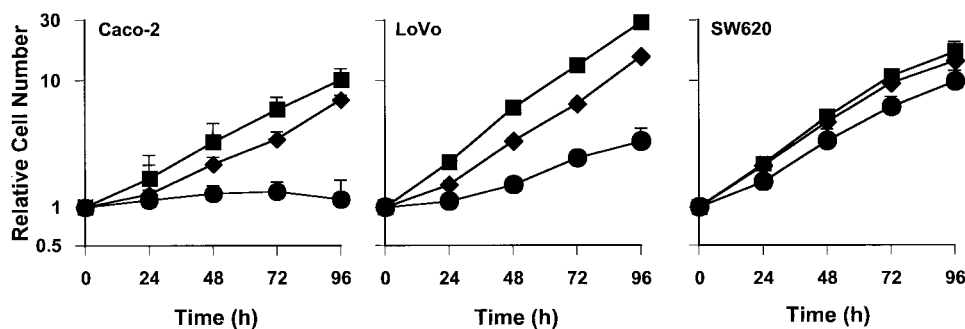


Fig. 3 Influence of CI-1033 on growth of Caco-2, LoVo, and SW620 cells. Cells were seeded in 60-mm plates and treated under control conditions (■), 1 μ M (◆), or 3 μ M (●) CI-1033 once, and adherent cells were counted every day for 4 days by Coulter Counter. Data are presented as the mean for three experiments; bars, \pm SE.

significantly at that time (see Fig. 2). Body weight and tumor measurements were taken twice a week. Tumor volume (TV) was calculated according to the equation for a prolate spheroid: $TV = (\pi/6; L \times W^2)$, where L and W are the longer and shorter dimensions of the tumor, respectively. Data are expressed as the ratio of tumor volume at various times after treatment compared with the first day of treatment (day 0). Measurements were made until day 15 (Caco-2), day 60, or until the tumor volume increased by approximately a factor of eight (LoVo), at which point the animals were sacrificed to avoid potential discomfort. On day 15 Caco-2 tumor-bearing animals were sacrificed, the tumor and skin tissue were harvested and frozen in Tissue-Tek OCT compound (Electron Microscopy Sciences, Washington,

PA), and stored below -70°C until immunostaining was performed. Animals were handled according to the established procedures of the University of Michigan Laboratory Animals Maintenance Manual.

Statistics. Results are presented as the mean \pm the SE of at least three experiments. For tumor growth data, Student's *t* test was used to compare treatment groups.

RESULTS

Effects of CI-1033 on the Receptor Tyrosine Kinases *in Vitro*. After performing pilot experiments with a variety of cell lines (data not shown), we selected three colon cancer cell lines for additional study, LoVo and Caco-2 cells, which expressed a prominent band at M_r 180,000 on a phosphotyrosine immunoblot, and SW620 cells, which expressed a minimal signal. To confirm the identity of this band, we performed immunoblotting with phospho-Y845 EGFR and phospho-Y1248 ErbB-2 antibodies (Fig. 1A). This confirmed the presence of phospho-EGFR and ErbB-2 in LoVo and Caco-2 cells, and the absence of phosphorylated EGFR and ErbB-2 in SW620 cells. We additionally confirmed the presence of constitutively phosphorylated EGFR and ErbB-2 by performing immunoprecipitation with EGFR and ErbB-2 antibodies followed by immunoblotting with an antiphosphotyrosine antibody (data not shown). Finally, the absence of EGFR in SW620 cells was confirmed at the message level by reverse transcription-PCR (Fig. 1B). We then assessed the dependence on concentration and duration of exposure of CI-1033 on the phosphorylated epidermal growth factor and ErbB-2 receptors in LoVo, Caco-2, and SW620 cells. These experiments were performed with 1–3 μ M CI-1033, as these levels are produced in plasma after a 20 mg/kg oral dose.⁴ Cells were treated with 1 or 3 μ M CI-1033 and subjected to phosphotyrosine immunoblotting from 30 min to 24 h after the beginning of drug exposure (Fig. 2). Both 1 and 3 μ M CI-1033 inhibited phosphorylation for prolonged time periods. Substantial inhibition of phosphorylation of receptor tyrosine kinases was observed by 30 min after exposure to 3 μ M CI-1033 and continued for up to 24 h in the presence of the drug. To provide a positive control as well as a context for the level of expression, MDA-MB-468 cells, which have an EGFR am-

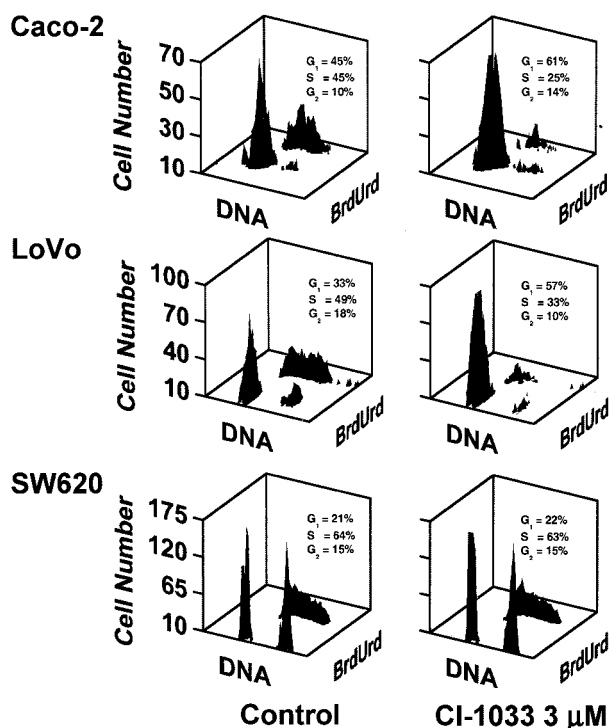


Fig. 4 Effect of CI-1033 on cell cycle distribution in Caco-2, LoVo, and SW620 cells. Cells were treated with 3 μ M CI-1033 for 24 h. At the end of the incubation the cells were exposed to bromodeoxyuridine, harvested, and processed for dual parameter flow cytometry as described in "Materials and Methods."

⁴Dr. Cho-Ming Loi, Pfizer Global Research and Development (Ann Arbor, MI), personal communication.

plification, were also assessed (30). Note that the extent of inhibition of phosphorylation by the drug was greatest in MDA-MB-468 and Caco-2 cells, whereas less inhibition was observed in LoVo cells (Fig. 2).

Effect of CI-1033 Treatment on Cell Growth and Survival. We next wished to determine whether the concentrations and duration of treatment with CI-1033 that affected receptor tyrosine kinases would influence cell growth and survival. We observed that LoVo and Caco-2 cells, which demonstrated elevated EGFR and ErbB2-phosphorylation compared with SW620 cells, tended to be responsive to CI-1033, with concentrations in the range of 1–3 μM producing significant growth retardation. In contrast, the growth of SW620 cells (which showed no EGFR and minimal ErbB-2 expression) was not inhibited by these concentrations of CI-1033 (Fig. 3) and was minimally affected by concentrations up to 10 μM for up to 4 days (data not shown). These latter findings suggest that nonspecific effects of CI-1033 are minimal below 10 μM .

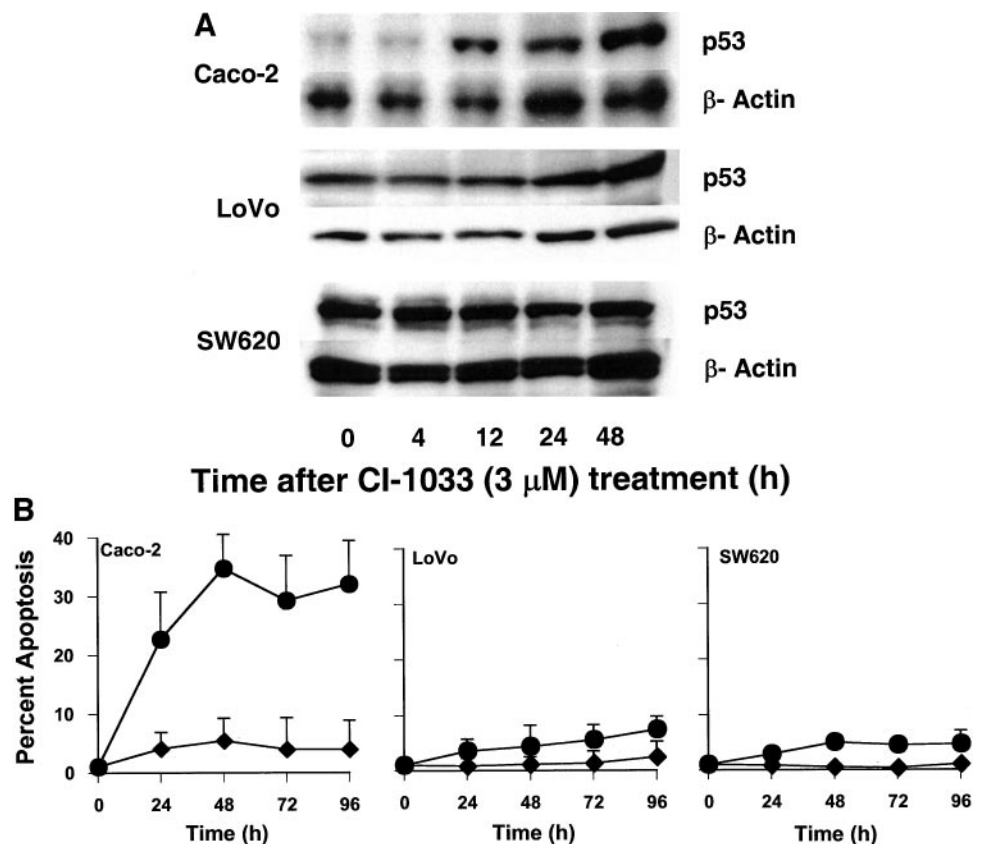
The finding of growth arrest in LoVo and Caco-2 cells suggested that CI-1033 affected cell cycle distribution. Therefore, we assessed cell cycle distribution after treatment with CI-1033 (3 μM) for 24, 48, and 72 h using two-parameter flow cytometry. We found that in LoVo and Caco-2 cells, 24 h of drug treatment resulted in growth arrest, an accumulation of cells in G₁, and a loss of cells from S phase, indicating that CI-1033 produced G₁ arrest (Fig. 4). This CI-1033-induced G₁ arrest was not observed in SW620 cells. Similar results were

obtained at 48 and 72 h after drug exposure (data not shown). To begin to determine the mechanism of G₁ arrest, we assessed p53 levels after drug treatment. We found that CI-1033 produced an elevation in p53 protein in LoVo and Caco-2 that was detectable as early as 4 h after initiation of treatment and was maintained for at least 48 h, whereas SW620 cells (p53 mutant) were not affected (Fig. 5A).

This inhibition of cell growth and the increase in p53 suggested that CI-1033 might be capable of causing apoptosis in addition to cell cycle arrest. To test this possibility, Caco-2, LoVo, and SW620 cells were treated with 1 or 3 μM CI-1033, harvested at 24 h intervals for 4 days, and assessed for apoptotic cell death. Induction of apoptosis was concentration and time dependent, with none detected after exposure to 1 μM for 4 h (data not shown), but increasing cell death was observed after exposure to higher concentrations and with longer times (Fig. 5B). Thus, the concentration of CI-1033 required to produce cell death in Caco-2 and LoVo cells was greater than that required to produce cell cycle arrest and was associated with the disappearance of phosphorylated receptor (see Fig. 2).

Effect of CI-1033 on Radiation Sensitivity *in Vitro* and *in Vivo*. We hypothesized that the cell cycle redistribution produced by CI-1033 might also be associated with an increase in cellular radiation sensitivity. To test this hypothesis, Caco-2, LoVo, and SW620 cells were treated with CI-1033 for 24 h (3 μM) and then irradiated (2–8 Gy). Twenty-four h after radiation treatment, cells were assessed for clonogenic survival. We

Fig. 5 Effects of CI-1033 on p53 levels and apoptosis in Caco-2, LoVo, and SW620 cells. A, cells were exposed to 3 μM CI-1033 for up to 48 h. Total cell lysates were assessed for p53 expression. B, cells were treated with 1 μM (\blacklozenge) or 3 μM (\bullet) CI-1033 for 4 days (see Fig. 3). They were then assessed for apoptosis as described in "Materials and Methods." Data are presented as the mean of three experiments; bars, \pm SE.



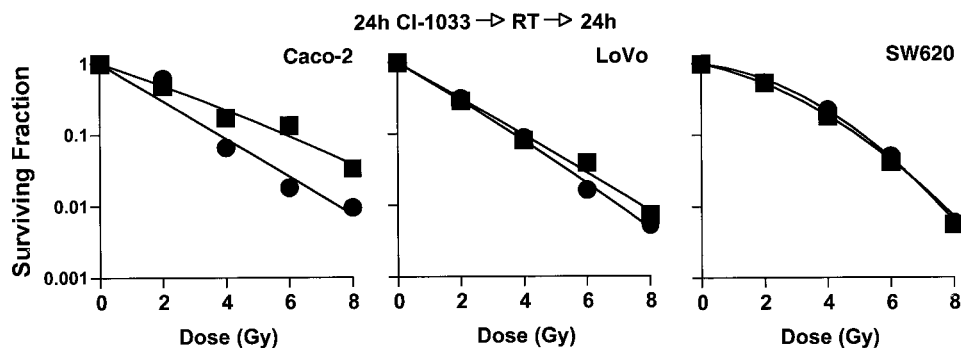


Fig. 6 Effect of CI-1033 on clonogenic survival after irradiation in Caco-2, LoVo, and SW620 cells. Cells were treated for 24 h under control conditions (■) or 3 μ M (●) CI-1033, irradiated, and then incubated for another 24 h. Cells were then assessed for clonogenic survival as described in "Materials and Methods."

found that CI-1033 produced a modest increase in radiosensitivity of Caco-2 (enhancement ratio = 1.4 ± 0.1) and LoVo cells (1.2 ± 0.04), whereas SW620 cells were not sensitized (Fig. 6).

Although there was only a modest interaction between CI-1033 and radiation *in vitro*, it seemed possible that the *in vivo* interactions might be more profound (see discussion). Thus, combined treatment with CI-1033 and radiation was initially tested in nude mice bearing flank tumors derived from LoVo cells. We conducted several pilot experiments, which demonstrated that oral doses of 20–40 mg/kg could be tolerated during a fractionated course of radiation and could suppress receptor-phosphotyrosine expression *in vivo* (Fig. 7). We then assessed in detail the combination of CI-1033 (20 mg/kg/day, which results in a plasma level of approximately 2.5–5 μ M at 24 h after oral administration⁴) and a clinically applicable course of radiation (15 daily 2 Gy fractions). We observed prolonged growth delay (4.2-fold at day 30 compared with controls) only when CI-1033 and radiation were combined (Fig. 8). This increased efficacy was associated with moderate but acceptable toxicity (maximum weight loss during treatment was $12.6 \pm 2.2\%$ on day 21; weight recovered completely in all animals by day 35). No increase in skin toxicity was observed (data not shown). Histological evaluation of treated LoVo xenografts revealed islands of viable tumor within specimens resected from tumors receiving radiation or CI-1033 alone, whereas the great majority of the tumor was necrotic after combined treatment (Fig. 9). These findings demonstrated that the effect of combining CI-1033 with

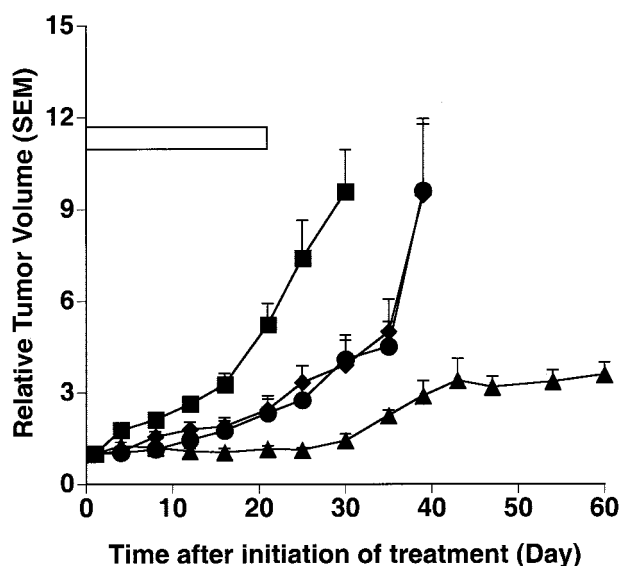


Fig. 8 Effect of CI-1033 treatment with or without radiation on the growth of LoVo flank tumors in nude mice. LoVo flank tumors were prepared as described in "Materials and Methods." When tumors reached 100 mm³, animals were randomly divided into four groups. Group I (■; $n = 6$) received vehicle alone. Group II received CI-1033 (20 mg/kg) orally Mon-Fri for 3 weeks (●; $n = 6$). Group III received radiation (15 daily 2 Gy fractions; ◆; $n = 6$) and Group IV received a combination of CI-1033 and radiation (▲; $n = 8$). The average volumes relative to those at day 0 are shown; bars, \pm SE. Horizontal bar, the treatment period.

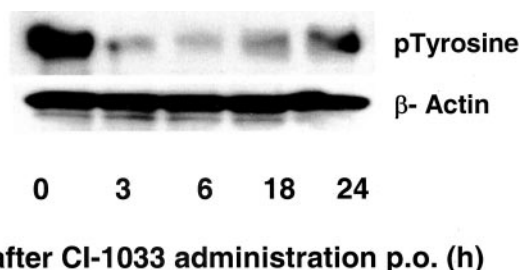
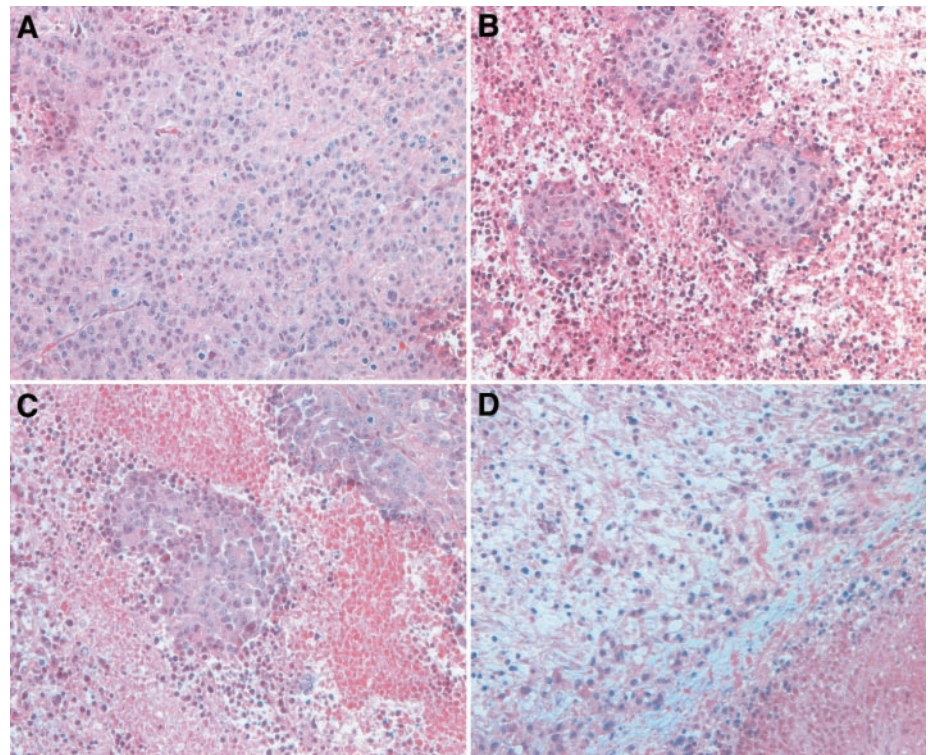


Fig. 7 Effect of CI-1033 on phosphotyrosine content in LoVo tumors. Nude mice were implanted with LoVo cells as described in "Materials and Methods." When tumors had grown to 100 mm³, animals received a single oral administration of CI-1033 (40 mg/kg). Tumors were assessed for phosphotyrosine content using immunoblotting.

radiation *in vivo* appeared to be substantially greater than we would have anticipated based purely on the modest radiosensitizing effects observed *in vitro*.

These observations led us to initiate short-term *in vivo* experiments to begin to elucidate the mechanism of radiosensitization. We chose Caco-2 cells for this experiment both to confirm that our observations were not limited to LoVo tumors and because we had observed that the CI-1033 inhibited the receptor tyrosine kinase activity for a longer duration in Caco-2 than LoVo (see Fig. 2). The initial tumor growth data were similar to those from the LoVo experiments (Fig. 10). These animals were sacrificed, the tumors were harvested for tissue microarray construction, and immunostaining was performed. In addition to markers involved in the cellular response to radiation

Fig. 9 Histological evaluation of LoVo tumors 60 days after initiation of the treatment. Animals received vehicle (A), CI-1033 (B), radiation (C), or a combination (D) as described in Fig. 8. A representative field for each condition is shown ($\times 250$).



(phospho-EGFR, phospho-ERK 1/2, and p53), we assessed the effect of treatment on the endothelial cells using CD31 antibody. Immunostaining indicated that in tumor from the combination treatment group, phosphorylated EGFR and pERK 1/2 and endothelial cell marker CD31 levels were reduced relative to either CI-1033 or radiation alone. On the other hand, p53 levels appeared to be elevated after combination therapy (Fig. 11).

DISCUSSION

In this study we have found that the small molecule kinase inhibitor CI-1033 (formerly known as PD183805), which specifically and irreversibly blocks tyrosine kinase activity of ErbB receptors, had substantial effects on human colorectal carcinoma cell lines that express activated forms of EGFR and ErbB-2. *In vitro*, CI-1033 blocked phosphorylation of EGFR and ErbB-2 at the same concentrations that led to G₁ cell cycle arrest, an increase in cell doubling time, apoptosis, and (modest) radiosensitization. These results were specific to cells that express EGFR and ErbB-2. CI-1033 had a minimal effect on the growth and survival of SW620 cells that do not express constitutively phosphorylated forms of these receptor tyrosine kinases. CI-1033 inhibited phosphorylation of receptor tyrosine kinases *in vivo*, and, although it produced only modest tumor growth inhibition on its own, it produced almost total inhibition of tumor growth when combined with a fractionated course of radiation *in vivo*.

Our finding, that modest *in vitro* radiosensitization could translate into substantial synergy *in vivo*, is consistent with those reported from the combination of radiation and cetuximab (15, 31, 32), as well as from investigations of other small molecular

tyrosine kinase inhibitors used with chemotherapy (33–35). There are several potential explanations for the apparent difference between *in vitro* and *in vivo* effects. The simplest explanation, which we proposed earlier (26), is that potent *in vivo*

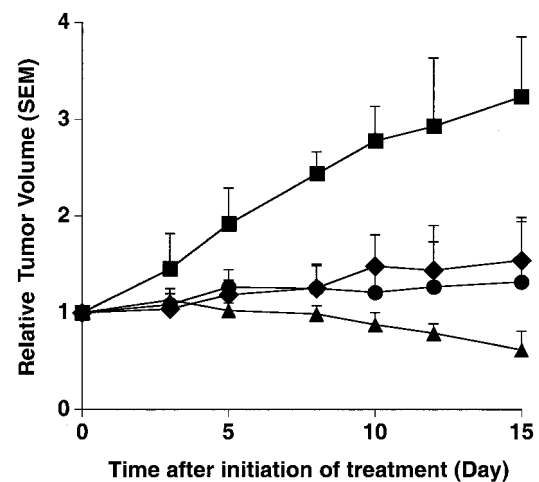


Fig. 10 Effect of CI-1033 treatment with or without radiation on the growth of Caco-2 flank tumors in nude mice. Caco-2 flank tumors were prepared as described in “Materials and Methods.” When tumors reached 100 mm³, animals were randomly divided into four groups. Group I (■; $n = 6$) received vehicle alone. Group II received CI-1033 (20 mg/kg) orally Mon-Fri for 2 weeks (●; $n = 6$). Group III received radiation (15 daily 2 Gy fractions; ◆; $n = 6$) and Group IV received a combination of CI-1033 and radiation (▲; $n = 6$). The average volumes relative to those at day 0 are shown; bars, \pm SE.

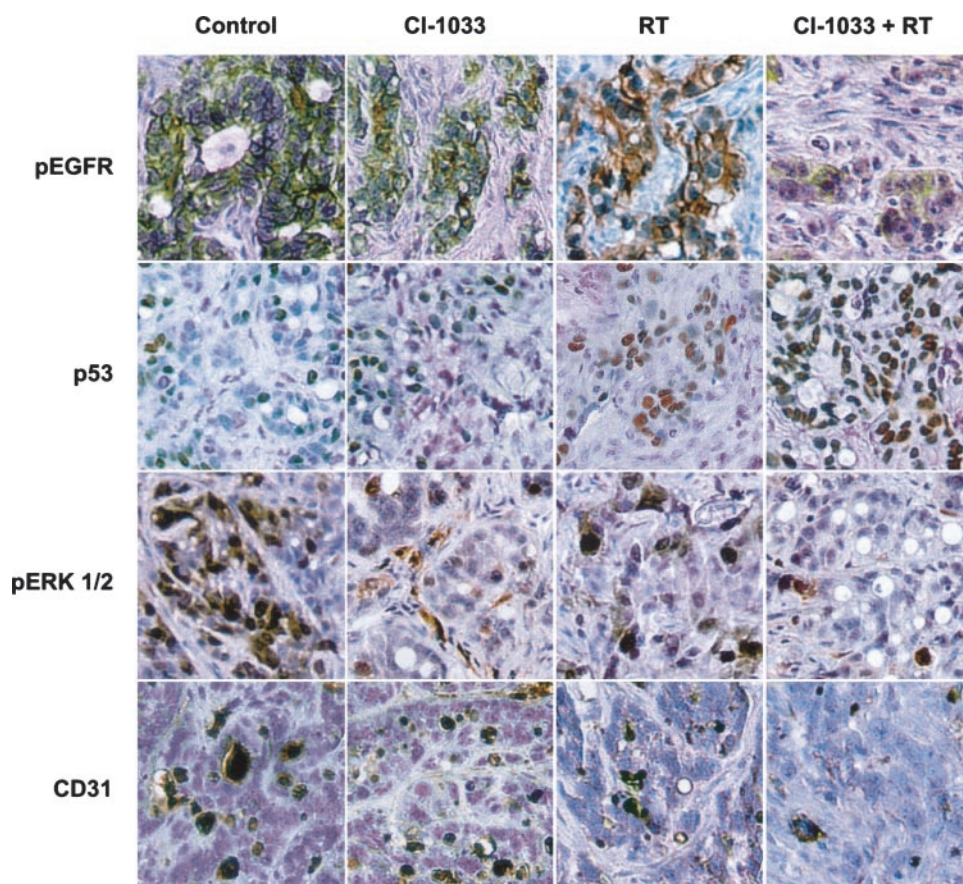


Fig. 11 Representative immunohistochemical staining of Caco-2 xenograft. Fifteen days after the initiation of treatment, described in Fig. 10, animals were sacrificed and the tumors were removed to prepare tissue microarray slides for additional analysis as described in "Materials and Methods." A representative field for each condition is shown ($\times 250$).

synergy might be achieved by the combination of slight radiosensitization, slight cytotoxicity, and inhibition of tumor cell growth resulting from G_1 arrest (which is not a radiation-sensitive phase of the cell cycle). Even modest sensitization of each fraction would produce substantial enhancement by the end of a course of treatment. For example, an enhancement ratio of 1.2/fraction for each of 15 fractions would produce a total enhancement ratio of 15.4 (1.2^{15}). An additional factor could include the blockage of EGFR stimulation. Schmidt-Ullrich *et al.* (36) have demonstrated that radiation can increase EGFR tyrosine kinase activity, and it has been hypothesized that this elevation of activity can produce clinical resistance through stimulation of survival pathways and/or tumor growth during treatment. It is possible that additional mechanisms operate, because other studies indicate that tyrosine kinase inhibitors may also block the secretion of angiogenic factors such as vascular endothelial growth factor and interleukin-8.

A better understanding of *in vivo* mechanisms is crucial in determining the optimal application of this category of drugs. The role of p53 in the response to CI-1033 is not clear. The cell lines that we chose with moderate to high activation of EGFR and ErbB-2 receptor tyrosine kinase contained wild-type p53. Although it is possible that p53 plays a crucial role, G_1 arrest and apoptosis have been produced by both cetuximab and small molecule tyrosine kinase inhibitors in cells that do not express p53 (16, 37). Likewise, the combination of cetuximab and

radiation can increase bax and decrease bcl-2, leading to apoptosis (37). Preliminary experiments demonstrate that p53 mutant MDA-MB-468 cells undergo growth arrest when exposed to low concentrations of CI-1033,⁵ suggesting that wild-type p53 function is not crucial to CI-1033-mediated growth arrest under all circumstances. It will be important to elucidate the pathways responsible for G_1 arrest and apoptosis, and their relative dependence on p53 in future studies.

The critical role of ERK 1/2 in the EGFR signal transduction pathway (38) suggests that it could play a role in the mechanism of radiosensitization by CI-1033. Thus, we examined the response of phospho-ERK 1/2 to the combination of CI-1033 and radiation both *in vitro* and *in vivo*. *In vitro*, 3 μM CI-1033 inhibited both proliferation and ERK 1/2 phosphorylation (data not shown), and *in vivo* (Fig. 11) a dramatic decrease in phospho-ERK 1/2 was observed with the combination of drug and radiation. These effects were seen at the drug concentrations that inhibit EGFR phosphorylation (see Ref. 4 and Fig. 2) and that can be achieved in patients. These data suggest that phospho-ERK 1/2 response may be a good candidate for a marker for determining which tumors will respond to therapy with this category of drugs.

⁵ M. K. Nyati, unpublished observations.

It is increasingly becoming clear that blockade of EGFR activation by cetuximab and by small molecule tyrosine kinase inhibitors results in a significant decrease in tumor-cell production of angiogenic growth factors such as basic fibroblast growth factor, vascular endothelial growth factor, and interleukin-8 (39). The decrease in the angiogenic growth factors, in turn, correlates with a significant decrease in microvessel density and an increase in apoptosis of endothelial cells in human tumor xenografts. Our finding that the combination of CI-1033 and radiation reduced the number of CD31 staining vessels is consistent with other studies and may explain, in part, the observed tumor growth delay when there is minimal *in vitro* radiosensitization (40).

In summary, we have found that Caco-2 and LoVo cells, which demonstrated high levels of EGFR and ErbB-2 tyrosine kinase activity, were affected by CI-1033, whereas SW620 cells, which had low levels of receptor tyrosine kinase activity, were unaffected by relatively high concentrations for long time periods. Furthermore, at least in the case of LoVo and Caco-2 cells, the extent of suppression of tyrosine kinase activity by CI-1033, rather than the amount of activity in untreated cells, seemed to be more closely associated with growth inhibition. Thus, the simple finding of receptor overexpression may not guarantee a particular response. Moreover, blocking an overexpressed receptor tyrosine kinase may not always inhibit cell growth, as a hallmark of cancer is aberrant cross-talk between pathways. For instance, initial results using cetuximab combined with chemotherapy for colon cancer (41) have not shown a relationship between receptor content (based on immunohistochemical staining) and response. Although some might interpret this result as indicating that these agents can be given to all patients with hope of success, the overall low clinical response rates (in the range of 20%) would suggest that a more useful direction would be to determine the appropriate target so as to better identify patients who could benefit from treatment.

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REFERENCES

- Hung, M. C., and Lau, Y. K. Basic science of HER-2/neu: a review. *Semin. Oncol.*, *26*: 51–59, 1999.
- Bacus, S. S., Zelnick, C. R., Plowman, G., and Yarden, Y. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. *Am. J. Clin. Pathol.*, *102*: S13–24, 1994.
- Dougall, W. C., Qian, X., and Greene, M. I. Interaction of the neu/p185 and EGF receptor tyrosine kinases: implications for cellular transformation and tumor therapy. *J. Cell. Biochem.*, *53*: 61–73, 1993.
- Moghal, N., and Sternberg, P. W. Multiple positive and negative regulators of signaling by the EGF-receptor. *Curr. Opin. Cell Biol.*, *11*: 190–196, 1999.
- Nicholson, R. I., Gee, J. M., and Harper, M. E. EGFR and cancer prognosis. *Eur. J. Cancer*, *37*(Suppl. 4): 9–15, 2001.
- Eissa, S., Khalifa, A., el-Gharib, A., Salah, N., and Mohamed, M. K. Multivariate analysis of DNA ploidy, p53, c-erbB-2 proteins, EGFR, and steroid hormone receptors for prediction of poor short term prognosis in breast cancer. *Anticancer Res.*, *17*: 1417–1423, 1997.
- Ioachim, E., Goussia, A., and Agnantis, N. J. Glycoprotein CD44 expression in colorectal neoplasms. An immunohistochemical study including correlation with cathepsin D, extracellular matrix components, p53, Rb, bcl-2, c-erbB-2, EGFR and proliferation indices. *Virchows Arch.*, *434*: 45–50, 1999.
- Nicholson, S., Wright, C., Sainsbury, J. R., Halcrow, P., Kelly, P., Angus, B., Farndon, J. R., and Harris, A. L. Epidermal growth factor receptor (EGFR) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. *J. Steroid Biochem. Mol. Biol.*, *37*: 811–814, 1990.
- Verbeek, B. S., Adriaansen-Slot, S. S., Vroom, T. M., Beckers, T., and Rijksen, G. Overexpression of EGFR and c-erbB2 causes enhanced cell migration in human breast cancer cells and NIH3T3 fibroblasts. *FEBS Lett.*, *425*: 145–150, 1998.
- Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.*, *19*: 183–232, 1995.
- Barker, F. G., 2nd, Simmons, M. L., Chang, S. M., Prados, M. D., Larson, D. A., Sneed, P. K., Wara, W. M., Berger, M. S., Chen, P., Israel, M. A., and Aldape, K. D. EGFR overexpression and radiation response in glioblastoma multiforme. *Int. J. Radiat. Oncol. Biol. Phys.*, *51*: 410–418, 2001.
- Fry, D. W. Inhibition of the epidermal growth factor receptor family of tyrosine kinases as an approach to cancer chemotherapy: progression from reversible to irreversible inhibitors. *Pharmacol. Ther.*, *82*: 207–218, 1999.
- Normanno, N., Tortora, G., De Luca, A., Pomato, G., Casamassimi, A., Agrawal, S., Mendelsohn, J., Bianco, A. R., and Ciardiello, F. Synergistic growth inhibition and induction of apoptosis by a novel mixed backbone antisense oligonucleotide targeting CRIPTO in combination with C225 anti-EGFR monoclonal antibody and 8-Cl-cAMP in human GEO colon cancer cells. *Oncol. Rep.*, *6*: 1105–1109, 1999.
- Wakeling, A. E., Barker, A. J., Davies, D. H., Brown, D. S., Green, L. R., Cartledge, S. A., and Woodburn, J. R. Specific inhibition of epidermal growth factor receptor tyrosine kinase by 4-anilinoquinazolines. *Breast Cancer Res. Treat.*, *38*: 67–73, 1996.
- Harari, P. M., and Huang, S. M. Radiation response modification following molecular inhibition of epidermal growth factor receptor signaling. *Semin. Radiat. Oncol.*, *11*: 281–289, 2001.
- Bonner, J. A., Raisch, K. P., Trummel, H. Q., Robert, F., Meredith, R. F., Spencer, S. A., Buchsbaum, D. J., Saleh, M. N., Stackhouse, M. A., LoBuglio, A. F., Peters, G. E., Carroll, W. R., and Waksal, H. W. Enhanced apoptosis with combination C225/radiation treatment serves as the impetus for clinical investigation in head and neck cancers. *J. Clin. Oncol.*, *18*: 47S–53S, 2000.
- Erllichman, C., Boerner, S. A., Hallgren, C. G., Spieker, R., Wang, X. Y., James, C. D., Scheffer, G. L., Maliepaard, M., Ross, D. D., Bible, K. C., and Kaufmann, S. H. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. *Cancer Res.*, *61*: 739–748, 2001.
- Gieseg, M. A., de Bock, C., Ferguson, L. R., and Denny, W. A. Evidence for epidermal growth factor receptor-enhanced chemosensitivity in combinations of cisplatin and the new irreversible tyrosine kinase inhibitor CI-1033. *Anticancer Drugs*, *12*: 681–682, 2001.
- Nelson, J. M., and Fry, D. W. Akt, MAPK (Erk1/2), and p38 act in concert to promote apoptosis in response to ErbB receptor family inhibition. *J. Biol. Chem.*, *276*: 14842–14847, 2001.
- Slichenmyer, W. J., Elliott, W. L., and Fry, D. W. CI-1033, a pan-erbB tyrosine kinase inhibitor. *Semin. Oncol.*, *28*: 80–85, 2001.
- Moulder, S. L., Yakes, F. M., Muthuswamy, S. K., Bianco, R., Simpson, J. F., and Arteaga, C. L. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res.*, *61*: 8887–8895, 2001.

22. Norman, P. OSI-774 OSI Pharmaceuticals. *Curr. Opin. Investig. Drugs*, 2: 298–304, 2001.
23. Lawrence, T. S., and Nyati, M. K. Small-molecule tyrosine kinase inhibitors as radiosensitizers. *Semin. Radiat. Oncol.*, 12: 33–36, 2002.
24. Waxman, E. S., and Herbst, R. S. The role of the epidermal growth factor receptor in the treatment of colorectal carcinoma. *Semin. Oncol. Nurs.*, 18: 20–29, 2002.
25. Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., and Cutsem, V. E. Cetuximab (C225) alone or in combination with irinotecan (CPT-11) in patients with epidermal growth factor receptor (EGFR)-positive, irinotecan-refractory metastatic colorectal cancer (MCRC). *Proc. Am. Soc. Clin. Oncol.*, 22: 252, 2003.
26. Rao, G. S., Murray, S., and Ethier, S. P. Radiosensitization of human breast cancer cells by a novel ErbB family receptor tyrosine kinase inhibitor. *Int. J. Radiat. Oncol. Biol. Phys.*, 48: 1519–1528, 2000.
27. Lawrence, T. S., Davis, M. A., Hough, A., and Rehemtulla, A. The role of apoptosis in 2', 2'-difluoro-2'-deoxycytidine (gemcitabine)-mediated radiosensitization. *Clin. Cancer Res.*, 7: 314–319, 2001.
28. Lawrence, T. S. Reduction of doxorubicin cytotoxicity by ouabain: correlation with topoisomerase-induced DNA strand breakage in human and hamster cells. *Cancer Res.*, 48: 725–730, 1988.
29. Fertil, B., Dertinger, H., Courdi, A., and Malaise, E. P. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. *Radiat. Res.*, 99: 73–84, 1984.
30. Filmus, J., Pollak, M. N., Cailleau, R., and Buick, R. N. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochem. Biophys. Res. Commun.*, 128: 898–905, 1985.
31. Huang, S. M., and Harari, P. M. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. *Investig. New Drugs*, 17: 259–269, 1999.
32. Saleh, M. N., Raisch, K. P., Stackhouse, M. A., Grizzle, W. E., Bonner, J. A., Mayo, M. S., Kim, H. G., Meredith, R. F., Wheeler, R. H., and Buchsbaum, D. J. Combined modality therapy of A431 human epidermoid cancer using anti-EGFR antibody C225 and radiation. *Cancer Biother. Radiopharm.*, 14: 451–463, 1999.
33. Solorzano, C. C., Baker, C. H., Tsan, R., Traxler, P., Cohen, P., Buchdunger, E., Killion, J. J., and Fidler, I. J. Optimization for the blockade of epidermal growth factor receptor signaling for therapy of human pancreatic carcinoma. *Clin. Cancer Res.*, 7: 2563–2572, 2001.
34. Bianco, C., Tortora, G., Bianco, R., Caputo, R., Veneziani, B. M., Damiano, V., Troiani, T., Fontanini, G., Raben, D., Pepe, S., Bianco, A. R., and Ciardiello, F. Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). *Clin. Cancer Res.*, 8: 3250–3258, 2002.
35. Raben, D., Helfrich, B. A., Chan, D., Johnson, G., and Bunn, P. A., Jr. ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. *Semin. Oncol.*, 29: 37–46, 2002.
36. Schmidt-Ullrich, R. K., Mikkelsen, R. B., Dent, P., Todd, D. G., Valerie, K., Kavanagh, B. D., Contessa, J. N., Rorrer, W. K., and Chen, P. B. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. *Oncogene*, 15: 1191–1197, 1997.
37. Huang, S. M., Bock, J. M., and Harari, P. M. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res.*, 59: 1935–1940, 1999.
38. Navolanic, P. M., Steelman, L. S., and McCubrey, J. A. EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review). *Int. J. Oncol.*, 22: 237–252, 2003.
39. Kedar, D., Baker, C. H., Killion, J. J., Dinney, C. P., and Fidler, I. J. Blockade of the epidermal growth factor receptor signaling inhibits angiogenesis leading to regression of human renal cell carcinoma growing orthotopically in nude mice. *Clin. Cancer Res.*, 8: 3592–3600, 2002.
40. Huang, S. M., and Harari, P. M. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin. Cancer Res.*, 6: 2166–2174, 2000.
41. Saltz, L., Rubin, M., Hochster, H., Tchekmeydian, N. S., Waksal, H., Needle, M., and LoBuglio, A. Cetuximab (IMC-C225) plus irinotecan (CPT-11) is active in CPT-11-refractory colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFR). *Proc. Am. Soc. Clin. Oncol.*, 7, 20: 3A, 2001.