Sequence-dependent effects of ZD1839 (‘Iressa’) in combination with cytotoxic treatment in human head and neck cancer

Elevated levels of epidermal growth factor receptor in head and neck cancer have been extensively reported, and are correlated with poor prognosis. The combination of cisplatin and 5-fluorouracil is a standard treatment regimen for head and neck cancer, with radiation representing another therapeutic option. Six head and neck cancer cell lines were used to study the cytotoxic effects of combining ZD1839 (‘Iressa’), a new selective epidermal growth factor receptor tyrosine kinase inhibitor, and radiation. Two of the cell lines were also used to study the combination of ZD1839 and cisplatin/5-fluorouracil. Cytotoxic effects were assessed by the MTT test. The results indicated that ZD1839 applied before radiation gave the best effects (P<0.002); an effect that was strongest in those p53-mutated cell lines that express the highest epidermal growth factor receptor levels. The effects of ZD1839 with cisplatin and/or 5-fluorouracil were sequence dependent (P<0.003), with the best results achieved when ZD1839 was applied first. For the triple combinations, ZD1839 applied before cisplatin and 5-fluorouracil resulted in a slight synergistic effect (P<0.03), although the effect was greater when ZD1839 was applied both before and during cytotoxic drug exposure. In conclusion, ZD1839 applied before radiation and before and/or during cisplatin/5-fluorouracil may improve the efficacy of treatment for head and neck cancer.

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Epidermal growth factor receptor (EGFR) has been particularly well studied in head and neck cancer (HNC) because of its association with the cellular mechanisms involved in tumour progression. High levels of EGFR expression have been correlated with poor prognosis (Santini et al, 1991; Dassonville et al, 1993). EGFR signalling has been found to control not only cell growth, but also angiogenesis and DNA repair (Wheeler et al, 1999; Schlessinger, 2000), and has recently been assessed as an innovative target in cancer therapy and particularly in HNC. To date, clinical studies in this area have involved the administration of cetuximab (C225, a chimeric monoclonal antibody), either alone or in combination with chemotherapy or radiotherapy, to patients with advanced head and neck squamous cell carcinoma (Ezekiel et al, 1999; Huang et al, 1999; Mendelsohn et al, 1999; Baselga et al, 2000; Hu and Harari, 2000; Milas et al, 2000). More recently, a wide range of small-molecule tyrosine kinase inhibitors have been developed, and one of the most advanced in this class of compounds is ZD1839 (‘Iressa’), which is a quinazoline derivative. This agent interacts specifically with the highly conserved ATP binding site of the tyrosine kinase domain of EGFR, resulting in inhibition of ligand-induced EGFR activation, and blockade of signal transduction pathways (Ciardiello et al, 2000; Strotnek et al, 2000).

The cisplatin/5-fluorouracil (5-FU) regimen is considered to be a standard chemotherapy regimen in the treatment of advanced HNC as part of an organ-conserving strategy (Taylor et al, 1997; Brizel et al, 1998; Calais et al, 1999). Another standard treatment is the combination of external beam radiation therapy with surgical extirpation (Bensadoun et al, 1998; Fu et al, 2000; Vokes et al, 2000). Despite the effectiveness of these treatments, the survival rates vary according to the tumour site and stage, and globally, prognosis remains poor (Vokes et al, 1999). Thus, one interesting and promising research direction for altering the natural history of HNC could be a molecular-targeted therapy against EGFR in association with one of the standard therapeutic strategies. Experimental data have indicated that application of EGFR-targeting agents can not only slow down cell proliferation, but also improve apoptotic capacities and decrease DNA repair. These observations suggest that EGFR targeting can lead to chemo- and radiosensitisation and recent experimental results tend to confirm this view (Huang et al, 1999).

The aim of this study was two-fold: firstly, to explore the sequence-dependent cytotoxic effects of combining ZD1839 with radiation using a panel of six human head and neck squamous cell carcinoma cell lines; and secondly, to investigate the sequence-dependent cytotoxic effects of combining ZD1839 with cisplatin and/or 5-FU on two of the cell lines from the panel. This preclinical work was undertaken to serve as a rationale to support ongoing clinical investigations of ZD1839 in HNC patients.

MATERIALS AND METHODS

Chemicals

ZD1839 was kindly provided by AstraZeneca. A 50 mM working solution in dimethylsulphoxide (DMSO) was prepared before use. Human recombinant $^{125}$I-EGF (ref IM 196, specific activity $4514 \times 10^{4}$ Bq mmol$^{-1}$, $9.25 \times 10^{5}$ Bq per 250 μl) and unlabelled human recombinant EGF (ref ARN 5100) were obtained from...
Amersham. Dulbecco’s modification of Eagle’s medium (DMEM), RPMI 1640 and glutamine were purchased from Whittaker (Verviers, Belgium). Foetal bovine serum (FBS) was obtained from Dutscher (Brumath, France). Penicillin and streptomycin were from Meyrieux (Lyons, France). Transferrin and insulin were purchased from Flow (Irvine, Scotland). Bovine serum albumin (BSA), 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were purchased from Sigma (St Quintin Fallavier, France).

Cell lines

Six HNC cell lines of human origin were used (CAL27, CAL33, CAL60, CAL166, Hep-2, Detroit562) (Table 1). Cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 600 μg l⁻¹ insulin, 500 μg l⁻¹ transferrin, 50,000 units l⁻¹ penicillin and 80 μM streptomycin in a fully humidified incubator (Sanyo, Japan) at 37°C in an atmosphere containing 8% CO₂.

EGFR assay

EGFR expression was assayed by competitive analysis and Scatchard plots as previously described in human cancer cell lines (Olivier et al, 1990). Cells were grown in 24-well plates (10⁶ cells per well) in 10% FBS-DMEM at 37°C. At 80–90% confluence, cells were rinsed three times with 500 μl RPMI 1640 containing 0.1% BSA at 2–4°C (plates placed on a tray with ice). Plates were then incubated for 30 min with the same medium (500 μl per well) at 4°C. Cells were first screened for their capacity to bind EGF specifically. Total binding was measured after incubation with 0.2 nM ¹²⁵I-EGF (3 h, 4°C, 0.1% BSA – RPMI); non-specific binding was measured in the presence of an excess of unlabelled EGF (20 nM). With no exception, the six tested cell lines exhibited EGFR binding and their precise EGFR content was then measured. Cells were incubated in the RPMI medium for 3 h, at 4°C in the presence of various concentrations of ¹²⁵I-EGF (0.01, 0.02, 0.04, 0.08, 0.12, 0.18, 0.20 nM); for higher EGF concentrations, cells were incubated with 0.2 nM ¹²⁵I-EGF with increasing concentrations of unlabelled EGF (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 20, 200 nM). Plates were placed on ice to stop the reaction, and the supernatant was removed from each well. Cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA (4°C, 500 μl per well). After removal of the supernatant, cells were solubilised in 1 m NaOH at 37°C (500 μl per well for 30 min). The radioactivity of each well was determined by gamma counting. Results were expressed in fmol per mg cell protein. Scatchard analysis was used to calculate the number of receptor sites per cell (N) and the dissociation constant (Kd). Each point of every Scatchard plot was performed in quadruplicate. Cells were counted in four wells run in parallel, resuspended in 200 μl PBS at room temperature and counted with a haemocytometer. Experiments were performed in duplicate only, because of the intrinsic reproducibility of the assay (coefficient of variance=7.3%, n=4).

Determination of p53 status

DNA was extracted from all head and neck cell lines of the panel. Exons 4 to 8 were screened for mutations by use of denaturing gradient gel electrophoresis in accordance with the method described by Hamelin et al (1993) for exons 5, 7, and 8, and the method of Gulberg et al (1997) for exons 4 and 6. Exon 9 was screened for mutations by the method described by Cabelguenne et al (2000). Polymerase chain reaction (PCR) amplification products were loaded onto a 6.5% polyacrylamide gel that contained an appropriate gradient of urea and formamide. After electrophoresis, gels were stained with ethidium bromide. Tumours that showed an electrophoresis variant pattern were amplified and sequenced for each variant exon. PCR products were purified with QiAquick PCR Purification Kit (QIAGEN SA, Courtabeuf, France) and sequenced on both strands on an ABI 310 genetic analyser (PE Applied Biosystems, Courtabeuf, France). A Big Dye Terminator sequencing kit (PE Applied Biosystems) was used in accordance to the manufacturer’s instructions; this step was followed by ethanol precipitation to remove non-incorporated dyes. Sequences were analysed by Sequence Analysis 3.0 (PE Applied Biosystems).

Evaluation of radiotoxicity

Cells were irradiated with gamma rays during exponential cell growth as monolayers in 96-well microtiter plates using a ⁶⁰Co unit at a dose rate of 1 Gy min⁻¹. Dose-effect curves were established for all six head and neck cell lines using a total of 10 doses (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 15 Gy). Sequence-dependent cytotoxic effects of binary combinations of ZD1839 (0.2–200 μM) with ionising radiation were then evaluated using eight doses of ionising radiation, ranging from 0.5 to 10 Gy for CAL27 and CAL33, and from 2 to 15 Gy for CAL60, CAL166, Hep-2 and Detroit562 (the radiation doses were selected from the above experiments to encompass more precisely the zone of sensitivity). Cells were maintained in DMEM supplemented with 10% FBS during all radiation exposures, which were performed at room temperature.

Determination of the cytotoxic effects of radiation alone

Cells were seeded in 96-well microtiter plates (100 μl per well) to obtain exponential growth for the whole duration of the experiment: initial cell densities were 2500 (CAL27 and Hep-2), 3000 (CAL33 and Detroit562), 4500 (CAL166) and 5000 (CAL60) cells per well.

The cells were exposed to ionising radiation 48 h later (dose previously described) and the MTT assay was performed 48 h after that. Control cells received no radiation treatment.

Determination of cytotoxicity using ZD1839 in combination with radiation

Cells were seeded in 96-well microtiter plates (100 μl per well) to obtain exponential growth for the whole dura-

Table 1 Head and neck cancer cell line characteristics

| Cell line | Origin | EGFR levels (fmol mg⁻¹ protein) | p53 status | Doubling time (h) | IC₅₀ radiation (Gy) | IC₅₀ cisplatin (μM) | IC₅₀ 5-FU (μM) | IC₅₀ ZD1839 (μM) |
|-----------|--------|-------------------------------|------------|-----------------|-------------------|-------------------|----------------|-----------------|
| CAL27     | CAL    | 8258 (31)                     | mutant (exon 6) | 18.2 (1.5) | 6.2 (1.1) | 2.4 (0.1) | 4.1 (0.8) | 17.5 (2.4) |
| CAL33     | CAL    | 33794 (624)                   | mutant (exon 5) | 17.5 (0.1) | 2.6 (0.4) | 5.7 (1.2) | 2.7 (1.9) | 6.07 (0.8) |
| CAL60     | CAL    | 2703 (101)                    | mutant (exon 7) | 11.8 (0.2) | 5.1 (0.1) | 2.2 (0.1) | 1.1 (0.2) | 11.4 (0.9) |
| CAL166    | CAL    | 3253 (126)                    | wild type     | 13.1 (1.0) | 6.2 (0.8) | 9.7 (0.6) | 5.4 (0.6) | 22.8 (4.8) |
| Hep-2     | ATCC   | 388 (27)                      | wild type     | 10.9 (1.0) | 9.8 (0.3) | 7.1 (1.7) | 5.6 (1.0) | 31.1 (2.4) |
| Detroit562| ATCC   | 668 (45)                      | mutant (exon 5) | 14.8 (0.4) | 7.7 (0.1) | 12.6 (1.1) | 31.7 (1.1) | 20.6 (1.5) |

*Mean values (standard deviation), at least two separate experiments done at distance for EGFR content, and three different experiments for other determinations; CAL, Center Antoine Lacassagne; ATCC, American Type Culture Collection, Rockville.*
tion of the experiment (initial cell density is given above). In each plate, one ZD1839 concentration and one radiation dose were tested. Only half of each plate was irradiated in order to measure on the same plate the effects of ZD1839 alone (20 wells), radiation alone (five wells), ZD1839 combined with radiation (20 wells) and a control (no radiation, no ZD1839; five wells).

Forty-eight hours after seeding the cells, three different sequences of radiation and 48-h incubations of ZD1839 were compared: (a) ZD1839 (48 h) followed by radiation, then medium for 48 h; (b) concomitant ZD1839 (48 h) and ionising radiation (radiation delivered in the middle of ZD1839 exposure), followed by medium for 48 h; (c) ionising radiation prior to ZD1839 (48 h), then medium for 48 h.

Growth inhibition was assessed 168 h after cell seeding by using the MTT test as described below (Carmichael et al., 1987). Cells were washed with PBS and incubated with MTT; after 2 h of exposure, MTT was released and fixed was revealed by the addition of DMSO (100 µl). Absorbance at 450 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Cell sensitivity to the tested drugs was expressed by IC50 (concentration leading to 50% cell survival). Triplicate determinations were made in separate experiments.

Evaluation of cisplatin and/or 5-FU cytotoxicity

Cells were seeded in 96-well microtitration plates (100 µl per well) to obtain exponential growth for the duration of the experiments (initial cell density was 2500 and 3000 cells per well for Hep-2 and CAL33, respectively). Forty-eight hours after seeding, cells were exposed to ZD1839 and cisplatin and/or 5-FU in a variety of sequences. In all cases, cells were exposed to ZD1839 for 48 h, and the cisplatin/5-FU sequence was constant (cisplatin applied for 2 h prior to exposure to 5-FU for 48 h). The sequence of ZD1839 varied: (a) ZD1839 (48 h) followed by cisplatin and/or 5-FU; (b) concomitant ZD1839 and cisplatin and/or 5-FU (the exposure to ZD1839 was 50 h); (c) ZD1839 prior to and during cisplatin/5-FU exposure (ZD1839 was given for 48 h before cisplatin/5-FU and then for 50 h concomitant with cisplatin/5-FU, making 98 h in total) followed by medium for 48 h; (d) cisplatin/5-FU prior to ZD1839 followed by medium for 48 h. Eleven concentrations were tested for each drug: ZD1839 0.2–200 µM; cisplatin 0.1–200 µM; 5-FU 0.22–220 µM. The cisplatin/5-FU combination was tested at a constant concentration ratio of the drugs for a given cell line, the ratio being dictated by the drug sensitivity and close to the ratio of the IC50 for each drug.

Thereafter, growth inhibition was assessed 48 h after the end of the experiment (in medium alone) by the MTT test described above. Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition) and separate experiments were performed in triplicate. The dose-effect curves were analysed using Prism software (GraphPad Software, San Diego, CA, USA).

Combination index (CI) calculations and determination of the potentiation factor

The cytotoxic effects obtained with the different ZD1839/cisplatin/5-FU combinations were analysed according to the Chou and Talalay (1984) method on CalcuSyn software (Biosoft, Cambridge, UK). Interaction between the double combinations (ZD1839 plus either cisplatin or 5-FU), or the triple combinations (ZD1839 plus cisplatin and 5-FU) was assessed by means of an automatically computed combination index (CI). CI was determined at 50 and 75% cell death, and was defined as follows:

\[ \text{CI}_{50,75} = \left[ \frac{\text{IC}_{50,75}}{\text{IC}_{50}} \right]_{\text{D}_{X}} = \left[ \frac{\text{IC}_{50,75}}{\text{IC}_{50}} \right]_{\text{D}_{X}} \]

where \( \text{IC}_{50,75} \) is the IC50 at a fixed final effect (F) for the combination of cytotoxic A and cytotoxic B.

\[ \text{D}_{X} = \frac{\text{cytotoxic A in the combination A + B giving an effect F}}{\text{cytotoxic B alone giving an effect F}} \]

The potentiation factor of ionising radiation by ZD1839 was defined as the ratio of the IC50 of ionising radiation alone to the IC50 of the combination (ZD1839 plus ionising radiation); higher potentiation factors indicate greater cytotoxicity. Potentiation factor I was obtained using sequence I (ZD1839 (48 h) followed by radiation, then medium for 48 h). Potentiation factor II used sequence II (concomitant ZD1839 (48 h) and ionising radiation, radiation delivered in the middle of the ZD1839 exposure), and potentiation factor III used sequence III (ionising radiation prior to ZD1839 (48 h), then medium for 48 h).

Statistical analysis

For the panel of cell lines, the relationship between EGFR expression and response to ionizing radiation was analysed by plotting IC50 values for radiation against the respective EGF content. The Friedman non-parametric rank test was used to analyse the impact of the sequence ZD1839/cisplatin/5-FU (CI values) or ZD1839/radiation and to compare the different sequences combining ZD1839 and radiation on the basis of the potentiation factors. The correlation coefficients (r) and the P-values were computed using SPSS software (Chicago, IL, USA). A P-value of less than 0.05 was considered to be statistically significant.

RESULTS

The study panel of human squamous cell carcinoma cells showed substantial variability in EGFR expression, ranging from 388...
Correlation between cell sensitivity to ionising radiation and EGFR content.

Figure 2 Correlation between cell sensitivity to ionising radiation and EGFR content.

Figure 1 Dose-survival curves following ionising radiation for Hep-2 and CAL33 cells.
the tumoural content of its specific ligands may play an important role in radiosensitivity. Cell doubling time and EGFR content were not found to be correlated in the present study. Thus the impact of EGFR on radiosensitivity is not explicable by EGFR-dependent modifications of cell kinetic characteristics, which could enhance the action of irradiation. The classical view of the cellular effects of ionising radiation consists of an initial event involving the induction of DNA damage, in addition to the activation of several intracellular signalling cascades that have commonly been regarded as mitogenic, including the Raf-MEK-Erk kinase cascade (Todd et al, 1999). DNA damage is modulated by metabolic DNA repair processes. Interestingly, Bandyopadhyay et al (1998) have shown that EGFR-mediated signalling may control the activity of DNA-PK, an enzyme directly involved in DNA damage repair; more

**Table 2** CI values* according to the different sequences of ZD1839/radiation

| Cell line | Sequence I CI | Sequence II CI | Sequence III CI | Sequence I CI | Sequence II CI | Sequence III CI |
|-----------|---------------|----------------|-----------------|---------------|----------------|-----------------|
| CAL27     | 0.4 (0.1)     | 1.5 (0.1)      | 3.1 (0.1)       | 0.8 (0.4)     | 1.5 (0.1)      | 3.1 (1.0)       |
| CAL33     | 0.5 (0.1)     | 1.1 (0.1)      | 1.9 (0.2)       | 0.8 (0.0)     | 1.6 (0.8)      | 2.8 (0.3)       |
| CAL60     | 0.7 (0.0)     | 0.7 (0.1)      | 1.4 (0.1)       | 0.5 (0.1)     | 0.9 (0.2)      | 1.7 (0.2)       |
| CAL166    | 0.9 (0.1)     | 2.1 (0.3)      | 1.8 (0.1)       | 2.2 (0.4)     | 1.5 (0.1)      | 1.8 (0.3)       |
| Hep-2     | 0.9 (0.7)     | 2.5 (0.0)      | 2.9 (1.2)       | 1.3 (0.3)     | 2.2 (1.0)      | 1.1 (0.2)       |
| Detroit562| 1.2 (0.1)     | 16.2 (3.2)     | 2.2 (0.4)       | 1.1 (0.2)     | 15.2 (0.2)     | 2.0 (0.3)       |

*50 and 75% mean (standard deviation) CI values (at least three separate experiments) computed at 50 and 75% of growth inhibitory effects; sequence I, ZD1839 applied before radiation; sequence II ZD1839 applied during radiation; sequence III, ZD1839 applied after radiation. The Friedman non-parametric rank test was used to analyse the impact of the sequence of treatment on the whole cell line panel, \( P=0.002 \).
Sequence-dependent effects of ZD1839

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Table 3

| Cell line | Potentiation factor I | Potentiation factor II | Potentiation factor III |
|-----------|-----------------------|------------------------|------------------------|
| CAL27     | 2.7 (0.2)             | 2.2 (2.0)              | 0.5 (0.1)              |
| CAL33     | 2.3 (1.5)             | 1.5 (1.4)              | 0.1 (0.1)              |
| CAL60     | 3.7 (0.1)             | 0.5 (0.1)              | 0.6 (0.1)              |
| CAL166    | 3.2 (0.4)             | 0.5 (0.2)              | 0.8 (0.1)              |
| Hep-2     | 7.5 (2.1)             | 2.0 (0.8)              | 1.0 (0.2)              |
| Detroit562| 3.1 (0.0)             | 2.5 (0.0)              | 0.8 (0.1)              |

Mean (standard deviation) potentiation factor values (at least three separate experiments); statistical analysis (Friedman non-parametric rank test) was used to compare the different sequences combining ZD1839 and radiation on the basis of the potentiation factors on the whole cell line panel, P<0.003.

Figure 4 Typical examples of CI/fractional effects curves for CAL33 cells: (a) sequence I, (b) sequence II, (c) sequence III.

Colonies of tumour cells were scored as described in the Methods section. Colonies were considered to be killed when the fractional effect of the combined drugs was less than 0.3, the number of colonies being less than 10% of control. Post-irradiation treatment with TEM was found to increase the number of colonies in each cell line, with TEM showing a trend to effectiveness in the presence of cytotoxic drugs.

A strong positive interaction between irradiation and ZD1839, when the ZD1839 was applied before the radiation, was demonstrated in the present study (Tables 2 and 3). This could be explained by an intrinsic activity of ZD1839 as radiosensitiser, as has previously been shown for cetuximab, another EGFR-targeting drug (Ezekiel et al, 1999; Huang et al, 1999; Mendelsohn et al, 1999; Baselga et al, 2000; Huang and Harari, 2000; Milas et al, 2000), and also for trastuzumab (Herceptin™), a HER-2-targeting drug (Pegram et al, 1998; Pietras et al, 1999; Pegram and Slamon, 2000). Trastuzumab increased the radiosensitivity of HER-2-over-expressing MCF7 breast cancer cells as measured by in vitro colony-forming assays, and the combination of trastuzumab and radiation showed synergistic tumour reduction in nude mice (Pietras et al, 1999). According to Pietras et al (1999) the mechanism of radiosensitization appears to involve both cell cycle regulation and DNA repair. Interestingly, the application of ZD1839 in our study was found to increase the percentage of cells in the G1 phase (results not shown), which are particularly sensitive to radiation (Teyssier et al, 1999). Thus, there are several potential mechanisms of radiosensitisation by inhibitors of the EGFR family; they may include favourable cell cycle reorganisation and/or abrogation or attenuation of signals required for survival during cell cycle arrest. Marked antagonistic effects were observed when exposing cells to ZD1839 after irradiation (sequence III). This observation may be related to the fact that irradiation is able to upregulate EGFR phosphorylation in a similar manner to EGF (Wan et al, 2001) and thus create the circumstances of opposite effects to ZD1839.

Two cell lines of the initial panel of six were considered for analysis of the association between ZD1839 and cytotoxic drugs. We have recently demonstrated an inverse correlation between p53 content (representative of p53 mutations) and EGFR levels in a group of HNC patients (Etienne et al, 1999). Thus, the two cell lines were selected because they are representative of human HNC characteristics on the basis of their p53 status and EGFR levels.

Precisely, from their work, high EGFR expression could maintain a high level of signalling, which would be associated with high basal levels of DNA repair capacity. However, this does not appear to explain the observations reported here. An approach used by Lewis et al (2000), examining the whole pattern of MAP kinase pathway signalling targets, could help to elucidate the molecular origins of the present observation of a radiosensitivity linked to high tumoural EGFR levels.

Two cell lines of the initial panel of six were considered for analysis of the association between ZD1839 and cytotoxic drugs. We have recently demonstrated an inverse correlation between p53 content (representative of p53 mutations) and EGFR levels in a group of HNC patients (Etienne et al, 1999). Thus, the two cell lines were selected because they are representative of human HNC characteristics on the basis of their p53 status and EGFR levels: CAL33 is p53 mutant and has high EGFR expression, and Hep-2 is p53 wild type and has low EGFR expression. We observed synergistic effects when ZD1839 was applied before cisplatin and/or 5-FU in both cell lines (Table 4). The impact of ZD1839 on the effects of the combined drugs was sequence dependent. The highest synergistic effects were obtained when ZD1839 was applied before and during exposure to cytotoxic drugs (Table 4). Previous in vivo and in vitro studies have shown that the anti-EGFR receptor monoclonal antibody C225 can potentiate the effects of a number of chemotherapeutic agents, including doxorubicin, paclitaxel and cisplatin (Ezekiel et al, 1999; Mendelsohn et al, 1999; Huang et al, 1999; Pegram et al, 1999).
The mechanisms by which receptor blockade by C225 augments the cytotoxic effect of anti-neoplastic agents was attributed by these authors to cell cycle effects and an enhanced capacity for apoptosis by C225. The antiproliferative activity of ZD1839 combined with a number of cytotoxic drugs (cisplatin, carboplatin, oxaliplatin, paclitaxel, docetaxel, doxorubicin, etoposide, topotecan and raltitrexed) has been recently assessed in a variety of human cancer cell lines (Ciardiello et al., 2000; Sirotnak et al., 2000). The co-administration of ZD1839 was found to enhance the growth-

### Table 4

CI results in Hep-2 and CAL33 cell lines corresponding to concentration of drugs leading to 50% cell survival

| Treatment | Hep-2 | Combination effect | CAL33 | Combination effect |
|-----------|-------|--------------------|-------|--------------------|
| Prior exposure to ZD1839 | | | | |
| ZD1839+cisplatin | 0.9 (0.2) | Additive | 0.8 (0.1) | Synergistic |
| ZD1839+5-FU | 0.6 (0.1) | Synergistic | 0.7 (0.3) | Synergistic |
| ZD1839+cisplatin+5-FU | 0.8 (0.1) | Synergistic | 0.7 (0.1) | Synergistic |

| Concomitant exposure to ZD1839 | | | |
| ZD1839+cisplatin | 1.1 (0.5) | Additive | 1.2 (0.6) | Additive |
| ZD1839+5-FU | 0.8 (0.3) | Synergistic | 1.5 (0.3) | Antagonistic |
| ZD1839+cisplatin+5-FU | 1.3 (0.4) | Antagonistic | 1.1 (0.5) | Additive |

| Prior and concomitant exposure to ZD1839 | | | |
| ZD1839+cisplatin | 0.6 (0.2) | Synergistic | 0.4 (0.2) | Synergistic |

| Exposure to ZD1839 after cisplatin and/or 5-FU | | | |
| ZD1839+cisplatin | 1.4 (0.3) | Antagonistic | 1.1 (0.2) | Additive |
| ZD1839+5-FU | 1.0 (0.2) | Additive | 1.3 (0.8) | Antagonistic |
| ZD1839+cisplatin+5-FU | 1.5 (0.4) | Antagonistic | 1.4 (0.8) | Antagonistic |

*At least three separate experiments; ZD1839 applied for 48 h; cisplatin for 2 h; 5-FU for 48 h.*

**Figure 5**

Typical dose-effect curves comparing sequences I and III for (A, B) Hep-2 and (C, D) CAL33. Sequence I consisted of ZD1839, followed by cisplatin/5-FU for 48 h; sequence III was ZD1839 applied first and also during the cisplatin/5-FU exposure, followed by medium for 48 h.
inhibitory effects of all cytotoxic drugs tested. The present data confirm this interesting property of ZD1839 as a strong radiosensitizer. In addition, the present study brings particular focus to the importance of the combination sequence: it is shown that synergy is not a rule and that some antagonistic effects may result from inappropriate sequences, e.g. where ZD1839 is confirm this interesting property of ZD1839 as a strong radio-

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Sequence-dependent effects of ZD1839

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Experimental Therapeutics

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for the design of future clinical trials combining ZD1839 and cyto-
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