Enhancement of Antitumor Activity of Ionizing Radiation by Combined Treatment with the Selective Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor ZD1839 (Iressa)\(^1\)

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ABSTRACT

Purpose: The epidermal growth factor receptor (EGFR) is expressed in the majority of human epithelial cancers and has been implicated in the development of cancer cell resistance to cytotoxic drugs and to ionizing radiation.

Experimental Design: We used ZD1839, a selective small molecule EGFR tyrosine kinase inhibitor currently in clinical development. We tested the antiproliferative and the proapoptotic activity of ZD1839 in combination with ionizing radiation in human colon (GEO), ovarian (OVAR-CAR-3), non-small cell lung (A549 and Calu-6), and breast (MCF-7 ADR) cancer cell lines. The antitumor activity of this combination was also tested in nude mice bearing established GEO colon cancer xenografts.

Results: With ionizing radiation or ZD1839, a dose-dependent growth inhibition was observed in all of the cancer cell lines growing in soft agar. A cooperative antiproliferative and proapoptotic effect was obtained when cancer cells were treated with iodinating radiation followed by ZD1839. This effect was accompanied by inhibition in the expression of the antiapoptotic proteins bcl-xL and bcl-2, and by a suppression of the activated (phosphorylated) form of akt protein. Treatment of mice bearing established human GEO colon cancer xenografts with radiotherapy (RT) resulted in a dose-dependent tumor growth inhibition that was reversible upon treatment cessation. Long term GEO tumor growth regressions were obtained after RT in combination with ZD1839. This resulted in a significant improvement in survival of these mice as compared with the control group (\(P < 0.001\)), the RT-treated group (\(P < 0.001\)), or the ZD1839-treated group (\(P < 0.001\)). The only mice alive 10 weeks after tumor cell injection were in the RT-plus-ZD1839 group. Furthermore, 10% of mice in this group were alive and tumor-free after 26 weeks. Similar results were obtained in mice bearing established human A549 lung adenocarcinoma xenografts. Finally, the combined treatment with RT plus ZD1839 was accompanied by a significant potentiation in the inhibition of transforming growth factor \(\alpha\), vascular epidermal growth factor, and basic fibroblast growth factor expression in cancer cells, which resulted in significant antiangiogenic effects as determined by immunohistochemical count of neovessels within the GEO tumors.

Conclusion: This study provides a rationale for evaluating in cancer patients the combination of ionizing radiation and selective EGFR tyrosine kinase inhibitors such as ZD1839.

INTRODUCTION

Growth factors control cellular proliferation and differentiation and are important in initiating and maintaining neoplastic transformation. Cancer cells exhibit a decreased requirement for exogenous growth factors as compared with normal cells. This is attributable in part to the ability of tumor cells to overexpress growth factors and/or their specific cell membrane receptors resulting in the autonomous activation of autocrine and para-crine growth pathways.

TGFR\(^3\) and its specific receptor, the EGFRs, have been

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\(^{3}\) The abbreviations used are: TGFR, transforming growth factor \(\alpha\); EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; bFGF, basic fibroblast growth factor; RT, radiation therapy.
implicated in the development and progression of the majority of human epithelial cancer types (1). TGFα and/or EGFR are expressed at high levels in different human cancers in which there is generally an association with advanced disease and poor prognosis (1). EGFR activation is not only critical for cell proliferation but EGFR-mediated signals also contribute to other processes that are crucial to cancer progression, including angiogenesis, metastatic spread, and the inhibition of apoptosis (2). High expression of EGFR is also associated with resistance to hormonal therapy, cytotoxic drugs, or radiotherapy (2–4). More specifically, high EGFR expression has been associated with reduced sensitivity or with resistance to ionizing radiation in several preclinical models (5). In this respect, Akimoto et al. (4) have found an inverse relationship between EGFR levels and radiocurability in several murine carcinomas of different histology. Treatment with ionizing radiation can induce the EGFR/ras/raf/mitogen-activated protein kinase (MAPK) proliferative pathway through the release of TGFα and the activation of the EGFR tyrosine kinase (6–7). EGFR activation could be a cell survival response to block apoptotic signals in cancer cells exposed to cytotoxic damage. This may have clinical relevance because it could represent a mechanism by which cancer cells escape radiation-induced cell death.

A large body of experimental and clinical work supports the view that the EGFR is a relevant target for cancer therapy. Different pharmacological and biological approaches have been developed for blocking EGFR activation and/or function in cancer cells. Several anti-EGFR blocking MAbs have been developed. C225 ( cetuximab), a chimeric human-mouse IgG1 MAb, has recently entered Phase II and Phase III clinical evaluation in cancer patients (8). A promising approach for the therapeutic blockade of EGFR signaling has been recently developed with the discovery of low-molecular-weight compounds that inhibit ligand-induced activation of the EGFR tyrosine kinase activity necessary for all receptor-activated intracellular signaling (2, 9). Among various quinazoline-derived agents that have been tested as anticancer drugs in preclinical models, ZD1839 (Iressa) is an orally active, selective EGFR-TKI, which is currently in Phase III clinical trials in patients with non-small cell lung cancer (9–13).

Previous preclinical data has demonstrated the enhancement of the antitumor activity of ionizing radiation by treatment with the anti-EGFR MAb C225 (14–17). In this study, we evaluated the antiproliferative and the proapoptotic activity of ZD1839 in combination with ionizing radiation in human colon (GEO), ovarian (OVCAR-3), non-small cell lung (A549 and Calu-6), and breast (MCF-7 ADR) cancer cell lines, that express both TGFα and EGFR (12). Furthermore, the antitumor activity, the effects on mice survival, and the potential effects on tumor-induced neovascularization of this combination were also tested in nude mice bearing established GEO colon cancer xenografts.

### MATERIALS AND METHODS

**Materials.** Clinical grade ZD1839 (Iressa) was kindly provided by AstraZeneca (Macclesfield, United Kingdom).

**Cell Lines.** Human GEO colon cancer, A549 and Calu-6 non-small cell lung cancer, and OVCAR-3 ovarian cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 ADR breast cancer cells were a generous gift of Dr. D. Del Bufalo, Istituto Nazionale Tumori “Regina Elena,” Rome, Italy. The cell lines were maintained in complete culture media consisting of DMEM (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, penicillin (100 UI/ml), streptomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

**Ionizing Radiation Treatment and Growth in Soft Agar.** Exponentially growing cancer cells were irradiated in 100-mm tissue-culture dishes (Becton Dickinson, Lincoln Park, NJ) using a 6-MV photon linear accelerator (General Electric). After irradiation, cells were trypsinized, and 10⁴ cells/well were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multidish cluster dishes (Becton Dickinson) and treated (in the combination treatment experiments) every day for a total of 4 days with different concentrations of ZD1839. In the experiments in which cancer cells were treated with ZD1839 alone, treatment was performed every day for a total of 4 days with different concentrations of ZD1839. After 10–14 days, the cells were stained with nitro blue tetrazolium (Sigma, Irvine, United Kingdom) and the percent of Induction of Apoptosis.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ionizing radiation IC₅₀ (cGy)</th>
<th>ZD1839 IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEO</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Calu-6</td>
<td>125</td>
<td>0.15</td>
</tr>
<tr>
<td>A549</td>
<td>150</td>
<td>0.8</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>85</td>
<td>0.15</td>
</tr>
<tr>
<td>MCF-7 ADR</td>
<td>110</td>
<td>0.05</td>
</tr>
</tbody>
</table>

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**Flow Cytometric Analysis of Cell Cycle Distribution and of Induction of Apoptosis.** To evaluate the induction of apoptosis, 10⁵ cells (GEO and OVCAR-3) were plated in complete medium in 60-mm tissue culture dishes (Becton Dickinson) and treated every day for a total of 3 days with different concentrations of ZD1839 or on day 1 with ionizing radiation. For the combined treatment, cells were treated on day one with the indicated concentration of ionizing radiation and every day for 3 days with the indicated concentration of ZD1839. After 4 days, both adherent and detached cells were harvested. Flow cytometric analysis of apoptotic cell death was performed on cell pellet that had been fixed in 70% ethanol, washed in PBS, and mixed with RNase (Sigma) and propidium iodide (Sigma) solution as reported previously(18). DNA content was analyzed by a FACScan flow-cytometer (Becton Dickinson, San Jose, CA) coupled with a Hewlett Packard computer, and the percentage of apoptotic cells was calculated by gating the hypodiploid region on the DNA content histogram using the LYSYS software (Becton Dickinson) as reported previously (18). Cell cycle

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data analysis was performed using the CELL-FIT software (Becton Dickinson) as reported previously (19).

**Immunoprecipitation and Western Blotting.** Total cell protein extracts were obtained as previously described (10). Protein extracts were immunoprecipitated with 528 anti-EGFR MAb (kindly provided by Dr. J. Mendelsohn, University of Texas M. D. Anderson Cancer Center, Houston, TX), as reported previously (10). For Western blot analysis, immunoprecipitates (50 μg/lane) were resolved by 7.5% SDS-PAGE and were probed with either an anti-human EGFR MAb (Transduction Laboratories, Lexington, KY) or the PY20 anti-P-tyrosine MAb (Transduction Laboratories). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom), as described previously (10). For Western blot analysis of bcl-2, bcl-xL, phospho-akt, or akt expression, 50 μg/lane were resolved by 12.5% SDS-PAGE and probed with an anti-human bcl-2 MAb, an anti-human bcl-xL MAb, an anti-human phospho-akt MAb, and an anti-human akt MAb.

**GEO and A549 Xenografts in Nude Mice.** Five- to 6-week-old female BALB/c athymic (nu/nu) mice were purchased from Charles River Laboratories, Milan, Italy. The research protocol was approved and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for 1 week before injection with cancer cells. Mice were given injections s.c. into their dorsal flank with 10^7 GEO or A549 cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). For each cancer cell line, after 7 days, when established tumors of ~0.1–0.3 cm^3 in volume were palpable, 10 mice/group were treated i.p. with ZD1839 (100 mg/kg/dose, days 1 to 5 each week for 4 weeks) and/or received RT treatment (10 Gy/dose daily, days 1–4). Tumor size was measured using the formula (π/6) × (larger diameter) × (smaller diameter)^2.

**Immunohistochemical Analysis.** Immunocytochemistry was performed on formalin-fixed, paraffin-embedded tissue sections (5 μm) of GEO xenografts as previously reported (12). After overnight incubation with the appropriate primary antibody at 4°C, sections were washed and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution, Vectastain ABC kit; Vector Laboratory, Burlingame, CA), washed again, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as described previously (12). The slides were then rinsed in distilled water, counterstained with hematoxylin and mounted. The following antibodies were used: an anti-Ki67 MAb (clone MIB1; DBA, Milan, Italy) used at 1:100 dilution; an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) used at 1:100 dilution; and an anti-EGFR MAb (clone 22C11; GenWay Biotech, USA) used at 1:100 dilution.
Biotechnologies, Santa Cruz, CA) used at 1:50 dilution; an anti-bFGF rabbit polyclonal antibody (Santa Cruz) used at 1:200 dilution; and an anti-human TGFα mouse MAb (Ab-2; Oncogene Science, Manhasset, NY) used at 1:100 dilution. To determine the percentage of positive cells, at least 1000 cancer cells per slide were counted and scored (12). New blood vessels were detected using a MAb raised against human Factor VIII-related antigen (Dako, Milan, Italy) at a dilution of 1:50 and were stained with a standard immunoperoxidase method (Vectastain ABC kit). Each slide was scanned at low power (×250 microscope magnification, 0.37 mm²). Five fields were analyzed, and for each of them, the number of stained blood vessels was counted. For individual tumors, microvessel count was scored by averaging the five field counts (12).

Statistical Analysis. The Student’s t test and the Mantel-Cox log-rank test were used to evaluate the statistical significance of the results. All of the Ps represent two-sided tests of statistical significance. All of the analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

RESULTS
Inhibition of Cell Growth in Soft Agar. As shown in Table 1, we first evaluated the effects of ionizing radiation or ZD1839 treatment on the cloning efficiency in soft agar of four human epithelial cancer cell lines. We selected human cancer cell lines of different histology with variable degrees of EGFR expression, ranging from ~40,000 (GEO and Calu-6) to 80,000 (A549) to 150,000 (OVCAR-3) to 250,000 (MCF-7 ADR) EGF binding sites/cell (10, 19). Ionizing radiation treatment caused a dose-dependent inhibition in soft agar growth in all of the cell lines with an IC₅₀ ranging from 85 to 125 cGy. Treatment with the EGFR-selective TKI ZD1839 resulted in a dose-dependent inhibition of colony formation with an IC₅₀ of ~0.05–0.15 µM in all of the cancer cell lines tested.

We next determined whether the combined treatment with ionizing radiation and ZD1839 could enhance the antiproliferative effect of single treatment. A supra-additive inhibitory effect on growth was observed in all of the cells at all of the doses of ZD1839 and ionizing radiation tested in combination. As an example, in GEO cells, single treatment with ionizing radiation, 50 cGy, or with ZD1839, 0.05 µM, each caused ~20–25% growth inhibition, whereas the combined treatment caused >75% inhibition of colony formation in soft agar (Fig. 1A). A similar degree of potentiation of growth inhibition was observed with the other three cancer cell lines (Fig. 1, B, C, and D).

Cell Cycle Disruption and Induction of Apoptosis. To evaluate whether ionizing radiation and/or ZD1839 treatment could cause any specific perturbation of the cell cycle, an analysis of GEO and OVCAR-3 cells treated with different doses of ionizing radiation and/or ZD1839 was performed (Table 2). Compared with control untreated cells, ZD1839 alone caused an accumulation of cells in the G₀-G₁ phases in both of the cell lines. For example, in GEO cells the percentage of G₀-G₁ cells increased from 61.4 to 81.2% after treatment with 1 µM ZD1839 (Table 2). In both of the cell lines, treatment with ionizing radiation induced an increase in G₂-M phases and a slight reduction in S phase, compared with controls. As an example, in OVCAR-3 cells, the percentage of G₂-M cells increased from 10.8 to 18.1% after exposure to 250 cGy of ionizing radiation (Table 2). A more complex cell cycle perturbation was caused in both of the cell lines by the combined treatment with ionizing radiation and ZD1839, with an accumulation of cancer cells in both G₀-G₁ and G₂-M and an accompanying reduction in S phase (Table 2). We next tested whether the combined treatment with ZD1839 and ionizing radiation was accompanied also by increased programmed cell death. Treatment with ionizing radiation increased apoptotic cell death in a dose-dependent manner in both GEO and OVCAR-3 cells.
The addition of ZD1839 at low doses (0.05–1.0 μM) to H9262 M, which induce little or no apoptosis-potentiated ionizing radiation, induced programmed cell death in both of the cell lines by 2- to 3-fold (Fig. 2).

We next evaluated whether the combination of radiotherapy and EGFR blockade could affect the expression and/or the activation of intracellular proteins that are involved in antiapoptotic signaling. As shown in Fig. 3A, ZD1839 (1 μM) alone, or in combination with ionizing radiation (100 cGy) for 6 or 24 h, induced a significant suppression of EGFR autophosphorylation in GEO cells, whereas no change was induced by radiotherapy alone. Although ionizing radiation (100 cGy) did not significantly affect the expression of bcl-2 and bcl-xL and did not change the content of the active, phosphorylated form of akt, ZD1839 (1 μM) treatment caused inhibition in the expression of the antiapoptotic proteins bcl-xL and bcl-2, and the suppression of phosphorylated akt (Fig. 3C–F). These changes were even more pronounced when GEO cells were treated with the combination of ionizing radiation and ZD1839.

**Inhibition of Xenograft Growth in Nude Mice.** We also evaluated the effects of ZD1839 and ionizing radiation in vivo. We have previously tested the effect of different novel therapeutic agents in nude mice bearing GEO colon cancer xenografts (10, 17–18). GEO cells form moderately differentiated adenocarcinomas that express both the EGFR and TGFα, when injected s.c. in immunodeficient mice (12). When GEO colon cancer cell xenografts were established in nude mice, the mice were given RT or were treated i.p. with ZD1839. As illustrated in Fig. 4, treatment with ZD1839 (100 mg/kg/dose daily for 4 weeks) and with ionizing radiation (10 Gy/dose daily, days 1–4 of the 1st week) caused a suppression of tumor growth in all of the mice. In mice that received ZD1839 plus RT, GEO
tumors grew very slowly for \( \sim 45-50 \) days after the end of treatment, when they resumed a growth rate similar to controls (Fig. 4A). The delayed GEO tumor growth in the ZD1839-plus-RT-treated group was accompanied by a prolonged life span of mice that was significantly different compared with controls \((P < 0.001)\), ZD1839-treated group \((P < 0.001)\), or RT-treated group \((P < 0.001)\). Furthermore, 10% of mice in this group were still alive without any histological evidence of tumor 26 weeks after GEO cancer cell injection (Fig. 4B and data not shown). Similarly, in nude mice bearing established human A549 lung adenocarcinoma xenografts, a cooperative antitumor effect was observed when ZD1839 was used in combination with ionizing radiation, with a significant reduction of tumor growth at the end of the treatment in all of the mice as compared with untreated mice or with single agent-treated mice (Fig. 5). Immunohistochemical evaluation of GEO tumor cell proliferation, as assessed by Ki67 nuclear staining, revealed a marked inhibition with both ZD1839 and RT treatment (Table 3; Fig. 6). Furthermore, only \( \sim 10\% \) of the cancer cells were Ki67 positive in GEO tumors from the group treated with ZD1839 plus RT (Table 3). Furthermore, expression of angiogenic growth factors, such as TGF, bFGF, and VEGF was significantly reduced in tumors from this group of mice (Table 3). Tumor-induced vascularization was quantified by immunohistochemistry as microvessel counts in the areas of most intense neovascularization, using an anti-Factor VIII-related-antigen MAb. ZD1839 treatment reduced the number of microvessels (from 18 to 10 microvessels/field as compared with control mice), whereas RT determined only a slight reduction (Table 3). An almost complete suppression of tumor microvessels was observed after combined treatment with ZD1839 and RT (Table 3; Fig. 6).

**DISCUSSION**

In the past 10 years, increasing experimental and clinical evidence has been accumulated on the possibility of combining conventional cancer therapies, such as cytotoxic drugs, hormonal therapy, or radiotherapy, with agents that selectively block
key proteins, such as the EGFR, which control cancer cell survival, proliferation, invasion, and metastasis (20–22). This seems a promising therapeutic approach for various reasons. First, because the targets for these drugs and their mechanism(s) of action are different from those of cytotoxic drugs and of ionizing radiation, it is conceivable that they can be combined without potential cross-resistance. Second, alterations in the expression and/or the activity of genes that regulate mitogenic signals not only can directly cause dysregulation of cell growth but also may affect the sensitivity of cancer cells to chemotherapy, hormonal therapy, or radiotherapy (20–22). High EGFR expression has also been found in cancer cells that are resistant to cytotoxic drugs and to ionizing radiation (4, 23). In human squamous A431 cancer cells that have an amplified EGFR gene and high EGFR protein expression, treatment with ionizing radiation induces the release of TGFβ and the activation of the EGFR tyrosine kinase (7). A recent report by Lammering et al. (24) has shown that overexpression of a functionally inactive, dominant-negative EGFR mutant gene in MDA-MB-231 cells causes down-regulation of EGFR phosphorylation with subsequent tumor cell radiosensitization. Taken together, these studies suggest that high EGFR expression and activation could be a survival response to counteract apoptotic signals in cancer cells exposed to ionizing radiation or to cytotoxic drugs (5).

In the present study, we have shown that treatment with the selective EGFR-TKI ZD1839 significantly potentiates the cytotoxic effects of ionizing radiation in human cancer cell lines that express functional EGFRs. The growth-inhibitory effect of the combined treatment with ZD1839 and ionizing radiation is accompanied by a perturbation of the cell cycle with a marked reduction of cells in S phase and an accumulation in both G0-G1 and G2-M phases. Moreover, ZD1839 treatment enhances radiation-induced apoptosis in these cells. These events are paralleled by inhibition in the expression and/or the function of intracellular signaling proteins that mediate antiapoptotic pathways such as Bcl-2, Bcl-xL and akt. Treatment of mice bearing GEO tumors with ZD1839 plus RT was started on day 7 after tumor cell injection, when tumor volume was ~0.2–0.3 cm3. Mice were treated i.p. daily from day 1 to day 5 with ZD1839 (2.5 mg/dose) and/or received RT treatment (10 Gy/dose daily, days 1 to 4 of the first week). Analysis was performed on day 21 after tumor cell injection. The percent (±SD) of specifically stained GEO cancer cells for Ki67, TGFβ, bFGF, or VEGF was recorded. To determine the percentage of positive cells, ~1000 cancer cells were scored. The number of microvessels for field (±SD) was measured using a mAb raised against the human Factor VIII-related antigen and was scored by averaging five field counts of three individual tumors for each group.

### Table 3 Immunohistochemical evaluation of GEO xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor size cm³</th>
<th>Proliferative activity (Ki67)</th>
<th>TGFβ</th>
<th>bFGF</th>
<th>VEGF</th>
<th>Microvessel counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.7 (±0.2)</td>
<td>70 (±5)%</td>
<td>65 (±7)%</td>
<td>55 (±5)%</td>
<td>60 (±4)%</td>
<td>18 (±3)</td>
</tr>
<tr>
<td>RT</td>
<td>0.26 (±0.08)</td>
<td>45 (±5)%</td>
<td>65 (±10)%</td>
<td>50 (±5)%</td>
<td>55 (±7)%</td>
<td>15 (±2)</td>
</tr>
<tr>
<td>ZD1839</td>
<td>0.24 (±0.05)</td>
<td>25 (±5)%</td>
<td>30 (±5)%</td>
<td>15 (±4)%</td>
<td>20 (±5)%</td>
<td>10 (±1)</td>
</tr>
<tr>
<td>Combination</td>
<td>0.05 (±0.01)</td>
<td>10 (±3)%</td>
<td>8 (±2)%</td>
<td>6 (±2)%</td>
<td>5 (±1)%</td>
<td>1 (±1)</td>
</tr>
</tbody>
</table>

**Fig. 6** Immunohistochemistry of GEO tumors. Mice bearing GEO tumor xenografts were treated as described in “Materials and Methods” and in the legend to Fig. 4. A, Ki67 nuclear staining in GEO control tumor; B, Ki67 nuclear staining in GEO tumor treated with the combination of ZD1839 plus RT; C, microvessel staining in GEO control tumor; D, microvessel staining in GEO tumor treated with the combination of ZD1839 plus RT. ×25.
established human GEO colon cancer xenografts with radiotherapy in combination with ZD1839 causes long-term GEO tumor growth regression with a significant improvement in mice survival and an ~10% cure. Finally, the combined treatment with RT plus ZD1839 was accompanied by a significant potentiation in the inhibition of TGFα, VEGF, and bFGF expression in cancer cells, which resulted in significant antiangiogenic effects as determined by immunohistochemical count of neovessels within the GEO tumors.

The results of the present study are in agreement and extend recent experimental work on the combination of the anti-EGFR blocking antibody C225 and ionizing radiation. Several groups including our own have reported studies demonstrating a potentiation of the antitumor activity in vitro and in vivo of ionizing radiation by treatment with C225 in several xenograft models of human vulvar squamous (A431), head and neck (SSC cell lines), or colon (GEO) cancers (14–17). Similar to results of the combined treatment with chemotherapy, the potentiation of radiotherapy effects after treatment with C225 is accompanied by an increase in apoptosis, a reduction in DNA repair mechanisms in cancer cells and by inhibition of tumor-induced angiogenesis in vivo (15–17). Recent data have been reported on the combination of ZD1839 and radiotherapy. ZD1839 treatment has an additive or synergistic effect when combined with ionizing radiation in several human non-small cell lung cancer cell lines and in the LoVo human colon carcinoma xenograft model (25–27).

The results of this study are of potential clinical interest. In fact, they provide a rationale for combining ZD1839 and radiotherapy in the treatment of human epithelial cancers expressing EGFR. ZD1839 is in advanced clinical development alone and in combination with cytotoxic drugs and can be safely administered as long-term oral treatment with very mild toxicity and without affecting the pharmacokinetics and the side effects of chemotherapy (28–30). Furthermore, preliminary clinical data suggest that the combination of ionizing radiation and other EGFR-targeted approaches, such as the anti-EGFR MA b C225 is feasible and has antitumor activity. In fact, a pilot study evaluating C225 in combination with radiotherapy has shown that, of 15 patients with locally advanced head and neck cancer, 13 patients experienced long-lasting complete responses and 2 had a partial response when treated with C225 and radiotherapy (median duration of response for all patients of 28 months; Ref. 31).

ACKNOWLEDGMENTS

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REFERENCES

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