Synergistic Effects of Gemcitabine and Gefitinib in the Treatment of Head and Neck Carcinoma

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Abstract

Although the combination of gemcitabine and radiation produces a high frequency of complete responses in the treatment of locally advanced head and neck cancer, substantial toxicity suggests that an improvement in the therapeutic index is required. The purpose of this study was to determine if gefitinib could improve the efficacy of gemcitabine and if drug schedule is important. We hypothesized that gemcitabine followed by gefitinib would be superior to the opposite order because of both cell cycle and growth factor signaling interactions. Using UMSCC-1 cells in vitro, we confirmed that gefitinib arrested cells in G1 and suppressed phospho-epidermal growth factor receptor (pY845EGFR) and that gemcitabine arrested cells in S phase and stimulated pY845EGFR. The schedule of gemcitabine followed by gefitinib caused arrest of cells in S phase. Gefitinib suppressed gemcitabine-mediated pS473AKT stimulation. This schedule caused decreased pS473AKT, increased poly(ADP-ribose) polymerase cleavage, and increased apoptosis compared with gemcitabine alone. The schedule of gefitinib followed by gemcitabine also caused suppression of pY845EGFR but arrested cells in G1. This schedule in which gefitinib was used first was associated with stable levels of pS473AKT and minimal poly(ADP-ribose) polymerase cleavage and apoptosis. These results were reflected in experiments in nude mice bearing UMSCC-1 xenografts, in which there was greater tumor regression and apoptosis when animals received gemcitabine followed by gefitinib during the first week of therapy. These findings suggest that the schedule of gemcitabine followed by gefitinib may increase the therapeutic index over gemcitabine alone and, combined with clinical data, encourage exploration of combination of gemcitabine, EGFR inhibitors, and radiation.

Introduction

The combination of radiation with concurrent chemotherapy has been convincingly shown to be superior to radiation alone in the treatment of advanced head and neck cancers (1, 2). However, this approach may have reached its maximum in therapeutic index for further dose escalation in cytotoxic chemotherapy and radiation has been associated with unacceptable (grade 4) acute mucositis and late pharyngeal fibrosis producing dysfunctional swallowing (3–6). These toxicities have served as a major barrier to intensifying therapy and thus to improving outcome in head and neck cancer.

To increase the therapeutic index, research strategies have focused on developing tumor-specific therapies to exploit biological differences between normal cells and cancer cells. One such promising target is the epidermal growth factor (EGF) receptor (EGFR), which is overexpressed in the majority of head and neck cancers and is associated with aggressive tumor behavior and poor clinical outcome (7, 8). In fact, a recent multivariate analysis showed that increased EGFR expression has a stronger effect on prognosis than the presence of a p53 mutation (9). Recently, a randomized trial for head and neck cancer showed that the combination of an EGFR inhibitor (C225, Cetuximab, Erbitux) and radiation produced a statistically significant improvement in local control and survival compared with radiation alone (10).

One tactic for targeting EGFR has been to develop small-molecule tyrosine kinase inhibitors that competitively inhibit the autophosphorylation of the catalytic domain of the ErbB family of receptors (11–13). Gefitinib (ZD1839, Iressa), which binds reversibly to the receptor tyrosine kinase of EGFR with high affinity, is a member of this class of compounds (14). Phase III trials have shown gefitinib to be generally well tolerated (15, 16) and two large randomized phase II studies showed gefitinib to be effective in some patients with previously treated non–small-cell lung cancer (17, 18). Although two phase III trials have not shown gefitinib to be effective when combined with chemotherapy in the treatment of non–small-cell lung cancer (15, 16), there is preclinical evidence that suggests that gefitinib may enhance the antitumor activity of a variety of cytotoxic drugs, such as cisplatin and fluorouracil (19–21).

Given the potential of gefitinib to enhance the effects of other chemotherapeutic agents, we decided to evaluate the combination of gefitinib with gemcitabine (2′,2′-difluorodeoxycytidine). We have shown that gemcitabine is an effective radiosensitizer in head and neck cancer in both preclinical and clinical studies (1, 22–24). Although two phase III trials have not shown gefitinib to be effective when combined with chemotherapy in the treatment of non–small-cell lung cancer (15, 16), there is preclinical evidence that suggests that gefitinib may enhance the antitumor activity of a variety of cytotoxic drugs, such as cisplatin and fluorouracil (19–21).

To test this hypothesis, we first needed to assess the efficacy and toxicity of combining gefitinib with gemcitabine before testing the combination of these two drugs with radiation.

Thus, in this study, we did in vitro studies to investigate the effects of combining gefitinib with gemcitabine on growth factor signaling, cell cycle progression, and apoptosis before testing the combination of these two drugs with radiation. We subsequently evaluated tumor response to this combination therapy compared with single-agent therapy in a mouse xenograft model (25). Because gefitinib arrests cells in the G1 phase of the cell cycle (26) and because gemcitabine requires entry into S phase to produce

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cytotoxicity and radiosensitization (25), we hypothesized that the schedule of drug administration would affect treatment efficacy. Additionally, a previous study has shown that the response of tumor cells to the combination of gemcitabine with a pan-erbB tyrosine kinase inhibitor, CI-1033, depends on the schedule of therapy (27). Therefore, within both our in vitro and in vivo systems, we also assessed if the order of drug delivery would affect the therapeutic response.

Materials and Methods

Reagents. Gemcitabine was provided by Eli Lilly and Co. (Indianapolis, IN). Gefitinib (Iressa, ZD1839) was provided by AstraZeneca (Macclesfield, Cheshire, United Kingdom). Phospho-EGFR (Y845), phospho-AKT (Ser473), total AKT, phospho-extracellular signal-regulated kinase (ERK; Thr202/Y204), and poly(ADP-ribose) polymerase antibodies were purchased from Cell Signaling Technology (Beverly, MA). EGFR antibody (sc-63) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). KI67 (clone 35) was from BD PharMingen (San Jose, CA), β-actin (clone AC-15) was from Sigma (St. Louis, MO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam (Cambridge, MA).

Cell culture. Human squamous cell carcinoma cell lines UMSCC-1, UMSCC-6, UMSCC-11B, UMSCC-14A, and UMSCC-38 were obtained courtesy of Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). The cell lines were grown in RPMI 1640 supplemented with 10% cosmic calf serum (Hyclone, Logan, UT). All the experiments were conducted in serum-containing media. Cultures were saved in Adobe Photoshop as uncompressed 8-bit gray-scale tiff files. For quantitation of protein levels, films were scanned and images plus (Amersham Biosciences, Piscataway, NJ) and exposing them to X-ray was detected by incubating membranes with enhanced chemiluminescence secondary antibody (Cell Signaling Technology, Beverly, MA) for 1 hour at TBS and probed with horseradish peroxidase–conjugated antirabbit (GAPDH) antibody was purchased from Abcam (Cambridge, MA).

In vitro cytometry. Floating cells were harvested from all plates and counted using a Coulter Counter (Beckman Coulter, Hialeah, FL) whereas adherent cells were trypsinized, counted, washed, and resuspended along with the corresponding floating cells. The combined cells were then pelleted and fixed by dropwise addition of 70% ice-cold ethanol and stored at 4°C overnight. Slides were then washed again in PBS, incubated in secondary antibody for 1 hour, washed, and prepared with a coverslip after a drop of ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) was added to each sample. Fluorescence images were acquired using a Zeiss LSM 510 META confocal microscope at the University of Michigan Cell and Developmental Biology core facility. For quantification of pY845EGFR staining, images were saved in Adobe Photoshop as uncompressed 8-bit gray-scale tiff files and analyzed using ImageJ 1.32 (NIH). For KI67 and terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, 200 cells in three random fields were counted.

In vivo tumor growth studies. UMSCC-1 cells (5 × 10⁶) were transplanted into the flanks of nu/nu CD-1 nude mice. When tumors reached an average volume of 100 mm³, the mice were randomized into five groups and treatment was initiated. Gemcitabine was dissolved in 0.9% sodium chloride and given via i.p. injection on days 1, 8, and 15 (days 7, 14, and 21 for animals receiving gefitinib first in combination with gemcitabine) at a dose of 400 mg/kg. Gefitinib was dissolved in water and given via oral gavage on days 2 to 6, 9 to 13, and 16 to 20 (1-5, 8-12, and 15-19 for animals receiving gefitinib first in combination with gemcitabine and those receiving gefitinib alone) at a dose of 100 mg/kg. Tumor volume was calculated according to the equation for a prolate spheroid: tumor volume = (π/6) × (L × W³), 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 56, at which point the animals were sacrificed. For immunostaining studies, mice were treated for one complete cycle (i.e., 1 week). Twenty-four hours after the completion of the treatment, animals were sacrificed; tumors were harvested and fixed in formalin for 24 hours before being stored in 70% ethanol. Animals were handled according to the established procedures of the University of Michigan Laboratory Animals Maintenance Manual.

Statistics. Results are presented as mean ± SE of at least three experiments. Student’s t test was used to test the statistical significance of differences. A significance level Threshold of P < 0.05 was used in this study.

Results

We initially screened six squamous carcinoma cell lines (UMSCC-1, UMSCC-6, UMSCC-11B, UMSCC-12, UMSCC-14A, and UMSCC-38) for EGFR phosphorylation (at the tyrosine-845 site) on EGF treatment (10 ng/mL; Fig. 1A). Among these six cell lines, UMSCC-1 cells showed a median level of EGFR activation. We thus selected UMSCC-1 cells for further study with the consideration that this cell line might represent a larger patient population. Next, we analyzed the effects of gefitinib concentration and duration on the phosphorylation of EGFR and its downstream target, ERK. Gefitinib produced a dose-dependent inhibition of both phosphorylated EGFR and phosphorylated ERK (Fig. 1B).

After establishing that EGFR in UMSCC-1 cells responded to EGF stimulation and was inhibited by gefitinib, we analyzed the effects of gefitinib and gemcitabine on cell growth and apoptosis in vitro. We tested various concentrations of gemcitabine (range, 10-1,000 nmol/L) and gefitinib (range, 1-30 μmol/L) to obtain IC₅₀ values (data not shown). As a first step, cells were exposed to 100 nmol/L gemcitabine and/or 10 μmol/L gefitinib for 24 to 96 hours in a number of combinations (as illustrated in Fig. 2A) to evaluate cell...
growth and apoptosis (Fig. 3 A and B). We observed that treatment with 10 μmol/L gefitinib alone caused significant growth arrest of UMSCC-1 cells at 72 hours compared with control conditions (Fig. 3 A) whereas treatment with 100 nmol/L gemcitabine alone resulted in about 60% cell death as measured by floating cells at 72 hours (Fig. 3 B). More than 90% of floating cells were dead as determined by trypan blue exclusion assay. As shown in Supplementary Fig. S1, the primary mode of cell death was apoptosis.

We were now in a position to investigate if the order of drug delivery would affect the efficacy of combination of gemcitabine and gefitinib. It is known that gemcitabine requires cells to enter S phase to produce cytotoxicity and radiosensitization (25). Conversely, gefitinib causes cell cycle arrest in G1 phase (26). Thus, we hypothesized that the greatest synergy would be seen if gemcitabine were administered before gefitinib. This hypothesis was assessed in UMSCC-1 cells in vitro using cell growth assays. In this assay, we observed a decrease in cell number of 39 ± 2% at 72 hours when gemcitabine (100 nmol/L) was administered 24 hours before gefitinib (10 μmol/L) from day 0. In contrast, we observed a relative increase in cell number of 34 ± 1% when gefitinib was administered first (Fig. 3 A). Gemcitabine-mediated toxicity was also inhibited by pretreatment with 10 μmol/L gefitinib at higher, toxic concentrations of gemcitabine (1 μmol/L), showing significant protection by gefitinib when it is administered before gemcitabine (data not shown).

Because we hypothesized that the sequence-dependent effects observed with gefitinib and gemcitabine may be related to the individual effects of these two drugs on the cell cycle, we did cell cycle analyses on UMSCC-1 cells that were treated according to the schema described in Fig. 2. Both gefitinib and gemcitabine affected the cell cycle of UMSCC-1 cells as expected (Fig. 3 C). In particular, after 24 hours of treatment with gefitinib, the G1 population increased markedly, from 40% at pretreatment to 70% at posttreatment. Cells remained in G1 phase for up to 72 hours in this treatment group. In contrast, exposure to gemcitabine blocked cells at the G1-S boundary at 24 hours, and by 72 hours posttreatment, 60% of cells were apoptotic as revealed by sub-G1 DNA content. A comparison of cell cycle distribution resulting from treatment with gefitinib followed by gemcitabine versus the reverse sequence revealed a striking difference (Fig. 3 C).
Specifically, treatment with gemcitabine first blocked cells at the G₁-S boundary. Cells that were subsequently exposed to gefitinib underwent apoptosis as indicated by the increase in sub-G₁ DNA content (Fig. 3B and C). In contrast, exposure to gefitinib first caused cells to accumulate in the G₁ phase of the cell cycle and this pattern of G₁ accumulation was not altered after subsequent treatment with gemcitabine. This finding suggests that gefitinib-induced G₁ arrest accounts for the protection from gemcitabine-mediated apoptosis observed in our cell growth and survival experiments.

Because our flow cytometry experiments had suggested that the combination of gemcitabine followed by gefitinib produced cell cycle arrest and apoptosis, we then focused on the relationships among drug effects on EGFR phosphorylation, molecular effectors of apoptosis, and cell cycle arrest. We found that 10 μmol/L gefitinib significantly inhibited phosphorylation of EGFR (pY845EGFR) for 24 hours. Interestingly, gemcitabine-treated cells showed an initial increase in pY845EGFR levels that lasted for 24 hours before decreasing to the basal level (Fig. 3D), which was blocked by treatment with gefitinib. At 72 hours post gemcitabine...
treatment, the decreased level of pY845EGFR was well correlated with increased cleavage of the poly(ADP-ribose) polymerase and was inversely related to the antiapoptotic protein pS473AKT. These findings suggest that EGFR activation may represent a survival response in tumor cells following gemcitabine treatment and that inhibition of EGFR phosphorylation by gefitinib may block this initial survival response and promote apoptosis.

As a prelude to investigating the combination of gefitinib and gemcitabine in an in vivo model, we first evaluated the inhibition of EGFR phosphorylation by gefitinib alone in an UMSCC-1 tumor xenograft model. After a single gavage of a 100 mg/kg dose of gefitinib in mice bearing UMSCC-1 tumor xenografts (~100-200 mm³), pY845EGFR was inhibited through 4 hours and returned to basal levels by 24 hours after treatment (Fig. 4A). This result suggested that daily doses of gefitinib are required to achieve longer inhibition of EGFR phosphorylation. Thus, in a second set of mice, we administered a daily dose of gefitinib (100 mg/kg via oral gavage) for 5 consecutive days. This schedule showed 70% inhibition of EGFR phosphorylation at 24 hours after the last treatment. More importantly, the level of EGFR phosphorylation did not recover fully even after 72 hours (40% inhibition; Fig. 4A). These findings suggest that administering gefitinib in consecutive daily doses extends the duration of gefitinib activity even after cessation of treatment.

After establishing the extent and duration of gefitinib-mediated EGFR inhibition in vivo, we investigated the effects of combining gefitinib and gemcitabine on tumor growth response in our UMSCC-1 tumor model. The individual and combination drug schedules were designed to reflect a clinically relevant approach as gemcitabine is administered once a week and gefitinib is given daily in the clinical setting (see Fig. 2B schema). Gemcitabine followed by gefitinib caused a marked reduction in tumor volume as early as 4 to 7 days after initiation of the treatment whereas all the other treatment regimens resulted in no significant differences (Fig. 4B). However, by the end of three treatment cycles (21 days), both schedules of combination therapy showed equivalent potency, with both causing significant tumor regression compared with all other treatments (Fig. 4C). The effect of the combination treatment lasted only for ~1 week after completion of the treatment; the tumors began to grow at the same rate as in untreated controls (Fig. 4C). Whereas the antitumor effects of both combination regimens were the same, the mice that received gefitinib before gemcitabine exhibited greater weight loss (10% more) when compared with the group who were receiving gemcitabine before gefitinib (Fig. 4D).

To elucidate in vivo the molecular mechanism of the drug interactions and their effects on pY845EGFR, tumor cell proliferation, and apoptosis, we analyzed tumors obtained after one cycle of treatment (7 days) by immunofluorescence for pY845EGFR, the primary target for gefitinib, and Ki67, a marker for cell proliferation; TUNEL staining was used to assess apoptosis (Fig. 5A). We found a 58 ± 5% decrease in pY845EGFR staining in gefitinib treated tumors when compared with the controls. This decrease was confirmed by immunoblotting (Fig. 5B). To analyze tumor cell proliferation and apoptosis, 200 cells were randomly scored for Ki67 and TUNEL positivity. Gefitinib treatment caused a
30% decrease in Ki67-positive cells and a 5% increase in TUNEL-positive cells. Unexpectedly, gemcitabine-treated tumors also resulted in a 29% decrease in pY845EGFR 6 days after a single dose. In this group, Ki67-positive cells were reduced to 60% and TUNEL-positive cells were increased to 26%. Tumor samples from the groups receiving combination therapy showed reductions in both pY845EGFR and Ki67 staining and an increase in TUNEL-positive cells compared with control (Table 1; Fig. 5A).

### Discussion

In this study, we have shown that the combination of gemcitabine followed by the small-molecule tyrosine kinase inhibitor gefitinib in vitro produced a significant increase in apoptosis compared with either agent alone. Drug schedule significantly affected the results of treatment, in that exposure to gefitinib first protected cells from subsequent treatment with gemcitabine. The importance of schedule seems to be a result of effects on both cell cycle progression and growth factor signaling. With respect to cell cycle progression, we observed that antagonism associated with initial exposure to gefitinib was correlated with the gefitinib-induced arrest of cells in G1. As S-phase entry is crucial for gemcitabine-mediated cytotoxicity, the G1 arrest was protective. In contrast, when cells were exposed to gemcitabine first, they entered S phase and, after gefitinib treatment, underwent apoptosis. These results are in agreement with and extend a previous study that examined the schedule dependence of gemcitabine and the pan-ErbB inhibitor CI-1033 (27).

In addition to this interaction at the level of the cell cycle, we found that schedule had an important effect on growth factor signaling. Gemcitabine produced an increase in pY845EGFR levels.

### Table 1. Effect of gemcitabine, gefitinib, and the combination treatment on pY845EGFR, Ki67, and TUNEL staining in UMSCC-1 xenografts

<table>
<thead>
<tr>
<th></th>
<th>pY845EGFR</th>
<th>Ki67</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>41±7</td>
<td>6±2</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>71±9*</td>
<td>25±6*</td>
<td>32±8*</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>42±5*</td>
<td>29±10*</td>
<td>11±3</td>
</tr>
<tr>
<td>Gemcitabine → gefitinib</td>
<td>57±11*</td>
<td>11±3*</td>
<td>58±6*</td>
</tr>
<tr>
<td>Gefitinib → gemcitabine</td>
<td>43±4*</td>
<td>13±2*</td>
<td>48±9*</td>
</tr>
</tbody>
</table>

NOTE: Values are percent control (pY845EGFR) or number of positively staining cells of 200 scored (Ki67 and TUNEL). Tumors were harvested after one cycle of treatment as outlined in Fig. 2B.

* Compared with the controls (P < 0.05).
† Compared with gemcitabine (P < 0.05).
‡ Compared with gefitinib (P < 0.05).

Figure 5. Immunohistochemical staining and immunoblotting of UMSCC-1 xenografts after treatment with gemcitabine and/or gefitinib. A, athymic nude mice bearing UMSCC-1 s.c. xenografts were treated with gemcitabine and/or gefitinib as shown in Fig. 2B for one full cycle. Tumors were harvested and fixed on day 7. Tumor sections were stained for pY845EGFR and Ki67 or TUNEL as described in Materials and Methods. a, untreated; b, gemcitabine alone; c, gefitinib alone; d, gemcitabine followed by gefitinib; e, gefitinib followed by gemcitabine. Magnification, ×40. B, tumor sections were prepared for immunoblotting as described in Fig. 4; pY845EGFR and β-actin (as a loading control) are shown.
beginning as early as 2 hours after treatment that lasted for 24 hours. Although the mechanism of this increase in p\(^{\text{Y845}}\)EGFR is not yet defined, it is possible that this is due to gemcitabine-mediated degradation of Cdc25A phosphatase (28), which is known to directly regulate EGFR phosphorylation (29). Regardless of the mechanism by which gemcitabine treatment increases p\(^{\text{Y845}}\)EGFR, subsequent exposure to gefitinib blocked this increase and was associated with a decrease in p\(^{\text{S473}}\)AKT, the appearance of poly(ADP-ribose) polymerase cleavage, and, as described above, apoptosis. In contrast, although exposure to gefitinib first led to a decrease in p\(^{\text{Y845}}\)EGFR as a direct effect of the drug, subsequent treatment with gemcitabine did not decrease p\(^{\text{S473}}\)AKT levels, and neither poly(ADP-ribose) polymerase cleavage nor substantial apoptosis was observed. Thus, the schedule of gemcitabine followed by gefitinib seemed to be superior to the reverse schedule based on factors related to both cell kinetics and growth factor signaling.

Based on our in vitro findings, we anticipated that the combination of gemcitabine followed by gefitinib would be more effective than the reverse combination schedule in controlling tumor growth. Although this hypothesis held true during the first week of the treatment period, as evidenced both by tumor regression and by immunohistochemistry showing increased apoptosis, tumor growth curves ultimately converged. This is likely due to the fact that by the second and third weeks, the differences in schedules become less distinct in that the last doses of gefitinib in cycle 1 of the “gemcitabine-gefitinib” schedule may affect the first dose of gemcitabine in cycle 2. It will be important in subsequent studies to determine the influence of time between cycles on the effectiveness of treatment.

Although we are unaware of previous studies combining gemcitabine and EGFR inhibitors in head and neck cancer, this combination has been evaluated in pancreatic cancer. For example, cetuximab suppresses the growth of orthotopically implanted pancreatic tumors in nude mice and potentiates the cytotoxic effects of gemcitabine with (30) and without (31) radiation. These results have been extended into clinical trials in pancreatic cancer. Xiong et al. (32) conducted a phase II trial of cetuximab in combination with gemcitabine in 41 EGFR-positive patients with locally advanced, metastatic, or recurrent pancreatic cancer. The 1-year survival rate of 32% was encouraging compared with the 18% 1-year survival reported in historical series of patients treated with gemcitabine alone (33). More recently, the National Cancer Institute of Canada Clinical Trials Group conducted a phase III trial with erlotinib plus gemcitabine versus gemcitabine alone (34). There was a small but significant improvement in overall survival that favored the erlotinib arm with a hazard ratio of 0.81 (95% confidence interval, 0.67-0.97). Erlotinib (OSI 774, Tarceva) did not add significantly to the rate of serious toxicity. Although modest in magnitude, these results represent the first improvement over gemcitabine as systemic therapy for advanced pancreatic cancer and provide proof of principle of the potential benefits of combining EGFR targeted therapy with gemcitabine.

Taken together, these preclinical and clinical findings suggest that the addition of EGFR targeted therapy to gemcitabine can improve the therapeutic index of tumors that are driven by constitutively activated EGFR. However, in both pancreatic studies and the current study, tumors regrew after treatment with gemcitabine and EGFR directed therapy, suggesting that intensification of treatment is required. With respect to head and neck cancer, the addition of radiation therapy seems logical, especially given the results of a recent clinical trial showing that cetuximab plus radiation therapy produces superior local control and survival compared with radiation therapy alone (10). Such preclinical studies are currently under way in our laboratory.

Acknowledgments

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