Combination Treatment with Ionising Radiation and Gefitinib ('Iressa', ZD1839), an Epidermal Growth Factor Receptor (EGFR) Inhibitor, Significantly Inhibits Bladder Cancer Cell Growth in vitro and in vivo

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Tyrosine kinase inhibitors/Radiotherapy/Invasive bladder cancer/Radiosensitizing agents.

Purpose: External beam radiotherapy (EBRT) is the principal bladder-preserving monotherapy for muscle-invasive bladder cancer. Seventy percent of muscle-invasive bladder cancers express epidermal growth factor receptor (EGFR), which is associated with poor prognosis. Ionising radiation (IR) stimulates EGFR causing activation of cytoprotective signalling cascades and thus may be an underlying cause of radioresistance in bladder tumours.

Materials and methods: We assessed the ability of IR to activate EGFR in bladder cancer cells and the effect of the anti-EGFR therapy, gefitinib on potential radiation-induced activation. Subsequently we assessed the effect of IR on signalling pathways downstream of EGFR. Finally we assessed the activity of gefitinib as a monotherapy, and in combination with IR, using clonogenic assay in vitro, and a murine model in vivo.

Results: IR activated EGFR and gefitinib partially inhibited this activation. Radiation-induced activation of EGFR activated the MAPK and Akt pathways. Gefitinib partially inhibited activation of the MAPK pathway but not the Akt pathway. Treatment with combined gefitinib and IR significantly inhibited bladder cancer cell colony formation more than treatment with gefitinib alone (p = 0.001–0.03). J82 xenograft tumours treated with combined gefitinib and IR showed significantly greater growth inhibition than tumours treated with IR alone (p = 0.04).

Conclusions: Combining gefitinib and IR results in significantly greater inhibition of invasive bladder cancer cell colony formation in vitro and significantly greater tumour growth inhibition in vivo. Given the high frequency of EGFR expression by bladder tumours and the low toxicity of gefitinib there is justification to translate this work into a clinical trial.

INTRODUCTION

Bladder cancer is a prevalent disease with 10,200 new cases and 5,000 deaths occurring annually in the UK.1 Prognosis depends on presenting tumour stage and grade, with higher stage, muscle-invasive tumours having a poorer prognosis. The results of radical surgery in patients with lower stage invasive disease are impressive,2 however, 5-year survival rates following radical treatment for tumours of all stages remain low at 40–50%.3,4 Given the morbidity and mortality associated with radical cystectomy,5 there remains a research drive to improve prognosis following bladder-sparing approaches, such as external beam radiotherapy or combination chemo-radiotherapy.

Epidermal growth factor receptor (EGFR) is a transmembranous receptor expressed by 70% of bladder tumours6 and expression is associated with tumour progression and diminished overall survival.7 It has previously been shown using A431 vulval squamous cells that ionising radiation (IR) can activate EGFR leading to cellular proliferation.8,9 Thus it is possible that radiation-induced EGFR activation is an underlying cause of bladder tumour radioresistance. EGFR blockade may therefore be a means of enhancing bladder tumour radio-responsiveness.

EGFR can be inhibited by several methods, including use of monoclonal antibodies (e.g. cetuximab), immunotoxin
conjugates (e.g., pseudomonas immunotoxin), small molecule tyrosine kinase inhibitors (e.g., gefitinib) and antisense oligonucleotides. Gefitinib, binds highly selectively to the ATP binding pocket of the intracellular tyrosine kinase domain of EGFR, preventing autophosphorylation of intracellular tyrosine residues and subsequently inhibiting downstream signalling.\(^{10}\) Disappointing results from large trials of monotherapy gefitinib in patients with chemorefractory lung cancer (INTACT1 and 2)\(^{11,12}\) has led to concentrated efforts to assess gefitinib’s efficacy in multimodality treatment regimens, in combination with more traditional anticancer therapies. Indeed, a recent phase III randomised controlled trial showed that combining high-dose fractionated radiotherapy with an alternative anti-EGFR therapy, cetuximab, significantly improved loco-regional disease control, progression-free and overall survival (hazard ratio for inclusion of cetuximab 0.68, 0.74 and 0.70 respectively), in patients with locally advanced squamous cell carcinoma of the head and neck.\(^{13}\)

We assessed the ability of IR to activate EGFR in bladder cancer cells, using Western blotting and immunoprecipitation techniques, in the presence and absence of gefitinib. We also assessed the effect of IR on the activation status of proteins downstream of EGFR (MAPK, Akt) and the potential of gefitinib to inhibit any radiation-induced activation of these proteins. Finally we used clonogenic assays in vitro and an athymic nude mouse model in vivo to assess the effect of combining gefitinib with IR in bladder cancer cells.

**MATERIAL AND METHODS**

**Cell culture techniques**

Six bladder cancer cell lines (HT1376, J82, RT112, RT4, T24 and UMUC3) were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and the American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured in flasks at 37°C in a 5% CO\(_2\) incubator in Dulbecco’s minimal essential medium with glutamine, sodium pyruvate and glucose additive, 1% non-essential amino acids and 10% fetal calf serum (Invitrogen, Paisley, UK). The use of antibiotic supplements was specifically avoided.

**Total EGFR status of bladder cancer cells**

Total EGFR expression of the 6 bladder cancer cell lines, and also of A431 cells, was assessed using Western blotting. Membranes were probed using polyclonal anti-EGFR antibody (Santa Cruz, California, USA). For all Western blots equivalent protein loading was confirmed using anti-\(\alpha\)-tubulin antibody (Sigma, St Louis, USA). The relative total EGFR expression (in relation to \(\alpha\)-tubulin) was quantified using densitometry software (Biorad Molecular Bioanalyst software, California, USA).

**Activation of EGFR by IR**

An *in vitro* irradiation model was established with A431 cells using a method previously described by Dent *et al.*\(^9\) Briefly, A431 cells were irradiated with 2 Gy IR (using a Pantak 300 irradiator) and lysed at sequential time points. Phosphorylated EGFR levels in the resulting lysates were quantified using Western blotting, with primary polyclonal anti-phospho-EGFR (tyr1086) antibody (Santa Cruz, California, USA). To ensure detection of phosphorylated EGFR a commercially available positive control (PC) was loaded into lane 1 of the Western blot. The technique was then applied to J82 and RT112 cells. Cells were pre-treated with 0.25 \(\mu\)M gefitinib or dimethylsulfoxide (DMSO, Sigma, St Louis, USA) carrier solution (controls) prior to irradiation. Cell lysis was performed prior to irradiation to assess basal phosphorylation levels (Pre) and at 0, 2, 3, 5, 10, 60, 120, 180, 240 and 300 min post-irradiation. Following estimation of lysate protein levels using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA), the two sets of lysates were separated and blotted in duplicate, using Western blotting. The duplicate membranes were probed for total and phosphorylated EGFR.

To confirm genuine detection of activated EGFR the experiment was repeated using immunoprecipitation in RT112 cells. Following irradiation the two treatment groups were lysed at the time points noted above. All phosphotyrosines were precipitated from the lysates using phosphotyrosine antibody (Pierce, Rockford, Illinois, USA).

**Fig. 1.** Total EGFR status of bladder cancer cells. Western blot showing A) the absolute levels of total EGFR expression in each of the 6 bladder cancer cell lines and B) relative expression of total EGFR assessed using densitometry in relation to alpha-tubulin expression.
rosine antibody (Sigma, St Louis, USA) and the membranes were stained with primary antibody to phosphorylated EGFR.

**Activation of proteins downstream of EGFR following bladder cancer cell irradiation**

Lysates prepared as described above were used to examine the total and phosphorylated levels of MAPK and Akt (primary antibodies from Cell Signalling, Massachusetts, USA), in the presence and absence of gefitinib.

**Effect of gefitinib and/or IR on bladder cancer cells in vitro**

Ninety percent confluent cells were trypsinised and seeded in 75 cm² flasks at densities of 100–1,500. Cells were permitted to adhere for 4 hours prior to application of gefitinib alone (0.01 to 10.0 μM), IR alone (1 to 6 Gy) or combined gefitinib/IR (0.01 to 10.0 μM and 1 Gy). In the combination treatment regimen, gefitinib was applied 4 hours after cell seeding and IR administered 2 hours later. Colonies took 8–18 days to develop and were stained with crystal violet (Sigma, St Louis, USA) and manually counted. Media containing fresh dilutions of gefitinib were changed daily. Clonogenic assays were internally controlled using untreated cells (DMSO carrier or 0 Gy IR). Relative plating efficiencies were expressed as percentages relative to the plating efficiency of untreated cells. All experiments were performed in triplicate.

**Fig. 2.** Activation of EGFR by ionising radiation. Western blots showing bimodal activation of EGFR following exposure to ionising radiation in A) A431 cells B) RT112 cells and C) J82 cells.

PC = commercially available positive control
Pre = cell lysate prepared prior to irradiation
p-EGFR = phosphorylated EGFR
T-EGFR = total EGFR

The relative expression of total EGFR determined using densitometry was correlated with the dose enhancement ratio (DER) achieved following application of 0.01 μM gefitinib and 1 Gy IR (DER = the magnitude of enhancement of radiotherapy achieved by addition of a radiosensitizing agent).

**Effect of gefitinib and/or IR on bladder cancer cells in vivo**

Authority to perform the *in vivo* work was granted by the Home Office, project license PIL 40/2556. Seeding density experiments were performed using J82 and RT112 cells in seven-week-old BALB-C athymic nude mice purchased from Harlan Laboratories, Bicester, UK. Cells resuspended in 100 μL matrigel solution (BD Biosciences, California, USA) were inoculated subcutaneously into the flank, under inhalational (isofluorane) general anaesthesia. Tumour volume was determined thus; \(\pi/6 \times (\text{tumour width})^2 \times \text{tumour length}\). Uniform tumour growth did not occur using RT112 cells therefore experiments were curtailed to J82 cells.

1 × 10^5 J82 cells were inoculated into the flanks of 24 mice. Once tumours had reached a volume of 100 mm^3 (day 1) mice were randomised into four treatment groups (6/group); control, gefitinib alone, IR alone or combined gefitinib/IR. Gefitinib was dissolved in carrier solution (0.1% aqueous TWEEN 80, Sigma, St Louis, USA) and administered at a dose of 50mg/kg by oral gavage on days 1–5 and 8–12. Mice not receiving gefitinib received carrier solution by oral gavage. IR was delivered as a single 5 Gy fraction under intraperitoneal (medotamine, Pfizer, New York, USA) general anaesthesia on day 1 (following administration of gefitinib/carryer solution). Anaesthetised mice were irradiated in a custom designed lead shield, which permitted irradiation of tumour tissue only. Mice not randomised to receive IR were anaesthetised and mock irradiated. Tumour measurements were taken weekly, except during active treatment, when they were performed thrice weekly to aid accurate gavage dosing. Mice with tumours exceeding 17mm diameter were culled in accordance with UKCCCR guidelines.

**Statistical analyses**

Statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, USA). Total EGFR levels and DER were correlated using Pearson’s correlation. Colony formation inhibition rates (*in vitro*) and tumour volumes (*in vivo*) were compared using Student’s t testing. The level of statistical significance was taken to be p = 0.05.

**Fig. 3.** Comparison of EGFR activation in RT112 cells, using Western blotting and immunoprecipitation techniques, in presence and absence of gefitinib. The bimodal effect of IR on EGFR activation detected by Western blotting (B) is confirmed using immunoprecipitation (A). The inhibitory effect of gefitinib on radiation-induced EGFR activation is demonstrated using both techniques.
Fig. 4. Activation of proteins downstream of EGFR following irradiation of bladder cancer cells. Western blots showing the effect of IR on the activation of proteins downstream of EGFR in the presence and absence of gefitinib (see text for more detailed description).
RESULTS

Total EGFR status of bladder cancer cells

Absolute and relative total EGFR expression is shown in Fig. 1.

Activation of EGFR by IR (Figs. 2 and 3)

Irradiation of A431 cells resulted in EGFR phosphorylation at the same time points as those previously reported. In J82 and RT112 cells the level of total EGFR did not vary following irradiation. Significant pre-irradiation levels of phosphorylated EGFR were present in both bladder cancer cell lines. Immediately following irradiation, phosphorylated EGFR levels fell (more so for J82 cells), suggesting transient inactivation of EGFR, prior to rising at similar time points (5–10 min, 180 min) to the peaks seen following irradiation of A431 cells. However, the magnitude of increase in phosphorylated EGFR was lower for bladder cancer cells than for A431 cells (Fig. 2). Administration of gefitinib pre-irradiation inhibited the previously detected radiation-induced EGFR activation back to basal phosphorylation levels (Fig. 3). Immunoprecipitation studies in RT112 cells showed the same general bimodal activation of EGFR to that detected using Western blotting (Fig. 3).

Activation of proteins downstream of EGFR following bladder cancer cell irradiation (Fig. 4)

Total MAPK and Akt levels did not vary following exposure to IR in either cell line, irrespective of pre-treatment with gefitinib. In RT112 cells phosphorylated MAPK levels were elevated 60–300 min post-irradiation and pre-treatment with gefitinib produced an overall reduction in phosphorylation levels. In J82 cells MAPK phosphorylation occurred 0–60 min post-irradiation and again pre-treatment with gefitinib caused an overall reduction in phosphorylation levels. In both cell lines Akt activation was detectable 120–300 min post-irradiation. Pre-treatment with gefitinib had no effect on radiation-induced Akt activation.

Effect of gefitinib and/or IR on bladder cancer cells in vitro (Figs. 5 and 6)

Treatment with gefitinib or IR alone caused dosedependent inhibition of colony formation in all cell lines (Fig. 5). Combination treatment using gefitinib and IR resulted in significantly greater inhibition of colony formation than either treatment alone (Fig. 6, Table 1). The increased inhibition in colony formation seen using combination treatment was at least additive and was reflected by a universal reduction in the IC50 dose for gefitinib, when used in combination with IR (Table 2). The DER achieved for all cell lines were positive (1.19–2.00) confirming the benefit of combining the two treatment modalities (Table 3). DER did not correlate with relative expression of total EGFR (Pearson’s correlation; r = 0.318, p = 0.539).

Effect of gefitinib and/or IR on bladder cancer cells in vivo (Fig. 7)

Treatment effects were calculated as percentage change in tumour volume relative to day 1 tumour volume.

A) Gefitinib

B) Ionising radiation

Fig. 5. Dose-response curves for treatment with A) gefitinib and B) IR. Inhibition of colony formation in each of the 6 bladder cancer cell lines following exposure to A) varying concentrations of gefitinib and B) varying doses of IR.
Fig. 6. Combination treatment with gefitinib and ionising radiation in bladder cancer cells. Histograms comparing the inhibition of colony formation following exposure of bladder cancer cells to either gefitinib alone (pale blue bars), combined gefitinib and IR (coloured bars) or 1 Gy IR alone (yellow bar).
J82 tumours grew rapidly, achieving maximum permitted diameter within 24 days. Treatment with gefitinib alone had no significant effect on tumour volume. Treatment with IR significantly retarded tumour growth relative to control tumours (Student’s t-test, p = 0.05). Combination treatment with gefitinib/IR significantly reduced tumour growth greater than treatment with IR alone (Student’s t-test p = 0.04). This effect persisted only for the duration of gefitinib administration, after which the growth curve for the combined treatment regimen reverted to the same gradient as that for untreated tumours, suggesting that gefitinib appeared to negate the effect of ionising radiation.

**DISCUSSION**

We have demonstrated that IR activates EGFR in bladder...
cancer cells at similar time points to those found by Dent \textit{et al.} using A431 cells.\textsuperscript{9} The magnitude of activation was lower than in A431 cells, however, was sufficient to result in activation of the MAPK and Akt pathways. We also showed that radiation-induced EGFR activation can be partially abrogated by gefitinib, thereby identifying a potential radiosensitizing agent in bladder cancer cells.

The overall mechanism of the bimodal radiation-induced EGFR activation is currently unknown. Dent \textit{et al.} showed the second peak of receptor activation occurs due to radiation-induced autocrine production of the EGFR ligand, TGF-\(\alpha\).\textsuperscript{9} However, the cause of early activation remains unclear. Bridges postulates that early radiation-induced ‘activation’ actually results from inhibition of intracellular phosphatases, which under normal homeostatic circumstances maintain EGFR inactive.\textsuperscript{14} Li \textit{et al.} recently reported that the early radiation-induced activation of EGFR is mediated via activation of Src (possibly by radiation-induced production of hydrogen peroxide), whilst confirming that the late activation is related to receptor autophosphorylation.\textsuperscript{15}

When assessing the activation status of proteins downstream of EGFR we found the late peak of EGFR activation resulted in MAPK and Akt activation. Pre-treatment with gefitinib did not affect Akt activation, whereas it globally reduced the level of radiation-induced MAPK activation, albeit at differing time points in differing cell lines. This implies that radiation-induced MAPK activation in bladder cancer cell lines is an EGFR-mediated phenomenon, whereas Akt activation is not wholly EGFR-dependent. Development of specific MAPK and Akt inhibitors for use in combination with IR may enable greater inhibition of cell proliferation and survival than that seen with small molecule tyrosine kinase inhibitors acting further upstream. Activation of downstream pathways irrespective of total EGFR expression level explains, in part, the lack of correlation we found between DER and total EGFR expression levels.

When assessing use of gefitinib and/or IR in bladder cancer cells in \textit{vitro} we found treatment with gefitinib alone yielded similar IC\textsubscript{50} doses to those determined by others assessing gefitinib monotherapy in bladder cancer cells, thereby validating our methodology.\textsuperscript{16} Subsequently we demonstrated that combining gefitinib and IR significantly inhibits bladder cancer cell proliferation rates greater than treatment with either monotherapy. The DER achieved by combining gefitinib with IR did not correlate with total EGFR expression and this finding concurs with the work of others assessing the relationship between total EGFR expression levels and chemosensitisation by gefitinib.\textsuperscript{17} Our rationale for selecting anti-EGFR therapies as radiosensitizing agents depends upon radiation-induced EGFR activation stimulating pro-proliferative downstream pathways. Thus one would expect any radiosensitisation achieved using gefitinib to correlate with the degree of radiation-induced EGFR activation achieved, rather than absolute total EGFR expression levels.

The radiosensitizing capacity of gefitinib has been assessed by others using \textit{in vitro} bladder cancer models. Maddineni \textit{et al.} showed combining gefitinib and IR significantly inhibited cell growth greater than use of IR alone, however gefitinib monotherapy had no effect the bladder cancer lines assessed (S40b and MGH-U1).\textsuperscript{18} Dominguez-Escrig \textit{et al.} assessed the radiosensitizing potential of gefitinib in RT112 and 253J B-V bladder cancer cells.\textsuperscript{19} Combination treatment significantly inhibited cell growth in 253J B-V cells but radiosensitisation only occurred at high, non-clinically relevant, doses of IR in RT112 cells. The radiosensitizing effect of tyrosine kinase inhibitors has also been demonstrated in a number of other cell lines.\textsuperscript{20} Lammering \textit{et al.} demonstrated that irradiating malignant glioblastoma cells treated with an alternative anti-EGFR therapy (transfection with dominant-negative EGFR) resulted in a DER of 1.8.\textsuperscript{21} Thus our results show the DER achieved using gefitinib with clinically relevant doses of IR in bladder cancer cell lines are of the same order of magnitude to those reported in other tumour systems.

We assessed the radiosensitizing potential of gefitinib \textit{in vivo} using an athymic nude mouse model. In J82 cells combining gefitinib and IR significantly reduced tumour growth rates relative to those for treatment with IR alone. Gefitinib monotherapy did not inhibit J82 tumour growth, mirroring the relative \textit{in vitro} insensitivity of J82 cells to gefitinib. Interestingly the inhibition of tumour growth \textit{in vivo} following combination treatment persisted only for the duration of gefitinib administration, suggesting a role for gefitinib in ongoing receptor suppression several days after the initial receptor stimulus. This concurs with data reported by Wakeling \textit{et al.} who found that gefitinib significantly reduced xenograft tumour growth in a range of cancer cell types which was only sustainable for the duration of drug administration.\textsuperscript{22}

Exactly how gefitinib exerts its radiosensitizing capacity is unclear. Several groups have addressed this question and it is likely that a combination of perturbation in cell cycle control, enhancement of radiation-induced apoptosis, inhibition of DNA repair mechanisms and inhibition of angiogenesis underlie the favourable interaction profile of the 2 treatments.\textsuperscript{20}

Overall our work supports the potential clinical role of combination treatment with tyrosine kinase inhibitors and IR in the treatment of invasive bladder cancer. Gefitinib enhances the effect of IR in bladder cancer cell lines both \textit{in vitro} and \textit{in vivo} and this effect is independent of total EGFR expression levels. Given the strength of the data presented here, the high prevalence of EGFR expression in invasive bladder tumours, recent clinical trials using anti-EGFR therapy in combination with radiotherapy\textsuperscript{15} and the low toxicity of gefitinib there is justification to translate this research into a clinical trial in patients with muscle-invasive bladder cancer.
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