Imatinib Radiosensitizes Bladder Cancer by Targeting Homologous Recombination

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Abstract

Radiotherapy is a major treatment modality used to treat muscle-invasive bladder cancer, with patient outcomes similar to surgery. However, radioresistance is a significant factor in treatment failure. Cell-free extracts of muscle-invasive bladder tumors are defective in nonhomologous end-joining (NHEJ), and this phenotype may be used clinically by combining radiotherapy with a radiosensitizing drug that targets homologous recombination, thereby sparing normal tissues with intact NHEJ. The response of the homologous recombination protein RAD51 to radiation is inhibited by the small-molecule tyrosine kinase inhibitor imatinib. Stable RT112 bladder cancer Ku knockdown (Ku80KD) cells were generated using short hairpin RNA technology to mimic the invasive tumor phenotype and also RAD51 knockdown (RAD51KD) cells to show imatinib’s pathway selectivity. Ku80KD, RAD51KD, nonsilencing vector control, and parental RT112 cells were treated with radiation in combination with either imatinib or lapatinib, which inhibits NHEJ and cell survival assessed by clonogenic assay. Drug doses were chosen at approximately IC40 and IC10 (nontoxic) levels. Imatinib radiosensitized Ku80KD cells to a greater extent than RAD51KD or RT112 cells. In contrast, lapatinib radiosensitized RAD51KD and RT112 cells but not Ku80KD cells. Taken together, our findings suggest a new application for imatinib in concurrent use with radiotherapy to treat muscle-invasive bladder cancer.

Cancer Res; 73(5); 1–10. ©2012 AACR.
80% in a Chinese cohort, published only in Chinese. Although KIT expression has been described in both upper tract tumors and small cell carcinomas of the bladder (11, 12), neither KIT nor ABL expression has been studied in transitional cell carcinomas of the bladder to our knowledge.

Ionizing radiation causes DNA damage, including base damage, single-strand breaks (SSB), and double-strand breaks (DSB). Unrepaired or misrepaired DSBs are lethal, resulting in cell death both in tumors and normal tissues (13, 14). Mammalian cells use 2 major pathways to repair DSBs, namely homologous recombination and nonhomologous end-joining (NHEJ; refs. 15, 16). Homologous recombination is an error-free pathway, predominantly used in the G2–S phase of the cell cycle, which requires the sister chromatid to act as a DNA template. A major homologous recombination protein is RAD51, which is involved in ATP-dependent DNA strand exchange. RAD51 expression is increased following ionizing radiation in tumor cells and induces the formation of RAD51 nuclear foci at sites of DSB (17). NHEJ is the major DSB repair pathway used in G0 and G1 (18) and involves the DNA-binding complex Ku70/Ku80 and the DNA-dependent protein kinase catalytic subunit (DNA PKcs; ref. 19). In addition to the classical DSB repair pathways, a less efficient Ku-independent pathway has been described in Ku-deficient yeast cells, which involves microhomology-mediated end-joining (MMEJ; ref. 20).

We have previously shown the MMEJ phenotype in vitro using cell-free extracts from muscle-invasive bladder tumors, and this is associated with reduced Ku–DNA binding and loss of TP53 function (21). Pucci and colleagues also showed reduced Ku–DNA binding in 5 advanced breast and muscle-invasive bladder tumors (22). This error-prone repair was not detected in normal human urothelial cell extracts (23), which suggests a therapeutic window that could be targeted by novel therapies. Negroni and colleagues (24) inhibited Ku80 expression by RNA interference (RNAi) using short hairpin RNA (shRNA) in RT112 bladder cancer cells and showed increased radiosensitivity and reduced Ku–DNA binding compared with parental RT112 vector-transfected cells.

The outcome of clinical radiotherapy depends, in part, upon the extent of DNA damage and how efficiently the cells can repair this damage. Selective targeting of DNA repair pathways could increase tumor cell kill while sparing normal tissues, thus increasing the therapeutic ratio. ABL upregulates RAD51 gene expression and imatinib reduces RAD51 protein expression and RAD51–chromatin binding (25) and reduces error-free homologous recombination efficiency (17). Imatinib also reduces the increased RAD51 expression induced by ionizing radiation and reduces the associated RAD51 nuclear focus formation in glioma cell lines (25), and in bladder, pancreatic, prostate, and lung carcinoma cell lines, imatinib increases cell kill in combination with ionizing radiation, due in part to mitotic catastrophe (17), unlike normal fibroblasts, where cell survival is unaffected. In xenograft studies, imatinib increases growth delay following fractionated radiotherapy in glioblastoma, epidermoid, and prostate carcinoma models with no apparent increase in toxicity (17, 26).

EGFR inhibitors, such as lapatinib, can act as radiosensitizers by targeting the intracellular signaling cascades (including Ras-MAPK and PI3K-AKT), which are triggered by binding of ligand to the transmembrane EGFR ligand-binding domain (27, 28). These cascades are normally activated by ionizing radiation in EGFR-overexpressing tumors, resulting in radioresistance (27). Moreover, they also act as radiosensitizers by repressing DNA repair in irradiated cells although this occurs via the NHEJ pathway, through inhibition of the phosphoinositide 3-kinase (PI3K)–mediated stimulation of DNA PKcs and by blocking of the nuclear interaction between EGFR and DNA PKcs normally induced by ionizing radiation (reviewed by Baumann and colleagues (27)).

We hypothesized that in muscle-invasive bladder cancer, it would be better to use an agent that targets the homologous recombination pathway rather than the NHEJ pathway. This would result in a form of "synthetic sickness" (see ref. 29 for recent review), where tumor cells already deficient in NHEJ would have reduced homologous recombination efficiency and repair, and thus increased ionizing radiation–induced lethality. As imatinib is known to target homologous recombination via RAD51 (17, 25), it may be one such agent, and the combination of imatinib and radiotherapy should result in an increased therapeutic ratio for muscle-invasive bladder cancer.

We therefore sought to see whether ABL, KIT, PDGFR, HER2, or EGFR are targets in bladder cancer by determining their role in radiotherapy response. We then moved to an experimental system to test the effectiveness of therapies that might be used in combination with radiotherapy to enhance radiosensitivity. This included targeting the ABL/RAD51 and EGFR pathways.

Materials and Methods

Tissue samples and immunohistochemistry

Ninety-one formalin-fixed paraffin-embedded bladder tumor biopsy samples were obtained from patients treated with radical radiotherapy for transitional cell carcinoma of the bladder at the Leeds Cancer Centre (West Yorkshire, United Kingdom) from 2002 to 2005. Details of the patients and radiotherapy treatments have been described previously (30). Patients gave informed consent for use of their tissues and local ethical approval was obtained from the Leeds (East) Research Ethics Committee (project 04/Q1206/62).

Antibody conditions were optimized by staining sections from control tissues, namely breast tumor for ABL, HER2, EGFR, and PDGFR and skin tumor for KIT. Then 4-μm sections from the bladder tumor specimens were heated, dewaxed, and hydrated in xylene, graded alcohols, and water. Antigen retrieval was achieved by boiling in EDTA (1 mmol/L at pH 8.0) or citric acid (pH 6.0) for 2 minutes of pressure cooking before quenching endogenous peroxidase activity with 3% H2O2 for 30 minutes. Endogenous protein-binding activity was blocked using avidin–biotin–blocking agent (Vector) and normal goat serum (Dako) before incubation with primary antibody; anti-ABL (1:250; NeoMarkers), anti-c-HER2 (1:200, Dako), anti-EGFR (1:100; Novocastra), anti-KIT (1:40; Novo-castra), and anti-PDGFRβ (1:25; Cell Signalling) diluted in dH2O (Dako) for 1 hour. Samples were then incubated for 30 minutes with secondary antibody conjugated to horseradish peroxidase using the Dako ChemMate Detection Kit (Dako). Immunoreactivity was revealed by incubation of...
sections with 3′-3′′-diaminobenzidine (DAB) for 10 minutes before washing, taking through graded alcohols, clearing in xylene and counterstaining with hematoxylin (VWR) before mounting in dibutylphthalate xylene (Leica). Digital images were captured within invasive tumor areas (3–10 images per slide, ×400 magnification) using an Olympus BX50 microscope and c-3030 camera.

Assessment was made of the percentage and intensity of tumor cells with membranous staining based on the recommendations for interpretation of the HercepTest: gastric cancer (31). Briefly, for all antibodies studied, complete, basolateral, or lateral membrane staining was scored as an intensity of 3+ for strong intensity and 2+ for weak to moderate intensity, where at least 10% of the tumors cells stained positive. Partial membrane staining or only faint/barely perceptible intensity staining in at least 10% of tumor cells was scored as a 1+. Staining intensity was scored independently in a blinded manner by 2 observers, discordant scores were reviewed, and a consensus was reached.

Cause-specific survival was defined from day 1 of radiotherapy until death from bladder cancer. Death from another cause was considered a censored observation. Kaplan–Meier curves were plotted for cause-specific survival and the log-rank statistic used to compare survival times across categories of protein expression.

Reagents
Imatinib was a generous gift from Novartis Pharma AG (Switzerland) and was later purchased from Stratech Scientific Ltd.; lapatinib was a generous gift from GlaxoSmithKline plc. For cell culture experiments, imatinib and lapatinib were dissolved in dimethyl sulfoxide (DMSO; Sigma) to a stock concentration of 10 mmol/L and stored in single-use aliquots at −20°C.

Cell culture conditions
The TP53 wild-type RT112 bladder transitional cell carcinoma cell line has been authenticated in M.A. Knowless’s laboratory by extensive genetic analysis [microsatellite typing, conventional karyotypic analysis, multiplex FISH (MFISH), array-based copy number analysis]. Cells were grown in RPMI-1640 (Sigma) supplemented with 10% v/v FBS (Sigma) and 2 mmol/L l-glutamine (Sigma) in a humidified atmosphere containing 5% CO2 at 37°C. Exponentially growing cells were used in all experiments.

Cell irradiation
Cells were harvested from exponential-phase cultures and diluted to 1,000 cells/mL. Five milliliter cell suspensions were then irradiated in tubes at a dose-rate of 1.0 Gy/min using an X-ray machine (Irradiator 320, NDT Equipment Services Ltd.), or cesium-137 source at 1.12 Gy/min using a Gamma-Service ray machine ( Irradiator 320, NDT Equipment Services Ltd.), or then irradiated in tubes at a dose-rate of 1.0 Gy/min using an X-cell irradiation used in all experiments.

Chemosensitivity studies
Exponentially growing cells were incubated in 75 cm2 flasks for 24 hours at appropriate drug concentrations (specified in the figure legends), and the cells then trypsinized and resuspended in medium containing drug as required.

Clonogenic assays
Following the necessary treatments, cells were plated at appropriate cell numbers in triplicate in 10-cm culture dishes containing 10 mL of fresh medium, with or without drug as required (see relevant figure legends). After 14 days incubation, the cells were stained with 1% methylene blue (Sigma) in 50% ethanol, and colonies with more than 50 cells were counted. The surviving fraction was determined as the total number of colonies formed divided by the total number of cells plated multiplied by the plating efficiency, as determined in untreated cells. Radiation survival curves were plotted after normalization for the cytotoxicity induced by control or drug alone, in GraphPad Prism, using the linear-quadratic model with the equation $SF = \exp\left(-aD + \frac{GSD^2}{D}\right)$. Each point on the survival curve represents the mean surviving fraction from at least 3 independent experiments.

RNA interference using short hairpin RNA
The siRNA expression vector pSilencer 2.1-U6 neomycin (Ambion), which contains a human U6 RNA polymerase III promoter able to transcribe shRNAs, was used in these experiments. Two constructs were made for each of Ku80 and RAD51 (see Supplementary Table S1 and Supplementary Fig. S1), whereby complementary oligonucleotides were used to encode hairpin siRNA inserts, designed to target a 21-mer sequence of human Ku80 coding region or 3′-untranslated region (UTR) mRNA, or RAD51 coding region mRNA, respectively. These were designed using the Ambion Insert Design Tool for pSilencer vectors and purchased from Sigma, and were then annealed and ligated into the linearized pSilencer vector. Circular negative control pSilencer neovector, that expresses a hairpin siRNA with limited homology to any known sequence in the human genome, was used in experiments as a nonspecific negative control.

The target sequences in the human Ku80 or RAD51 gene were determined empirically and analyzed by BLAST search, to confirm a lack of homology to other coding sequences, as per manufacturer’s recommendations. The target sequences of clones used for clonogenic assays were Ku80 5′-AAC TCC ATT CCT GGT ATA GAA-3′ (Ku80 coding region target sequence 1) and for RAD51 5′-AAT CAC TAA TCA GGT GGT AGC-3′ (RAD51 coding region target sequence 2; Supplementary Table S1). The corresponding targeting oligonucleotide sequences were for Ku80: top strand 5′-GAT CCG CCT CAT TCC TGG TAT AGA ATT CAA GAG ATT CTA TAC CAG GAA TGG AGT TTT TGG GAA A-3′ and bottom strand 5′-AGC TTT TCC AAA AAA CTC CAT TCC TGG TAT AGA ACG TCT TGA ATT CTA TAC CAG GAA TGG AGC G-3′, and for RAD51: top strand 5′-GAG CTC GCA CTA CTA AGG TGG TAG CTT CAC TCT TCA AGG TTT TGG GAA A-3′ and bottom strand: 5′-AGC TTT TCC AAA AAA TCA CTA ATC AGG TGG TGG TAG CTC TCT TGA AGC TAC CAC CTT AGT AGT TTT TGG GAA A-3′.

Stable transfection
RT112 cells were seeded into 75-cm2 flasks and the following day transfected at 60% confluence with pSilencer-Ku80,
pSilencer-RAD51, or circular negative control pSilencer neo-vector, using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 24 hours, fresh medium was added containing the selection reagent G418 (600 µg/mL; Gibco, Invitrogen). Selection was continued for 14 days, with the medium refreshed every other day. Single clones were picked and tested for Ku80 and RAD51 expression, respectively, by Western blotting and subsequently tested for ionizing radiation sensitivity using clonogenic assays.

**Western blot analysis**

Cells were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer (Sigma) with 1% of protease inhibitor and phosphatase inhibitor (Sigma). The cells were allowed to swell on ice for 20 minutes; then the lysate was centrifuged for 30 seconds at 12,000 × g. The supernatant was carefully removed and stored at −20°C. Total protein concentration in cell lysates was determined by the method of Bradford (Sigma). Thirty to 50 µg of protein was resolved on 4% to 20% polyacrylamide gels and transferred onto nitrocellulose membranes. The resulting membranes were incubated with blocking buffer (Li-cor) and primary antibodies. The antibodies used were rabbit polyclonal ABL (2862; Cell Signal), rabbit polyclonal Rad51 (ab63801; Abcam), rabbit polyclonal EGFR (sc-03; Santa Cruz Biotechnology), mouse monoclonal anti-Ku80 (Ab-2; Neomarkers), mouse monoclonal β-tubulin (clone TUB2.1 TT4026; Sigma), and mouse monoclonal β-actin (ab8226; Abcam).

Fluorochrome-conjugated secondary antibodies (Li-cor) were used and detected by infra red scanning densitometry using the Li-cor Odyssey Infrared Detection System (Li-cor Biosciences UK Ltd.). Quantification was based on normalization to β-actin. The immunoblotting experiments were carried out at least 3 times.

**Cell-cycle analysis**

Propidium iodide (PI) staining and flow cytometry were used to determine cell-cycle stages. Cells from the batches used for clonogenic assays were washed with PBS, fixed in ice-cold 70% ethanol, and stored at 4°C before analysis. Cells were spun down and resuspended in PI solution (50 µg/mL PI, 0.5 mg/mL RNase; Applied Biosystem) and incubated at room temperature in the dark at least for 30 minutes. DNA content was detected by flow cytometry (Beckman FACScan system). The relative proportions of cells in the G1, S, and G2–M phases of the cell cycle were determined using ModFit LT 3.2 software.

**Statistical analysis**

All statistical analyses were conducted using SPSS16.0 software. Clonogenic assays were conducted in triplicate at least 3 times, with the results expressed as mean ± SD as appropriate. Western blot analyses were conducted at least 3 times independently. Results were considered statistically significant at a P < 0.05, using a two-tailed unpaired Student t test. Sensitizer enhancement ratios (SER) were calculated at a surviving fraction of 0.1 (10% survival). Cause-specific survival was defined from day 1 of radiotherapy until death of the patient from bladder cancer. Death from another cause was considered a censored observation. Kaplan–Meier curves were

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**Figure 1.** Immunohistochemistry studies. A, representative images, at high-power magnification (×40): ABL, 1+, 2+, and 3+; EGFR, 1+, 2+, and 3+; HER2, 1+, 2+, and 3+; KIT-negative and -positive samples; PDGFR-B example of blood vessel staining but absent tumor cell staining. Scale bars represent 75 µm lengths. B, Kaplan-Meier survival curves from radiotherapy for: (i) ABL scoring comparing 3+ (solid line) and 2+ or lower (dashed line); (ii) EGFR scoring comparing 3+ (solid line) and 2+ or lower (dashed line); and (iii) HER2 scoring comparing 3+ (solid line) and 2+ or lower (dashed line).
plotted for cause-specific survival and the log-rank statistic used to compare survival times across categories of protein expression.

Results

Expression of tyrosine kinases in bladder tumor samples

We sought to estimate the percentage of muscle-invasive bladder tumors expressing the tyrosine kinases of interest (imatinib: ABL, KIT, and PDGFR-β; lapatinib: HER2 and EGFR), on the basis that this is likely to represent the cohort of patients for which the addition of a TKI to their radiotherapy may be beneficial. We also wished to determine whether these factors were prognostic in these patients, who had not received such treatments in addition to radiotherapy. The Dako scoring system for the HercepTest was applied to our tyrosine kinases of interest in 91 formalin-fixed paraffin-embedded bladder tumor samples (Fig. 1A). A sample was classified as positive if there was membranous staining in at least 10% of cells, which met the threshold criteria for intensity of staining (2+ or 3+).

In 75 of 91 (82%) cases there was positive immunostaining for ABL. In contrast, KIT expression was undetectable in most of the cases but showed weak staining in 8 cases, and PDGFR-β was expressed in endothelial cells and smooth muscle cells but was undetectable in bladder tumor cell membranes. There were 87 of 91 cases (96%) positive for EGFR staining and 86 of 90 (96%) cases HER2-positive.

We also correlated tyrosine kinase expression with patient survival, to look for prognostic significance of high expression. We classified patients into those with low tumor tyrosine kinase expression (equal or less than 2+ or 3+) and patients with high (3+) expression levels (as there were insufficient tumors scoring 0/1+ for meaningful comparison with 2+/3+). Neither ABL, EGFR, nor HER2 were significantly correlated with patient survival (Fig. 1B).

Effects of imatinib and lapatinib on RT112 cell proliferation

Before combining drug and radiation treatments, it was first necessary to determine the growth-inhibitory activity of the drugs in RT112 cells. Figure 2 shows cell viabilities by clonogenic assay following 14 days treatment with varying concentrations of imatinib or lapatinib. The IC50 value for imatinib in RT112 cells was 8 μmol/L and for lapatinib 3.5 μmol/L.

Inhibition of Ku80 or RAD51 expression in RT112 cells by siRNA

To generate NHEJ-deficient and homologous recombination-deficient RT112 cells, we used a siRNA-based strategy to reduce Ku80 or RAD51 expression in RT112 cells. We tested the effectiveness of 221-mer siRNAs targeting different sites within the exons or 3′-UTR regions of Ku80 and RAD51 (Supplementary Table S1), using pSilencer2.1-U6 neomycin, which drives expression of a shRNA from the human U6 promoter. The hairpin RNA is then processed into an siRNA, which induces RNAi of the target gene. RT112 cells were stably transfected with each of the vectors and clones picked and tested using Western blotting and ionizing radiation clonogenic assays. An individual clone for each of Ku80 (C13, coding region) and RAD51 (795J) was then selected for drug-ionizing radiation clonogenic assays. Densitometric analysis of representative
Western blot analyses revealed a 30% reduction in Ku80 protein expression in C13 cells and 75% reduction in RAD51 expression in 795J cells compared with parental RT112 cells and pSilencer neovector negative control cells (Supplementary Fig. S1).

**Ku80 or RAD51 interference causes a decreased viability of RT112 after X-ray exposure**

We examined the radiosensitizing effect of siRNA-mediated downregulation of Ku80 and RAD51 expression in RT112 cells using clonogenic assays. We found a statistically significant increase in radiosensitivity for the Ku80KD (\(P = 0.01\)) and RAD51KD (\(P = 0.02\)) cells as compared with parental RT112 and RT112-pSilencer vector control cells (Fig. 3).

**Imatinib significantly radiosensitized Ku80KD RT112 cells**

We then tested our hypothesis that imatinib radiosensitizes NHEJ-deficient Ku80KD cells, whereas having less effect on the parental RT112 cells with an intact NHEJ pathway, due to targeting the ionizing radiation-induced increase in RAD51. We conducted clonogenic assays on RT112, Ku80KD, and RAD51KD cells using relevant drug–ionizing radiation combinations. Cells were incubated for 24 hours with or without drug, before irradiation and plating followed by 14 days incubation, before staining and counting. Drug doses were chosen at approximately IC\(_{50}\) (high) and at IC\(_{40}\) nontoxic (low) levels.

Radiation survival curves were generated for each cell line after normalization for the level of cell killing induced by drug alone. As shown in Fig. 4A–C, 3 \(\mu\text{mol/L}\) imatinib had a significant radiosensitizing effect on Ku80KD cells (SER 1.27; \(P = 0.03\)) but no significant effect in parental RT112 cells (SER = 1.07; \(P = 0.051\)) or RAD51KD cells. At 6 \(\mu\text{mol/L}\) concentration, equivalent to approximately IC\(_{50}\), imatinib produced an SER of 1.69 in Ku80KD cells (\(P = 0.03\)) but only an SER of 1.34 in the RT112 parental cell line (\(P = 0.03; P = 0.046\) compared with Ku80KD 6 \(\mu\text{mol/L}\) imatinib survival curve) and 1.11 in the RAD51KD cells (\(P = 0.03; P = 0.04\) compared with Ku80KD 6 \(\mu\text{mol/L}\) imatinib survival curve).

In contrast, lapatinib radiosensitized RT112 cells in a dose-dependent manner (Fig. 4D; SER = 1.13 and 1.30 for 0.6 \(\mu\text{mol/L}\); \(P = 0.02\) and 2.4 \(\mu\text{mol/L}\); \(P = 0.01\)) but had no radiosensitizing effect on Ku80KD cells at either low (0.6 \(\mu\text{mol/L}\)) or high (2.4 \(\mu\text{mol/L}\)) concentrations (Fig. 4E). However, in contrast to imatinib, lapatinib had a dramatic radiosensitizing effect in RAD51KD cells, even at low dose (SER = 1.27; \(P = 0.02\)), with the high dose resulting in inhibition of RAD51KD cell colony formation, even without ionizing radiation (Fig. 4F).

Figure 4. Clonogenic survival of cells under ionizing radiation (IR) with or without drug exposure. The radiation survival curves were generated for each cell line after normalization to cell killing by drug alone. Bars, mean survival of at least 3 independent experiments ± SD. A, RT112, ionizing radiation/imatinib (3 \(\mu\text{mol/L}\); \(P = 0.051\); 6 \(\mu\text{mol/L}\); \(P = 0.03\)); B, Ku80KD, ionizing radiation/imatinib (3 \(\mu\text{mol/L}\); \(P = 0.03\); 6 \(\mu\text{mol/L}\); \(P = 0.03\)); C, RAD51KD, ionizing radiation/ imatinib (3 \(\mu\text{mol/L}\); \(P = 0.08\); 6 \(\mu\text{mol/L}\); \(P = 0.03\)); D, RT112, ionizing radiation/lapatinib (0.6 \(\mu\text{mol/L}\); \(P = 0.02\); 2.4 \(\mu\text{mol/L}\); \(P = 0.01\)); E, Ku80KD, ionizing radiation/lapatinib (0.6 \(\mu\text{mol/L}\); \(P = 0.60\); 2.4 \(\mu\text{mol/L}\); \(P = 0.86\)); F, RAD51KD, ionizing radiation/lapatinib (0.6 \(\mu\text{mol/L}\); \(P = 0.02\)).
Cell-cycle effects of imatinib and lapatinib

To determine whether the drug-mediated enhancement of radiosensitivity was due to cellular synchronization into a radiosensitive phase of the cell cycle, PI staining and flow cytometry were used to determine the cell-cycle phase distribution of samples used in each independent clonogenic assay. Neither imatinib nor lapatinib caused obvious cell-cycle arrest in treated cells of each cell type, except for a small increase in G1 fraction in RAD51KD cells treated by lapatinib at both drug concentrations (Fig. 5; \( P = 0.02 \) low dose; \( P = 0.04 \) high dose).

Effects of imatinib and lapatinib on RAD51 and EGFR

We also measured expression of ABL, EGFR, HER2, and RAD51 using Western blotting (Fig. 6). Cells were incubated with or without drug for 24 hours and then irradiated to 8 Gy or left untreated. Forty-eight hours later, cells were lysed for Western blotting. RT112, Ku80KD (C13) and RAD51KD (759J) cells all had detectable baseline levels of ABL, EGFR, and RAD51 although RAD51 levels were markedly reduced in the RAD51KD cells. HER2 was not detectable in any of the cell lines. We found no major increase in RAD51 levels following 8 Gy ionizing radiation. Imatinib treatment was associated with reduced RAD51 levels in RT112 and Ku80KD cells both alone and following 8 Gy ionizing radiation but no effect on ABL levels; lapatinib had no effect on RAD51 levels.

Discussion

Radiotherapy is a valid option in the radical treatment of muscle-invasive bladder cancer, with similar survival rates to cystectomy in our recent study (2). Cytotoxic chemotherapy is often combined with radiotherapy to improve survival rates (3–5), but this can be at the expense of late side effects. Moreover, many patients with bladder cancer who elect to have radiotherapy are elderly with poor renal function, and therefore cannot tolerate cisplatin-based chemotherapy regimens. There is therefore an urgent need to identify less toxic agents suitable for these patients.

Figure 5. Cell-cycle phase distribution in cells with or without drug exposure for 24 hours. Column, mean of at least 3 independent experiments; error bars represent SD. A, RT112, Ku80KD, and RAD51KD cells treated with or without imatinib (Stratatech) for 24 hours; B, RT112, Ku80KD and RAD51KD cells treated with or without lapatinib for 24 hours.
Our immunohistochemistry data show that ABL is expressed in more than 80% of muscle-invasive bladder tumors, suggesting that imatinib might be useful in such patients. EGFR and HER2 staining was positive in more than 95% of bladder tumors. While the Kaplan–Meier survival curves (Fig. 1B) showed no prognostic significance, our sample numbers were relatively small, with only 73% power to detect a HR of 0.4, with 35 cause-specific survival events at \( P = 0.05 \). Also, we used a scoring system developed for another tumor type and have not validated our findings in an independent patient cohort, so results should be treated with caution. As we did not have access to a patient cohort treated with a TKI and radiotherapy, we cannot comment on the potential predictive value of such markers.

We established that both imatinib and lapatinib are cytotoxic in the RT112 bladder cancer cell-line, which has modest ABL and EGFR expression levels (Fig. 2). Choudhury and colleagues (17) found an IC\(_{50}\) value of 20 \( \mu \)mol/L for imatinib in RT112 cells, whereas ours was 8 \( \mu \)mol/L; McHugh and colleagues found the IC\(_{50}\) for lapatinib in RT112 cells was 1.1 \( \mu \)mol/L MTT assay (32), ours being 3.5 \( \mu \)mol/L on clonogenic assay, perhaps reflecting differences in experimental systems. We then successfully knocked-down both Ku80 and RAD51, using shRNA technology, in \( T\beta 5\beta \) wild-type RT112 cell lines (Fig. 3). Negroni and colleagues (24) used the same method to knockdown Ku80 in RT112 cells and showed radiosensitivity and reduced Ku–DNA binding. We previously observed the latter in muscle-invasive bladder tumor extracts.

We then showed the radiosensitizing effects of both imatinib and lapatinib in RT112, Ku80KD, and RAD51KD cells (Fig. 4). Imatinib was an effective radiosensitizer in Ku80KD cells but less effective in parental RT112 cells. Although it did not affect ABL expression levels (Fig. 6) in both parental RT112 cells and Ku80KD cells, imatinib treatment was associated with reduced RAD51 expression levels both with and without ionizing radiation. As imatinib had no radiosensitizing effect at low dose and only limited effects at high dose in RAD51KD cells, this seems to support our hypothesis that imatinib works through the homologous recombination pathway but not the NHEJ pathway. In contrast, lapatinib had no radiosensitizing effect on Ku80KD cells but a marked effect in RAD51KD cells and also sensitized parental RT112 cells, consistent with lapatinib acting via NHEJ rather than homologous recombination. Recently, Myllynen and colleagues (33) showed that both homologous recombination and NHEJ are involved in regulation of DSB repair by EGFR, using an I-SceI–based reporter system, with reduction of homologous recombination by the TKI erlotinib at 0.5 \( \mu \)mol/L and less so by the monoclonal antibody cetuximab at 30 nmol/L concentration. However, our experiments suggest that lapatinib does not act on homologous recombination at 0.6 or 2.4 \( \mu \)mol/L. Other than a small effect on G\(_1\) arrest for lapatinib at both concentrations in RAD51KD cells, neither drug affected cell-cycle progression at 24 hours. Lapatinib has been found to induce G\(_1\) arrest in bladder cancer (RT112, 45%–65% G\(_1\) cells after 72 hours of 1.1 \( \mu \)mol/L lapatinib; ref. 34) and gastric cancer cell lines (58% G\(_1\) to 70% after 24 hours of 1 \( \mu \)mol/L lapatinib; ref. 35). Treatment with imatinib (5–6 \( \mu \)mol/L) for 48 to 72 hours caused a slight increase in the number of cells in G\(_1\)-phase in head-and-neck squamous carcinoma cell lines (47% G\(_1\) to 58%; ref. 36), ovarian cancer cell lines (80% G\(_1\) to 94%; ref. 37) but not in small-cell lung carcinoma cell lines (38).

Our data support the role of DNA repair in both imatinib and lapatinib’s radiosensitizing effects. They suggest that imatinib...
may be a useful radiosensitizer in muscle-invasive bladder cancer, as our tumors so far have all shown the MMEJ phenotype, with defective Ku–DNA binding and defective TP53 function, whereas superficial tumors had intact NHEJ (21). We are currently testing further tumor sample extracts. If some muscle-invasive tumors show intact NHEJ, imatinib would not be appropriate as a radiosensitizer in these patients, and there would be a need to develop a preradiotherapy predictive end-joining assay for use in patients with muscle-invasive bladder cancer. An advantage of imatinib as a radiosensitizer in this context, unlike lapatinib, is that we would expect normal tissue sparing and an increase in therapeutic ratio.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B. Qiao, M. Kerr, R.M. Phillips, A.E. Kilte
Development of methodology: B. Qiao, M. Kerr, R.G. Bristow, A.E. Kilte
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Qiao, A.E. Kilte
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Acknowledgments
The authors thank Ms. Filomena Estevez for her expert help with immuno-histochemistry staining and Drs. Sameer Chilka for outlining the muscle-invasive tumor areas, Paul Manley of Novartis and Daniel Ridley of Glaxo Smith Kline for critical reading of the article.

Grant Support
This study was supported by Yorkshire Cancer Research project grant L336 and Cancer Research UK; Yorkshire Cancer Research Project Grant L336 (B. Qiao); Slovene Human Resources Scholarship Fund (B. Groselj); Yorkshire Cancer Research Project Grant L350 (M.T.W. Teo); Cancer Research UK Program grant C6228/A7675 (M.A. Knowles); Canadian Foundation for Innovation and Canadian Cancer Society and a Canadian Cancer Research Society Career Scientist Award (R.G. Bristow); and Cancer Research UK Program Grant C255/S12678 (M. Kerr and A.E. Kilte).

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Received April 2, 2012; revised December 7, 2012; accepted December 18, 2012; published OnlineFirst January 9, 2013.


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Cancer Res  Published OnlineFirst January 9, 2013.

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doi:10.1158/0008-5472.CAN-12-1170

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