

Lapatinib, a dual inhibitor of ErbB-1/-2 receptors, enhances effects of combination chemotherapy in bladder cancer cells

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Received December 10, 2008; Accepted January 30, 2009

DOI: 10.3892/ijo_00000244

Abstract. Survival rate of patients diagnosed with the invasive form of bladder cancer is low suggesting an urgent need to implement novel treatments. GTC (gemcitabine, paclitaxel and cisplatin) is a new chemotherapeutic regimen, which has shown promise in clinical trials. Given that receptor tyrosine kinases of the ErbB family are overexpressed in a high proportion of metastatic bladder tumours, approaches involving small-molecule inhibitors of ErbB receptors in combination with conventional cytostatic drugs are of potential interest. Here, we show that the dual inhibitor of ErbB receptors, lapatinib, enhances cytostatic and induces cytotoxic effects of GTC in two bladder cancer cell lines which differ with regard to expression levels of proteins taking part in the ErbB pathway. Lapatinib inhibited phosphorylation of ErbB receptors and also reduced the level of phosphorylated AKT. Flow cytometry analysis demonstrated that GTC treatment affects cell cycle distribution differently in the presence or absence of lapatinib. In RT112 cells, which express high levels of ErbB receptors and harbour wild-type p53, combined GTC/lapatinib treatment resulted in the phosphorylation of p53 at Ser46 and accumulation of sub-G₁ cell populations. Our data indicate that a combinatorial

approach involving GTC and lapatinib may have therapeutic potential in a subset of bladder tumours depending on the genetic context.

Introduction

Bladder cancer is a worldwide disease with poor clinical outcome, especially among patients with the muscle invasive form of the disease (1). Several combinations of chemotherapeutic agents are used in treatment of metastatic transitional cell carcinoma (TCC) of the bladder. The combination of methotrexate, vinblastine, doxorubicin and cisplatin was previously considered the 'gold standard' chemotherapy treatment of TCC, although the median survival was only one year (2). Administration of the gemcitabine and cisplatin regimen has resulted in decreased toxicity, but has not improved survival rates (3). At present, a combination of gemcitabine, paclitaxel (taxol) and cisplatin (GTC) with median survival of 22 months is considered as a promising approach (4). In view of the continuing modest survival rates, novel targeted therapies for the treatment of advanced TCC of the bladder are urgently required. Much interest currently centres on modifying the activity of epidermal growth factor receptor (EGFR) family members (5-7). This family, which has been implicated in human cancers, comprises four receptors, ErbB-1 (HER1, EGFR), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4) (8). High expression of ErbB-1/ErbB-2, which has been described in TCC of the bladder, is an independent predictor of reduced survival (9-12). In contrast, high expression of ErbB-3/ErbB-4 is associated with favourable clinical outcome in TCC patients (13,14).

The ErbB receptors are composed of an extracellular ligand-binding region, transmembrane domain, and a cytoplasmic region containing a tyrosine kinase domain and autophosphorylation sites (15). Ligand activation of three receptors (ErbB-1, ErbB-3 and ErbB-4) causes them either to homodimerise or to form heterodimers with other ErbB receptors leading to the tyrosine kinase-mediated autophosphorylation. To date, eleven ErbB ligands, growth factors containing EGF-like domains, have been characterised. ErbB-2, a preferential partner for all other receptors (16), has

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Abbreviations: GTC, gemcitabine, paclitaxel and cisplatin; TCC, transitional cell carcinoma; TKI, tyrosine kinase inhibitors; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription; MTT, microtetrazolium; EGF, human epidermal growth factor; HRG1 β , human EGF domain of heregulin1- β 1; FBS, fetal bovine serum; IC₅₀, 50% inhibition of cell viability

Key words: lapatinib, ErbB receptor, gemcitabine, paclitaxel, cisplatin

no ligand. It is activated by heterodimerisation with every other member of the ErbB receptor family strongly augmenting signalling. ErbB-3 has no associated intrinsic kinase activity and ligand binding induces tyrosine phosphorylation only when heterodimers with other ErbB family members are formed (15). Analysis of the levels of ErbB ligands in TCC of the bladder revealed expression of ligands for only ErbB-1 and ErbB-4 but not ErbB-3 receptors (17). In addition to the canonical mode of ErbB activation, receptors of this family can be activated in a ligand-independent manner via phosphorylation by c-Src. This mode of ErbB-1 activation has been observed in various cancer cell lines treated with chemotherapeutic agents such as cisplatin, 5-fluorouracil, paclitaxel or gemcitabine (18-21).

Activation of ErbB tyrosine kinases stimulates a complex network of signalling events, which is involved in control of cell proliferation, differentiation or cell survival depending on genetic context. Signal transduction cascades activated by ErbB include the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K/AKT), signal transducer and activator of transcription (STAT) and phospholipase C pathways (15). Activation of the antiapoptotic ErbB/PI3K/AKT pathway has previously been linked to chemoresistance, making it an attractive marker when studying the action of chemotherapeutic agents (22).

There are two promising therapeutic approaches to block ErbB receptor activity; humanised monoclonal blocking antibodies (an anti-ErbB-2 antibody herceptin or trastuzumab) and small molecule tyrosine kinase inhibitors (TKIs) (8). TKIs inhibit the activity of tyrosine kinase in the intracellular component of ErbB receptors, acting at the ATP binding site, thus preventing receptor autophosphorylation and subsequent activation. TKIs exhibit various specificities; previous TKIs, Gefitinib (Iressa, ZD1839) or Erlotinib (Tarceva, OSI-774) are selective for ErbB-1, while more recently developed TKIs are dual inhibitors of both ErbB-1 and ErbB-2 or Pan-ErbB (8,23). The dual inhibitor lapatinib (GW572016) forms a slow off-rate complex with the kinase domain of EGFR resulting in prolonged repression of ErbB-1/-2 receptor activity (24). Heterodimers composed of ErbB-3 and a breast cancer-specific truncated form of ErbB-2 are also effectively inhibited by lapatinib (25). Lapatinib-mediated inhibition of ErbB-regulated signalling pathways in cell lines and in tumour xenografts resulted in partial cell cycle arrest and apoptosis (22,26,27). We and others have found that the effect of lapatinib on cell growth in various tumour cell lines is independent of ErbB-1/-2 expression levels (7,28,29).

In this study, we analysed the effects of the chemotherapeutic cocktail GTC in the presence or absence of lapatinib in bladder cancer cell lines. Firstly, we analysed the expression and ligand-dependent activation of ErbB receptors in six bladder cancer cell lines (a range from grade 1 to grade 3 tumours). We selected two cell lines to study the effect of lapatinib on the ligand- and GTC-induced stimulation of ErbB signalling. In any cell line, lapatinib modulated activity of ErbB receptors and inhibited AKT phosphorylation. Finally, studies were undertaken to assess the effect of GTC alone or in combination with lapatinib on cell growth, cell-cycle phase distribution and p53 phosphorylation.

Materials and methods

Reagents. Microtetrazolium (MTT) reagent was purchased from Sigma (St. Louis, MO). Recombinant human epidermal growth factor (EGF) and recombinant human EGF domain of heregulin1- β 1 (HRG1 β) were obtained from R&D Systems (Minneapolis, MN, USA). Cisplatin (Platinex™ 1.0 mg/ml) and paclitaxel (Taxol™ 6 mg/ml) were from Bristol-Myers Squibb Pharmaceuticals (Hounslow, UK). Gemcitabine (Gemzar® 200 mg) was from Lilly Pharmaceuticals (Basingstoke, UK). Lapatinib was provided by GlaxoSmithKline (Research Triangle Park, NC), and 10 mM stock solution was prepared in DMSO.

Antibodies. Antibodies to EGFR, p-EGFR (Tyr1110), ErbB-3, ErbB-4, PTP1B and mouse anti-goat Ig-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to p-EGFR (Tyr845), p-EGFR (Tyr992), p-EGFR (Tyr1045), p-EGFR (Tyr1068), ErbB-2, p-ErbB-2 (Tyr1248), p-ErbB-3 (Tyr1289), AKT, p-AKT (Ser473), p-AKT (Thr308), p42/44 MAPK, p-p42/44 MAPK (Thr202/Tyr204), PTEN, STAT1, p-STAT1 (Tyr701), STAT3, p-STAT3 (Tyr705) and all anti-p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit-Ig-HRP and rabbit anti-mouse-Ig-HRP were purchased from DakoCytomation (Glostrup, Denmark). Antibodies to E-cadherin (BD Transduction Laboratories, San Jose, CA), α -tubulin (Sigma) were used.

Cell culture. The human bladder cancer cell lines (RT4, HT1376, RT112, J82, UMUC3, T24) and the human squamous cell carcinoma A431 cell line (American Type Culture Collection and the European Collection of Cell Cultures) were maintained in high glucose-contained Dulbecco's modified essential media (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France) and 1% non-essential amino acids (bladder cancer cell lines) at 37°C with 5% CO₂.

Cell proliferation MTT assay. To determine the concentration of lapatinib or chemotherapeutic agents, which are required for 50% inhibition of cell viability (IC₅₀), MTT assay was performed as described (7). Briefly, cells were maintained in medium with 1% FBS (1000 cells/well in a 96-well plate). Treatment was performed for 48 h with different concentrations of reagents in 10 parallel wells in medium containing 1% FBS. As control, 5 parallel wells with basic medium or with 0.1% DMSO were used. IC₅₀ was calculated using Graphpad Prism software. Reagents were used at doses ranging between 0.01 and 100 μ M for lapatinib, 0.01 and 100 μ M for gemcitabine, 0.1 and 100 nM for paclitaxel and 0.01 and 100 μ M for cisplatin. Calculated IC₅₀ values for lapatinib in RT112 and J82 cells were respectively 1.1 and 1.22 μ M; for gemcitabine; 0.04 and 0.022 μ M; for paclitaxel, 3.4 and 5 nM, and for cisplatin, 29.2 and 3.55 μ M.

To study the effect of lapatinib in combination with chemotherapeutic agents, cells were seeded and starved as above. The IC₅₀ dose of lapatinib was added for 24 h. Gemcitabine, paclitaxel and cisplatin were mixed at their IC₅₀ doses, and then added to cells at different concentrations (1/8xIC₅₀ - 1xIC₅₀).

MTT assays were performed as above 48 h after addition of chemotherapeutic agents.

Preparation of protein lysates. Cells at 50% confluence were serum-depleted for 24 h in the presence or absence of lapatinib (IC₅₀ dose). Cells were treated with EGF (100 ng/ml) for 5 min, HRG1β (40 ng/ml) for 15 min or GTC (IC₅₀ dose of each reagent) for 20 h. Protein lysates were prepared in standard Laemmli buffer. Total protein concentration was measured by using the BCA protein assay kit (Pierce, Rockford, IL).

Western blot analysis. The total protein (25 μg) was separated in 8% SDS-PAGE, and transferred to Immobilon-P Transfer Membrane (Millipore Corp., Billerica, MA). The membranes were incubated in blocking buffer (Tris-buffered saline containing 0.1% Tween-20, 5% non-fat milk) for 1 h at room temperature, followed by incubation with the primary antibodies [dilution 1/1000, except for EGFR, (1/4000), and α-tubulin (1/8000)] and the secondary HRP-conjugated antibodies for 1 h. For detection, SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), or ECL Western Blot Analysis System (GE Healthcare, UK) kits were used. Expression of α-tubulin was used as a protein loading control.

Flow cytometry (FACS) analysis. Cells at 50% confluence were starved for 24 h in the presence or absence of lapatinib (IC₅₀ dose), followed by 48 h of treatment with GTC at IC₅₀ dose for each component. For the analysis, cells were harvested, fixed in 70% ethanol, treated with ribonuclease A (1 mg/ml, Sigma) and stained with propidium iodide (50 μg/ml, Sigma). The cellular DNA content was evaluated using a FACS flow cytometer and analysed by ModFit software.

Results

ErbB signalling in bladder cancer cell lines. An important role for ErbB family members in bladder cancer has been demonstrated previously (9-12,17). However, expression and activity of the proteins activated by ErbB signalling has never been addressed comprehensively in significant numbers of cell lines. We analysed expression of proteins involved in ErbB signalling in six different bladder cancer cell lines of varying grades. As a control, we used the vulval epidermoid carcinoma cell line, A431, known to express an artificially high level of EGFR. Morphologically, these cell lines represented two distinct types, epithelial (RT4, RT112, HT1376 and A431) and mesenchymal (T24, UMUC3 and J82). Cells belonging to the first group exhibited typical polarised epithelial morphology, formed tight cell-cell contacts and expressed high levels of the epithelial marker, E-cadherin (Fig. 1). Cells of the second group had elongated fibroblastoid phenotype and were E-cadherin-negative. ErbB-1 and ErbB-2 were highly expressed in epithelial cells, and their levels strongly correlated with the differentiation status (Fig. 1). In contrast, expression of ErbB-3 was nearly equal and the expression of ErbB-4 was hardly detectable in all bladder cell lines. AKT, a protein kinase responsible for ErbB-mediated anti-apoptotic response, was expressed at higher levels in mesenchymal cell lines, with two of these (J82 and UMUC3) expressing very

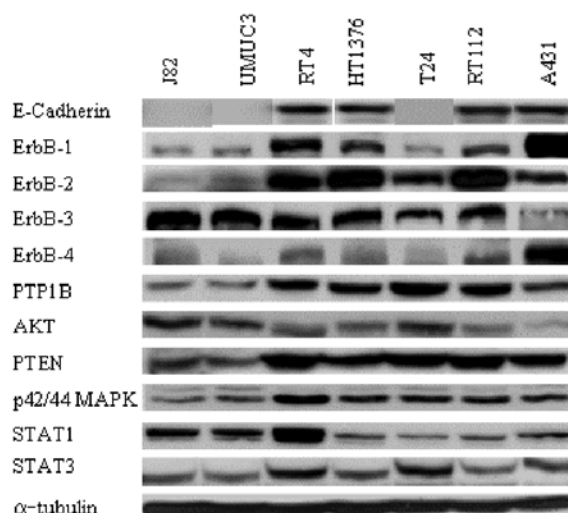


Figure 1. Expression levels of proteins involved in ErbB signalling in bladder cancer cell lines and in epidermoid carcinoma A431 cells. Cells were cultured in the presence of serum. Protein expression was analysed by Western blotting with indicated antibodies.

low levels of PTEN. Expression of other proteins involved in ErbB signalling and examined in this study (p42/44 MAPK, PTP1B, STAT1 and STAT3) did not correlate with morphological characteristics of cells (Fig. 1).

Next, we examined the activation of the ErbB pathway by the ErbB prototypical ligand, EGF. Short exposure of all cell lines to EGF resulted in strong phosphorylation of ErbB-1 tyrosine residues (Figs. 2 and 3). Phosphorylation of ErbB-2 was prominent in epithelial cells, but hardly detectable in mesenchymal cell lines (Fig. 2). In general, the level of ErbB-1 and ErbB-2 phosphorylation in response to EGF was much higher in epithelial cells expressing more of these receptors (Figs. 1 and 2). Even in the absence of EGF, all cell lines expressed detectable levels of phosphorylated ErbB-3 with the higher p-ErbB-3 levels in epithelial cells (Fig. 2). Whereas in A431 and RT112 cells, EGF enhanced phosphorylation of ErbB-3, in two remaining epithelial cell lines, RT4 and HT1376, phosphorylation of this receptor was not affected by EGF (Fig. 2). Interestingly, in mesenchymal cells, EGF even decreased the basal level of p-ErbB-3 expression, possibly, as a result of the replacement of ErbB-3 by ErbB-1 and ErbB-2 receptors in ErbB heterodimers. EGF treatment resulted in the activation of p42/44 MAPK and phosphorylation of AKT in RT112, A431 and in all mesenchymal cell lines, but not in RT4 or HT1376 cells. Activation of the STAT pathway was considerably less prominent. All mesenchymal non-stimulated cell lines expressed phosphorylated STAT1 and STAT3 independently of EGF. Activation of STAT1 and STAT3 by EGF was not detected in epithelial cell lines (Fig. 2). Overall, treatment with EGF induced receptor phosphorylation in all cell lines analysed. However, while two epithelial cell lines, RT4 and HT1376, appeared to be incompetent in transducing signals to the downstream targets, in other cells, p-p42/44 MAPK and AKT pathways were activated in response to EGF.

To address the effectiveness of lapatinib in the repression of the ErbB pathway, we selected two cell lines, RT112 and J82 capable of transducing EGF signals to downstream

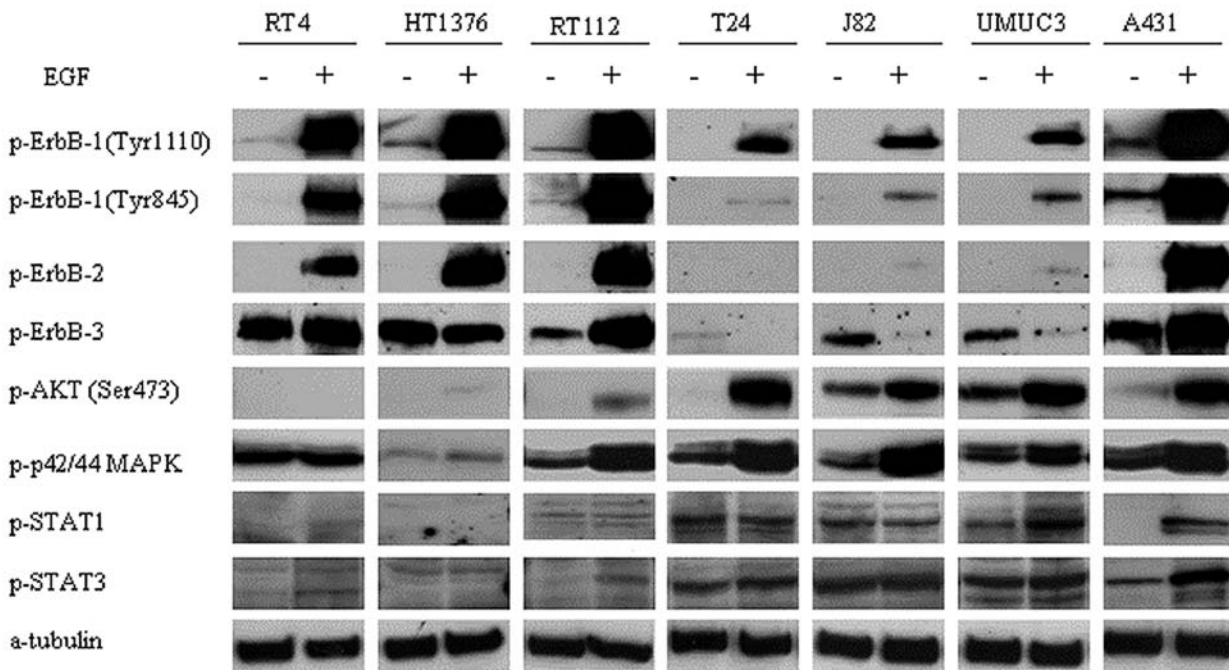


Figure 2. EGF treatment activates the ErbB pathway in bladder cancer cells. Phosphorylation of proteins activated by EGF was analysed with indicated phospho-specific antibodies by Western blotting. Cells were serum-depleted for 24 h and treated with EGF as described in Materials and methods.

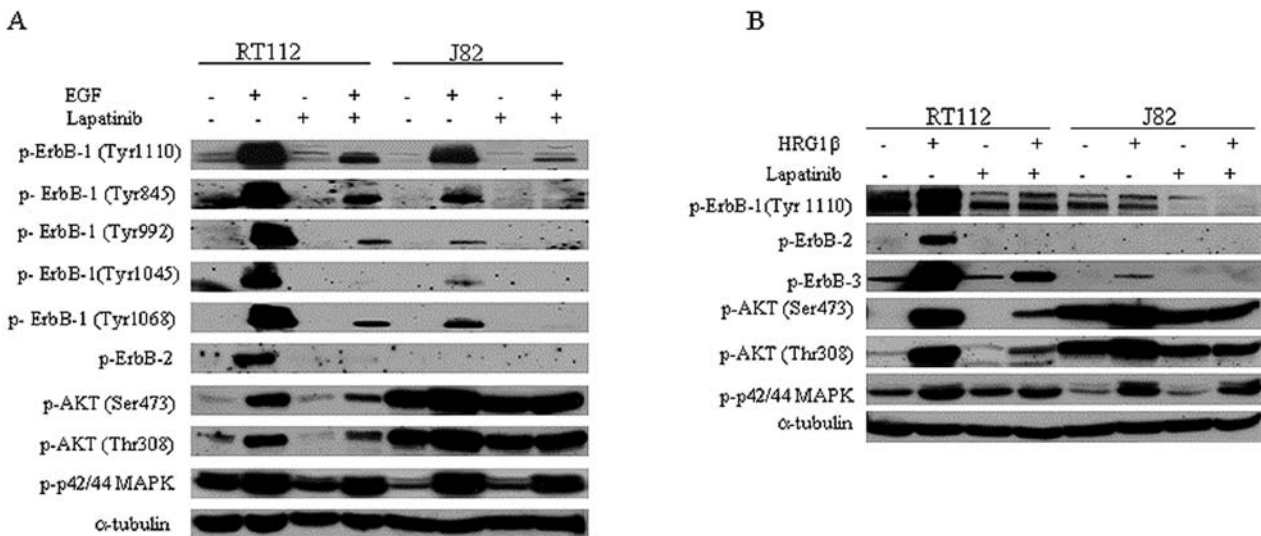


Figure 3. Lapatinib partly suppresses ErbB signalling in RT112 and J82 cells. Cells were maintained in the presence or absence of lapatinib at the IC_{50} dose and then stimulated with EGF (A), or HRG1 β (B) or mock-stimulated as indicated. Western blot analysis was performed with indicated phospho-specific antibodies.

targets. These cell lines belong to different morphological types and differ in ErbB-1 and ErbB-2 expression levels.

Lapatinib inhibits EGF- and HRG1 β -induced activation of ErbB receptors and downstream pathways. We aimed to examine whether EGF-induced phosphorylation of proteins involved in ErbB signalling is sensitive to lapatinib. Since the ErbB-3 receptor is expressed in all bladder cancer cell lines analysed (Fig. 1) and can form heterodimers with ErbB-2, HRG1 β (an ErbB-3-specific ligand) was also included in

the analysis. Semi-confluent cultures of RT112 and J82 cells were pre-treated with lapatinib and then stimulated by either EGF or HRG1 β . Lapatinib was used at IC_{50} value (7). As expected, EGF-mediated phosphorylation of ErbB-1 at several tyrosine residues was significantly inhibited by lapatinib (Fig. 3A). Similarly, lapatinib also strongly reduced HRG1 β -dependent phosphorylation of ErbB-3 (Fig. 3B). Of note, basal phosphorylation of ErbB-1 was only partly decreased. Treatment by either ligand activated lapatinib-sensitive phosphorylation of ErbB-2 in RT112 cells. Sub-

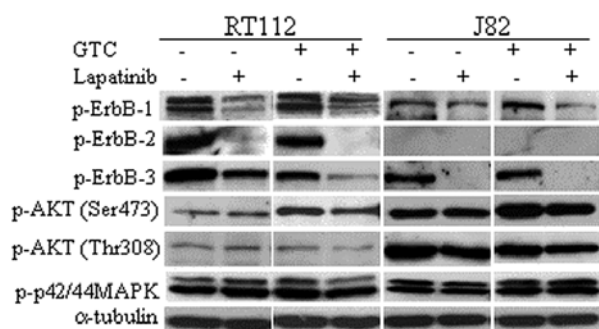


Figure 4. Effects of individual or combined treatments with lapatinib and GTC on the expression levels of phosphorylated ErbB-1, ErbB-2, ErbB-3, AKT and p42/p44 MAPK. Cells were serum-starved for 24 h in the presence or absence of lapatinib (IC_{50} dose) and treated with the combination of chemotherapeutic agents for 20 h. All agents were used at IC_{50} doses (see Materials and methods). Protein expression was analysed by Western blotting with the indicated antibodies as shown.

sequent activation of AKT at Ser473 or Thr308 by either ligand was sensitive to lapatinib in both RT112 and J82 cell lines. However, the effect of lapatinib was not competent of inhibiting pAKT below the basal level, suggesting that constitutive activation of PI3K-AKT pathway is independent of the ErbB pathway (Fig. 3). Interestingly, lapatinib produced only minimal effect on ligand-induced phosphorylation of p42/44 MAPK (Fig. 3). This may indicate that in these cell lines activation of downstream signalling pathways requires different degrees of receptor activation and in lapatinib-treated cells the level of constitutively active ErbB-1 is sufficient for p42/44 MAPK but not AKT phosphorylation.

Lapatinib inhibits the effect of GTC on ErbB signalling. To address whether inhibition of ErbB signalling by lapatinib sensitises bladder cancer cells to GTC-combined chemotherapy, we firstly analysed how the combined lapatinib/GTC treatment affected phosphorylation of ErbB receptors and downstream signalling. RT112 and J82 cells were incubated overnight with or without IC_{50} dose of lapatinib and then GTC (combined IC_{50} doses of the individual drugs) was applied for 20 h. Phosphorylation of ErbB receptors, p42/44 MAPK and AKT was analysed. Only a small increase in the levels of phospho-ErbB-1 was identified in both cell lines after 20 h of GTC treatment (Fig. 4). GTC slightly inhibited p-ErbB-2 level in RT112 and had no effect on p-ErbB-2 in J82 cells. Most prominently, lapatinib inhibited ErbB-3 phosphorylation in GTC-treated RT112 cells. Interestingly, GTC treatment induced phosphorylation of AKT at Ser473 but not at Thr308 in both cell lines (Fig. 4). The phosphorylation of p42/44 MAPK was not affected by GTC. GTC-dependent AKT phosphorylation on Ser473 in both cell lines was reduced by lapatinib indicating that ErbB signalling was, at least in part, responsible for AKT activation by GTC.

Lapatinib enhances GTC-sensitising effect in RT112 and J82 cells. We employed the MTT assay to test whether lapatinib enhances the growth inhibitory effect of GTC in RT112 and J82 cells. IC_{50} doses of combination chemotherapy (see Materials and methods) were added to the untreated cells or

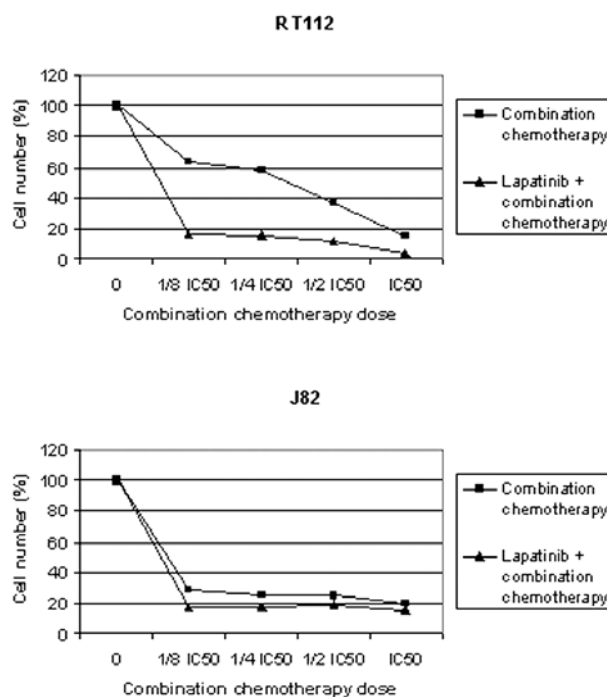


Figure 5. Lapatinib differently affects growth of RT112 and J82 cells treated with different doses of GTC. Cell growth was analysed by MTT assay as described in Materials and methods.

to the cells pre-treated for 24 h with the IC_{50} dose of lapatinib (Fig. 5).

In 48 h, MTT assay was performed to determine cell viability. J82 cells appeared to be more sensitive to GTC applied at the concentration of 1/8 IC_{50} (1/8 IC_{50} dose inhibited cell growth by 71% versus 36% inhibition in RT112 cells). When lapatinib was applied in combination with GTC, the growth inhibition reached 82% in both cell lines demonstrating the sensitising effect of lapatinib to the combination chemotherapy (Fig. 5). This effect was particularly evident in the GTC-resistant, RT112 cells.

Lapatinib and combination chemotherapy affect the cell cycle differentially in RT112 and J82 cells. The reduction in the growth rate of a cell population following treatment with an anti-cancer drug is the sum of cytostatic (cell cycle arrest) and cytotoxic (apoptosis or necrosis) effects of this drug. Having demonstrated the enhanced growth inhibition by combined lapatinib and GTC treatment of RT112 and J82 cells, we wished to examine the effect of these drugs on cell cycle distribution. Flow cytometry analysis of the effects of lapatinib alone on cell cycle distribution revealed a significant shift (~20% increase) into the G_1 phase of the cell cycle in both cell lines (Fig. 6). In contrast, the effect of combination chemotherapy was cell line-specific. In J82 cells treated with GTC, the majority of cells accumulated in S phase with decreased proportion of cells in G_1 or G_2/M . Adding GTC to the cells pre-incubated with lapatinib also demonstrated accumulation of cells in S phase. However, this treatment revealed a trend towards restoration of higher proportions of the cells accumulated in G_1 and G_2/M phases as compared with the cells treated with GTC only.

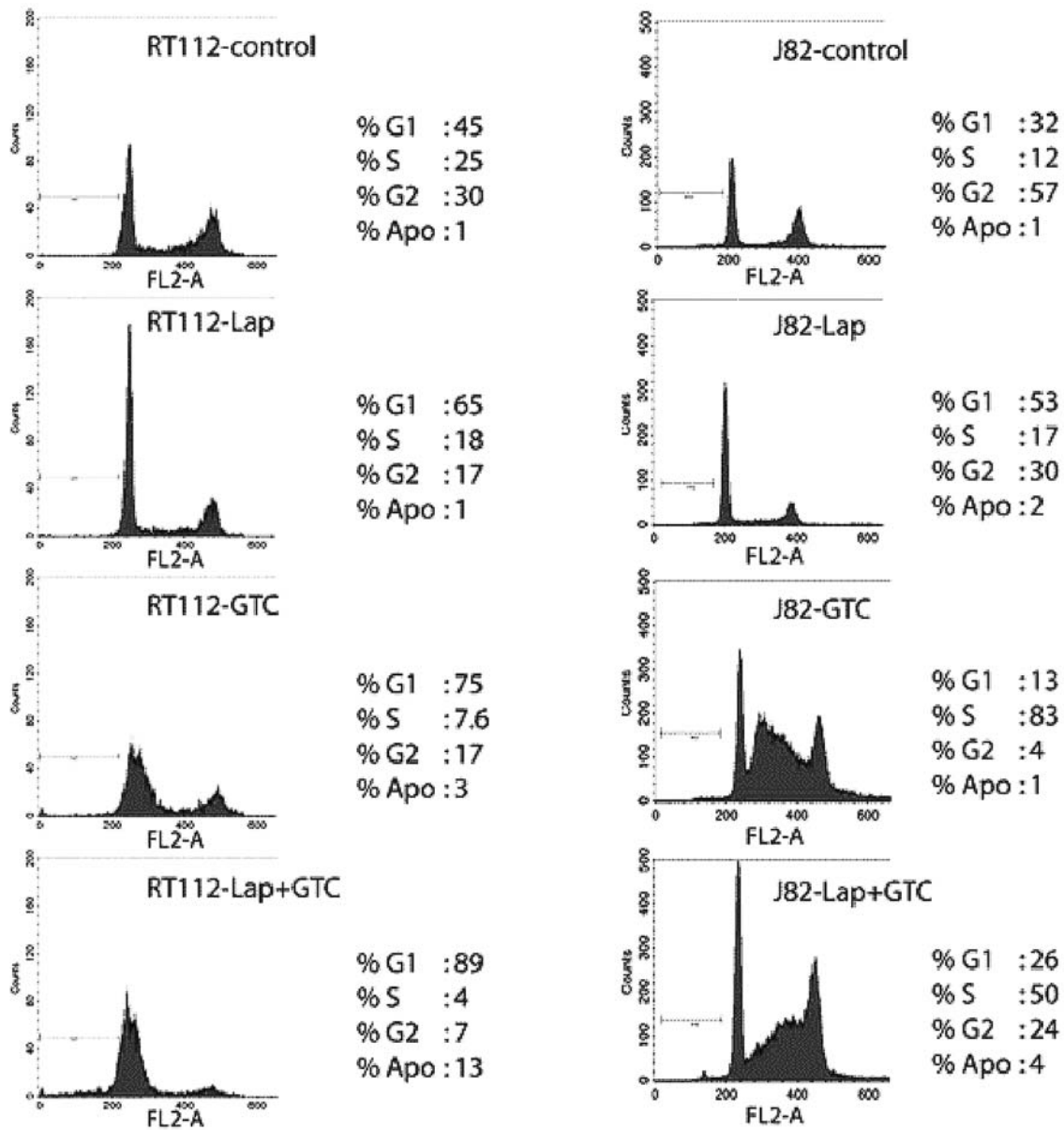


Figure 6. Cell cycle distribution of RT112 and J82 cells treated by GTC in the presence or absence of lapatinib. Cells were maintained in the presence or absence of lapatinib and then treated by combined chemotherapeutic agents for 48 h or mock-treated. All agents were used at their IC_{50} concentrations (see Materials and methods). FACS analysis was performed as described in Materials and methods.

In RT112 cells, GTC treatment resulted in an increased proportion of cells in the G_1 /early S phase (75%). Combined lapatinib/GTC treatment further strengthened this tendency. In fact, 89% of the cell population accumulated in the G_1 phase peak explaining the strong inhibitory effect of these drugs on the growth of RT112 cells (Fig. 6). In cells treated either with lapatinib or GTC, we observed no or a very low proportion of cells accumulated in sub- G_1 , indicating lack of apoptotic response (Fig. 6). In contrast, combined lapatinib/GTC treatment resulted in an increased amount of apoptotic cells especially in RT112 cell population (13%). The difference in the proportion of apoptotic RT112 and J82 cells observed after combined treatment may reflect the difference in p53 status in these cell lines. Surprisingly, the level of total p53 was similar in both cell lines and was not affected by lapatinib or GTC (Fig. 7). We observed no

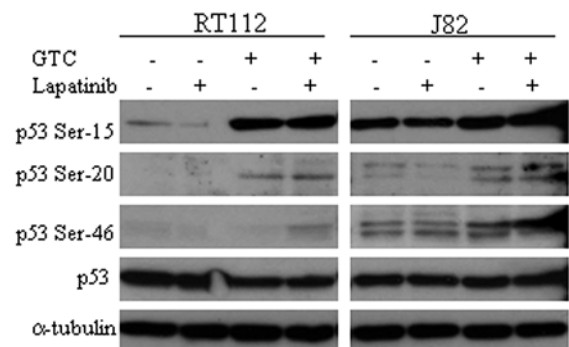


Figure 7. Effects of lapatinib and GTC treatments on phosphorylation of p53. Cells were maintained in the presence or absence of lapatinib and then treated with combined chemotherapeutic agents for 20 h (IC_{50} dose) or mock-treated. Expression levels and phosphorylation of p53 was analysed with indicated antibodies.

regulation of p53 phosphorylation in J82 cells, suggesting p53 is mutated in this cell line, as described by Matsui *et al* (30). On the other hand, Ser15, Ser20 and Ser46 were phosphorylated in RT112 cells upon treatment with GTC and/or GTC/lapatinip (Fig 7), which was shown to contain wild-type p53 (30). Of note, Ser46 phosphorylation, which facilitates p53-induced apoptosis (31), was only detected in RT112 cells undergoing lapatinip/GTC-treatment. Lapatinip alone did not alter the total or phosphorylated levels of p53 in both cell lines. These data indicate potential involvement of p53 in GTC/lapatinip-induced apoptotic response in RT112 cells.

Discussion

A major challenge in developing effective cancer therapies involves implementation of more effective treatment regimes which overcome the high toxicity of many conventional chemotherapeutic approaches. In recent years, new strategies, based on chemosensitisation of tumour cells with relatively low toxicity TKIs have gained much attention. These approaches involve application of decreased doses of toxic agents in combination with a non-toxic TKI complementing the efficacy of the treatment. Promising targets for such combinatorial treatment of bladder cancer patients are tyrosine kinase receptors of ErbB family, ErbB-1 and ErbB-2, whose expression in bladder tumours has high prognostic value. In this study, we performed an initial preclinical evaluation of the dual inhibitor of ErbB-1 and ErbB-2 receptors, lapatinib, as a sensitiser for a novel chemotherapeutic cocktail GTC currently being evaluated in a phase III clinical trial (4). All six bladder cancer cell lines analysed expressed ErbB-1, ErbB-2 and ErbB-3 but not ErbB-4 receptors (Fig. 1). Expression levels of ErbB-1 and ErbB-2 proteins were significantly lower in E-cadherin-negative mesenchymal cell lines, which likely represent later stages of tumour progression and tend to exhibit more aggressive behaviour in *in vivo* and *in vitro* assays. EGF effectively induced receptor phosphorylation in all six cell lines (Fig. 2). In all mesenchymal cell lines and in RT112 cells, EGF stimulated phosphorylation of ErbB downstream targets, p42/44 MAPK and AKT (Fig. 2). However, in two remaining epithelial cell lines, RT4 and HT1376, phosphorylation of ErbB targets was not detected, probably to genetic defects downstream of receptors.

We analysed the inhibitory effect of lapatinib in RT112 and J82 cells representing epithelial and mesenchymal cell phenotypes respectively. Lapatinib effectively blocked activation of ErbB receptors by EGF or HRG1 β in both cell lines. Surprisingly, the inhibitory effect of lapatinib was detected only on the AKT pathway and not on MAPK kinase (Fig. 3). Of note, lapatinib IC₅₀ values were similar in lines, (RT112 - high ErbB-1/2 and J82 - low ErbB-1/2 expression), as 1.1 and 1.2 μ M respectively (7). Taken together, these data indicate that ErbB composition and expression levels cannot be used as the sole criteria for evaluation of the sensitivity of these cancer cells to lapatinib.

All three components of the GTC chemotherapeutic regimen examined in this study target the cell cycle. Gemcitabine, is incorporated into DNA leading to masked chain termination and causes depletion of the dNTP pool, which leads to the cell cycle arrest in S phase (32). Paclitaxel

causes a mitotic (G₂/M) arrest by suppressing microtubule dynamics (33). The anti-tumour effect of cisplatin complexes results from their ability to generate various types of DNA adducts and activate intra-S phase check-point (34). Genotoxic insults have been shown to activate receptors of the ErbB family and affect downstream signalling (18-21). However, in RT112 and J82 cell lines, the effect of GTC on the phosphorylation of ErbB receptors was minimal, except for the apparent GTC-mediated decrease in phospho-ErbB-3 level in RT112 cells. In addition, in both cell lines GTC-dependent phosphorylation of AKT (Ser473, but not Thr308) was observed, possibly representing a survival mechanism, which contributes to chemoresistance. Of note, in both cell lines GTC-mediated activation of AKT at Ser473 required ErbB activity and was inhibited by lapatinib (Fig. 4). Lapatinib-mediated inhibition of GTC-induced AKT pathway may be at least in part responsible for the efficient chemosensitising effect observed in these cell lines (Fig. 5).

Surprisingly, J82, a mesenchymal cell line harbouring p53 mutation appeared to be significantly more sensitive to cisplatin, than the epithelial cell line RT112 expressing wild-type p53 (IC₅₀ doses were 3.55 versus 29.2 μ M) (7). J82 cells exposed to GTC underwent S-phase arrest which is under control of ATM/Chk2/Cdc25A/Cdk2 pathway activated under different DNA damage-inducing conditions including exposure to cisplatin (35). Consistent with the lapatinib-mediated accumulation of J82 cells in the G₁ phase, combined lapatinib/GTC treatment produced a shift from early towards late S-phase as compared with the effect of GTC alone. This treatment significantly inhibited cell growth with little increase in apoptosis (Fig. 6) suggesting that the combined treatment of J82 slows down progression through the cell cycle. In RT112 cells, GTC produced a different effect on cell-cycle phase distribution. Cells accumulated at the G₁/S border suggesting involvement of the replication check-point and resembling the effect of gemcitabine alone in squamous lung cancer cell lines (36). The pattern of cell cycle distribution in GTC-treated RT112 cells may indicate that this line acquired additional genetic lesions in the ATM/Chk2/Cdc25A/Cdk2 pathway leading to the escape from inter-S phase checkpoint control. Combined GTC/lapatinib treatment enhanced the effect of GTC alone; it lead to the accumulation of the vast majority of the cells in G₁ or at the G₁/S phase border and activated the p53-dependent apoptotic pathway (Figs. 6 and 7). These data are in agreement with the finding that lapatinib increases the growth inhibitory effect of GTC applied at 1/8 of the IC₅₀ concentration from 36 to 82% (Fig. 6). They also demonstrate that pre-treatment of RT112 with lapatinib allows the dose reduction of the highly toxic GTC cocktail to be reduced by 8-fold in order to reach 82% of growth inhibition.

These data provide a rationale for investigating lapatinib in the clinical setting. However, additional studies are required to gain further insights into the processes that influence the antitumour activity of lapatinib. In particular, determination of genetic backgrounds (mutations in the components of the pathways controlling G₁ and S phase checkpoints) of tumour cells responsive to lapatinib will be the focus of further studies. This will allow identification of patients who will

benefit from therapies based on combined lapatinib/GTC treatment.

Acknowledgements

This work was supported by the British Urological Foundation and MRC grant no. GO100872. We thank GlaxoSmithKline for lapatinib used in this study.

References

- Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74-108, 2005.
- Saxman SB, Propert KJ, Einhorn LH, Crawford ED, Tannock I, Raghavan D, Loehrer PJ Sr and Trump D: Long-term follow-up of a phase III intergroup study of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma: a cooperative group study. *J Clin Oncol* 15: 2564-2569, 1997.
- Von der Maase H, Hansen SW, Roberts JT, Dogliotti L, Oliver T, Moore MJ, Bodrogi I, Albers P, Knuth A, Lippert CM, Kerbrat P, Sanchez Rovira P, Wersall P, Cleall SP, Roychowdhury DF, Tomlin I, Visseren-Grul CM and Conte PF: Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of a large, randomized, multinational, multicenter, phase III study. *J Clin Oncol* 18: 3068-3077, 2000.
- Ecke TH, Bartel P, Koch S, Ruttloff J and Theissig F: Chemotherapy with gemcitabine, paclitaxel, and cisplatin in the treatment of patients with advanced transitional cell carcinoma of the urothelium. *Oncol Rep* 16: 1381-1388, 2006.
- Dominguez-Escrig JL, Kelly JD, Neal DE, King SM and Davies BR: Evaluation of the therapeutic potential of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib in preclinical models of bladder cancer. *Clin Cancer Res* 10: 4874-4884, 2004.
- Hussain MH, MacVicar GR, Petrylak DP, Dunn RL, Vaishampayan U, Lara PN Jr, Chatta GS, Nanus DM, Glode LM, Trump DL, Chen H and Smith DC: Trastuzumab, paclitaxel, carboplatin, and gemcitabine in advanced human epidermal growth factor receptor-2/neu-positive urothelial carcinoma: results of a multicenter phase II national cancer institute trial. *J Clin Oncol* 25: 2218-2224, 2007.
- McHugh LA, Kriajevskaja M, Mellon JK and Griffiths TR: Combined treatment of bladder cancer cell lines with lapatinib and varying chemotherapy regimens - evidence of schedule-dependent synergy. *Urology* 69: 390-394, 2007.
- Bubli EM and Yarden Y: The EGF receptor family: spearheading a merger of signaling and therapeutics. *Curr Opin Cell Biol* 19: 124-134, 2007.
- Mellon K, Wright C, Kelly P, Horne CH and Neal DE: Long-term outcome related to epidermal growth factor receptor status in bladder cancer. *J Urol* 153: 919-925, 1995.
- Chow NH, Chan SH, Tzai TS, Ho CL and Liu HS: Expression profiles of ErbB family receptors and prognosis in primary transitional cell carcinoma of the urinary bladder. *Clin Cancer Res* 7: 1957-1962, 2001.
- Kruger S, Weitsch G, Buttner H, Matthiensen A, Bohmer T, Marquardt T, Sayk F, Feller AC and Bohle A: Overexpression of c-ErbB-2 oncoprotein in muscle-invasive bladder carcinoma: Relationship with gene amplification, clinicopathological parameters and prognostic outcome. *Int J Oncol* 21: 981-987, 2002.
- Rajjayabun PH, Keegan PE, Lunec J and Mellon JK: ErbB receptor expression patterns in human bladder cancer. *Urology* 66: 196-200, 2005.
- Memon AA, Sorensen BS, Melgard P, Fokdal L, Thykjaer T and Nexø E: Expression of HER3, HER4 and their ligand heregulin-4 is associated with better survival in bladder cancer patients. *Br J Cancer* 91: 2034-2041, 2004.
- Memon AA, Sorensen BS, Meldgaard P, Fokdal L, Thykjaer T and Nexø E: The relation between survival and expression of HER1 and HER2 depends on the expression of HER3 and HER4: a study in bladder cancer patients. *Br J Cancer* 94: 1703-1709, 2006.
- Linggi B and Carpenter G: ErbB receptors: new insights on mechanisms and biology. *Trends Cell Biol* 16: 649-656, 2006.
- Tzahar E, Waterman H, Chen X, Levkowitz G, Karunakaran D, Lavi S, Ratzkin BJ and Yarden Y: A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* 16: 5276-5287, 1996.
- Amsellem-Ouazana D, Bieche I, Tozlu S, Botto H, Debre B and Lidereau R: Gene expression profiling of ErbB receptors and ligands in human transitional cell carcinoma of the bladder. *J Urol* 175: 1127-1132, 2006.
- Benhar M, Engelberg D and Levitzki A: Cisplatin-induced activation of the EGF receptor. *Oncogene* 21: 8723-8731, 2002.
- Sumitomo M, Asano T, Asakuma J, Asano T, Horiguchi A and Hayakawa M: Zd1839 modulates paclitaxel response in renal cancer by blocking paclitaxel-induced activation of the epidermal growth factor receptor-extracellular signal-regulated kinase pathway. *Clin Cancer Res* 10: 794-801, 2004.
- Van Schaeckbroeck S, Karaiskou-McCaul A, Kelly D, Longley D, Galligan L, van Cutsem E and Johnston P: Epidermal growth factor receptor activity determines response of colorectal cancer cells to gefitinib alone and in combination with chemotherapy. *Clin Cancer Res* 11: 7480-7489, 2005.
- Chun PY, Feng FY, Scheurer AM, Davis MA, Lawrence TS and Nyati MK: Synergistic effects of gemcitabine and gefitinib in the treatment of head and neck carcinoma. *Cancer Res* 66: 981-988, 2006.
- Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens G, Allgood KJ and Spector NL: Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/ErbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 21: 6255-6263, 2002.
- Rusnak DW, Affleck K, Cockerill SG, Stubberfield C, Harris R, Page M, Smith KJ, Guntrip SB, Carter MC, Shaw RJ, Jowett A, Stables J, Topley P, Wood ER, Brignola PS, Kadwell SH, Reep BR, Mullin RJ, Allgood KJ, Keith BR, Crosby RM, Murray DM, Knight WB, Gilmer TM and Lackey K: The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer. *Cancer Res* 61: 7196-7203, 2001.
- Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Ellis B, Pennisi C, Horne E, Lackey K, Allgood KJ, Rusnak DW, Gilmer TM and Shewchuk L: A unique structure for epidermal growth factor receptor bound to GW572016 (lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res* 64: 6652-6659, 2004.
- Xia W, Liu LH, Ho P and Spector NL: Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. *Oncogene* 23: 646-653, 2004.
- Zhou Y, Li S, Hu YP, Wang J, Hauser J, Conway AN, Vinci MA, Humphrey L, Zborowska E, Willson JK and Brattain MG: Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma cells to apoptosis. *Cancer Res* 66: 404-411, 2006.
- Xia W, Husain I, Liu L, Bacus S, Saini S, Spohn J, Pry K, Westlund R, Stein SH and Spector NL: Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers. *Cancer Res* 67: 1170-1175, 2007.
- Coley HM, Shotton CF, Ajose-Adeogun A, Modjtahedi H and Thomas H: Receptor tyrosine kinase (rtk) inhibition is effective in chemosensitising EGFR-expressing drug resistant human ovarian cancer cell lines when used in combination with cytotoxic agents. *Biochem Pharmacol* 72: 941-948, 2006.
- Budman DR, Soong R, Calabro A, Tai J and Diasio R: Identification of potentially useful combinations of epidermal growth factor receptor tyrosine kinase antagonists with conventional cytotoxic agents using median effect analysis. *Anticancer Drugs* 17: 921-928, 2006.
- Matsui Y, Ueda S, Watanabe J, Kuwabara I, Ogawa O and Nishiyama H: Sensitizing effect of galectin-7 in urothelial cancer to cisplatin through the accumulation of intracellular reactive oxygen species. *Cancer Res* 67: 1212-1220, 2007.
- Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y and Taya Y: P53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by ser-46-phosphorylated p53. *Cell* 102: 849-862, 2000.

32. Gregoire V, Hittelman WN, Rosier JF and Milas L: Chemo-radiotherapy: radiosensitizing nucleoside analogues (review). *Oncol Rep* 6: 949-957, 1999.
33. Jordan MA and Wilson L: Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 4: 253-265, 2004.
34. Sedletska Y, Giraud-Panis MJ and Malinge JM: Cisplatin is a DNA-damaging antitumour compound triggering multifactorial biochemical responses in cancer cells: importance of apoptotic pathways. *Curr Med Chem Anticancer Agents* 5: 251-265, 2005.
35. Agner J, Falck J, Lukas J and Bartek J: Differential impact of diverse anticancer chemotherapeutics on the Cdc25A-degradation checkpoint pathway. *Exp Cell Res* 302: 162-169, 2005.
36. Pauwels B, Korst AE, Andriessen V, Baay MF, Pattyn GG, Lambrechts HA, Pooter CM, Lardon F and Vermorken JB: Unraveling the mechanism of radiosensitization by gemcitabine: the role of TP53. *Radiat Res* 164: 642-650, 2005.