EGFR family inhibition identifies p38 MAPK as a potential therapeutic target in bladder cancer

Regina Mora Vidal¹, Sergio Regufe da Mota¹, Annette Hayden¹, Hannah Markham², James Douglas¹, Graham Packham¹, Simon J. Crabb¹

¹ Cancer Sciences Unit, University of Southampton, Southampton, UK
² Department of Histopathology, University Hospital Southampton NHS Foundation Trust, Southampton, UK

Corresponding author: Dr Simon Crabb, Cancer Sciences Unit, University of Southampton, Southampton General Hospital, Mailpoint 824, Tremona Road, Southampton SO16 6YD, UK, s.j.crabb@southampton.ac.uk, Tel +44 (0)23 8120 5170, Fax +44 (0)23 8120 5152

* Joint first authors

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Abstract

Objective
To investigate perturbations in downstream signalling pathway activation and potential resistance mechanisms to EGFR and/or HER2 inhibition in cell line models of bladder cancer.
Methods
We undertook a structured screening approach by phosphokinese array, followed by validation steps, to detect activated downstream signalling pathway nodes after therapeutic inhibition of EGFR and/or HER2 in bladder cancer cell lines.

Results
Erlotinib treatment of RT112 cells induced phosphorylation of 9 activated phospho-protein targets (p38 MAPK (Thr180/Tyr182), GSK-3α/β (Ser21/9), MEK1/2 (Ser218/222, Ser222/226), Akt (Ser473), TOR (Ser2448), Src (Tyr419), p27 (Thr198), p27 (Thr157) and PLCγ-1 (Tyr783)) whereas STAT4 (Tyr693) phosphorylation was reduced. Of these, p38 MAPK phosphorylation was confirmed to occur in response to inhibition of either EGFR and/or HER2 signalling through multiple validation steps including differing bladder cancer cell lines (RT112, UM-UC-3 and T24) and methods of receptor pathway inhibition (erlotinib, lapatinib, siRNA depletion of EGFR or HER2). Chemical inhibition of p38 MAPK with SB203580 led to inhibition of proliferation in RT112, UM-UC-3 and T24 cell lines (IC\textsubscript{50} 20.85 µM, 76.78 µM and 79.12 µM respectively). Fractional effect analyses indicated a synergistic interaction for inhibition of cell proliferation when combining SB203580 with lapatinib.

Conclusion
p38 MAPK is a potential therapeutic target in bladder cancer and this strategy warrants further development in this disease. It may also allow for combination therapy strategies to be developed in conjunction with EGFR and/or HER2 inhibition.

Introduction
Transitional cell carcinoma (TCC) of the bladder accounts for 10,000 new diagnoses and 5,000 deaths annually in the UK. Cisplatin based combination chemotherapy, in those fit enough to receive it, is the standard of care for initial systemic therapy. This results in a median survival of approximately 14 months as palliative treatment for metastatic TCC or an absolute survival advantage of 5-6% in the neoadjuvant setting prior to radical cystectomy or radiotherapy.\textsuperscript{1} Immunotherapy has recently been demonstrated to be effective as a second line palliative treatment but the disease is rapidly lethal in these circumstances with a median survival of only 10 months.\textsuperscript{2} Furthermore, no molecularly targeted or stratified treatment strategy has yet been established for this disease. A significant unmet need therefore exists to develop new therapeutic approaches based on underlying disease biology.\textsuperscript{1}

The human epidermal growth factor receptor (HER) family are cell surface tyrosine kinase receptors which include the epidermal growth factor receptor (EGFR), HER2, HER3 and HER4. Cognate ligand binding induces homo- or hetero-dimerisation between HER family members followed by diverse downstream signalling pathway activation. Although their oncogenic activity is believed to be mediated primarily through the RAS/mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, other pathways may also be involved including the signal transducer and activator of transcription (STAT) and phospholipase C and SRC/FAK pathways. Aberrant HER family activation in various cancers induces cellular proliferation, migration, attenuation of apoptosis, invasion and metastasis. EGFR and/or HER2 are established as clinical therapeutic targets for breast cancer, gastric cancer, non-small cell lung cancer, head and neck cancer and colorectal cancer.\textsuperscript{1, 3}
EGFR and HER2 protein over-expression in TCC/bladder cancer is poorly defined at between 35-96% and 8-98% respectively.\textsuperscript{4-9} Increased expression of either has been linked to TCC grade, stage and survival outcomes.\textsuperscript{4-6, 8-11} Prognostic impact of EGFR and HER2 expression may link to their co-expression status with respect to each other and also with HER3 and HER4.\textsuperscript{7, 11} EGFR and/or HER2 inhibition have been demonstrated to be active in pre-clinical models and have been tested in early phase TCC clinical trials, either as single agents, or in combination with cytotoxic chemotherapy.\textsuperscript{1, 3, 12-15} Single agent clinical activity in response to targeted inhibition of either EGFR or HER2 is established but randomised data and clear demonstration of efficacy that would warrant routine use is awaited. Furthermore the requirement for a patient selection strategy and how this should be undertaken are currently unknown.

Understanding of factors linked to response to EGFR and/or HER2 inhibition in TCC will likely be critical to patient selection for these approaches and may elucidate strategies for future clinical evaluation. We hypothesised that interrogation of downstream signalling pathway activation following chemical inhibition of EGFR and/or HER2 would allow for identification of critical molecular ‘nodes’ relevant for therapeutic efficacy. We demonstrate that p38 mitogen-activated protein kinase (MAPK) is activated in response to EGFR/HER2 inhibition in pre-clinical models for TCC/bladder cancer and that it represents a potential new therapeutic target.
Methods

Reagents and cell lines
Antibodies used were goat anti-EGFR, rabbit anti-HER4 (Santa Cruz Biotechnology, Texas, US); mouse anti-HER3 (Thermo Scientific, Basingstoke, UK); rabbit anti-actin (Sigma, Gillingham, UK); rabbit anti-HER2, rabbit anti-phospho Akt (Ser473), rabbit anti-Akt, rabbit anti-phospho ERK1/2 (Thr202/Tyr204), mouse anti-ERK1/2, rabbit anti-phospho p38 MAPK (Thr180/Tyr182) and rabbit anti-p38 MAPK (New England BioLabs, Hitchin, UK). Horseradish peroxidase (HRP)-conjugated secondary antibody was from GE Healthcare (UK). Erlotinib, lapatinib and SB203580 (Selleckchem, Munich) were dissolved in dimethyl sulfoxide (DMSO) and stored as stock solutions at -20°C. Cell lines (HT1376, T24, RT112, RT112CP, UM-UC-3 and UM-UC-6) were maintained in Dulbecco’s modified Eagle medium or in Roswell Park Memorial Institute 1640 media (RPMI) supplemented with 10% (v/v) fetal bovine serum, 2mM L-glutamine, 100μg/ml penicillin G and 100μg/ml streptomycin (PAA Laboratories GmbH, Austria). All cell lines were obtained from the European Collection of Cell Cultures. RT112CP were a kind gift from Prof John Masters at University College London.16

Immunoblotting
Immunoblotting was performed as previously described.17 Representative examples of a minimum of 3 separate experiments are shown.

Cell proliferation assays and assessment of synergistic interactions for combination chemical inhibition
Cell proliferation was measured as previously described using CellTiter 96 AQueous
One Solution Reagent (Promega, Southampton, UK) or by using the CellTiter-Glo®
2.0 assay (Promega, Southampton, UK) according to the manufacturer’s
instructions. IC50 values calculated for SB203580 in RT112, UM-UC-3 and T24
cells using GraphPad Prism software. Synergism for inhibition of cell proliferation as
a result of combination drug exposure was assessed using fractional effect analyses
using CalcuSyn software (version 2.0, Biosoft, Cambridge, UK) according to the
manufacturer’s instructions and as previously described.

siRNA forward transfection
RNA interference was performed with ON-TARGETplus siRNA SMARTpools against
EGFR or HER2 and non-targeting controls (GE Healthcare, UK) transfected with
INTERFERin transfection reagent (Polyplus Transfection Inc, New York, USA)
according to the manufacturer’s instructions. Briefly, 1.5 – 3x10^5 cells per well were
incubated overnight. The following day, 500 μl of Opti-MEM reduced serum medium
(Invitrogen, UK) was mixed with 5 μl INTERFERin and 1.5 μl siRNA for 15 minutes at
room temperature and the siRNA/INTERFERin complex was added to the cells for a
final siRNA concentration of 50 nM.

Human phosphokinase array
A human phospho-kinase membrane-based sandwich immunoassay (Proteome
Profiler Human Phospho-Kinase Array Kit ARY003, R&D Systems, Abingdon, UK)
was used to detect simultaneously the relative phosphorylation levels of 46 protein
kinase phosphorylation sites of potentially relevant signalling pathways downstream
of EGFR and/orHER2. 3x10^6 cells were cultured overnight before treating for one
hour with the relevant drug or DMSO solvent control. Following EGFR and/or HER2 inhibition the array was processed according to the manufacturer’s protocol.

Phosphorylated protein was detected using ECL reagent on a ChemiDoc-It Imaging System (Ultra-Violet Products, UK) using VisionWorksLS Image Acquisition and Analysis software (UVP Ltd).

**Phosphokinase array data analysis**

Signals from the phosphokinase array were quantified using Quantity One software. Data were then analysed by each of three different methods: i) as raw data (pixel density), ii) as a percentage of the array positive control mean signal, and iii) as a percentage of the global mean of all signals on the array. Only phosphokinase changes that reached statistical significance (p<0.05) by each of these three analysis approaches were taken forward for subsequent investigation and validation.

Statistical analysis was performed using Student’s t-test and presented for p values <0.01 and <0.05.
Results

Erlotinib and lapatinib response in bladder cancer cell lines

Mechanisms for resistance to inhibition of EGFR and/or HER2 might include alterations to downstream signalling pathway activation. To investigate this hypothesis in bladder cancer cell lines we undertook an initial screen to assess activation, in terms of kinase phosphorylation status, through a phosphokinase array. We utilised prospectively determined criteria for subsequent selection and validation of altered downstream signalling pathway nodes (Additional file 1: Figure S1). Our approach was to incorporate both the EGFR inhibitor erlotinib and the dual EGFR/HER2 inhibitor lapatinib with the intention to detect downstream signalling impacts that were common to both drugs. RT112 cells were chosen for initial analyses, as they expressed relatively high levels of both EGFR and HER2 amongst a panel of bladder cancer cell lines assessed (Fig. 1A) and were sensitive to both erlotinib (IC\textsubscript{50} 6.6 µM) and lapatinib (IC\textsubscript{50} 6.1 µM) in cell proliferation experiments. In this cell line, downregulation of phospho-ERK1/2 was robust following exposure to the EGFR inhibitor erlotinib by 1 hour (Fig. 1B) and we therefore selected this time point for initial studies.

1st phosphokinase array screen of erlotinib treatment of RT112 cells

The phosphokinase array was used to interrogate RT112 cells after treatment with erlotinib for 1 hour at a dose of 5 times the IC\textsubscript{50} value determined in cell proliferation experiments (data not shown). Increased expression was seen for 9 phospho-protein targets in erlotinib treated compared to control cells (Fig. 1C and Additional file 1: Figure S2 and S3), with consistent results achieved with each of the three data analysis methods used (Additional file 1: Figure S4A, B). These were p38 MAPK
(Thr180/Tyr182), GSK-3α/β (Ser21/9), MEK1/2 (Ser218/222, Ser222/226), Akt (Ser473), TOR (Ser2448), Src (Tyr419), p27 (Thr198), p27 (Thr157) and PLCγ-1 (Tyr783). In addition STAT4 (Tyr693) phosphorylation was reduced. Discrepancy was seen with the phosphorylation status of p53 (Ser392) (upregulated when analysed by pixel density, but downregulated when analysed by the percentage of the positive control or percentage of the global mean of signals) and so it was excluded, based on our pre-determined analysis plan, from subsequent validation steps.

Validation step 1 (2nd phosphokinase array) of lapatinib treatment of RT112 cells

We next undertook a validation step by retesting only the 10 phosphokinase targets confirmed to change on exposure to erlotinib by undertaking a second phosphokinase array experiment in RT112 cells but with the dual EGFR/HER2 inhibitor lapatinib, again at 5 times its IC\text{50} concentration for cell proliferation. In doing so, we validated 3 of the 10 phospho-proteins assessed that were again found to alter in their expression status. These were p38 MAPK (Thr180/Tyr182) and GSK-3α/β (Ser21/9) which were upregulated and STAT4 (Tyr693) which was downregulated (Fig. 1D and Additional file 1: Figure S4c, 4d, S5 and S6).

Validation step 2, individual phosphokinase experiments

To further validate the two rounds of phosphokinase array results, we undertook Western blot analyses for phosphorylation status of p38 MAPK (Thr180/Tyr182), GSK-3α/β (Ser21/9) and STAT4 (Tyr693) in response to erlotinib or lapatinib exposure. We found consistent increases in phosphorylation of p38 MAPK in
response to either drug (Fig. 2A, B). However, no difference was confirmed in the phosphorylation status of GSK-3α/β or STAT4 (data not shown). The increase of p38 MAPK phosphorylation in response to inhibition of EGFR or EGFR/HER2 was confirmed both in RT112 cells and also in T24 bladder cancer cells (Fig. 2A, B, C, D).

We then extended the validation of our findings by replicating the impact on p38 phosphorylation in response to either EGFR or HER2 depletion by siRNA in RT112 cells (Fig. 2E). Depletion of either receptor increased p38 phosphorylation.

**Combination inhibition of p38 MAPK and EGFR +/- HER2 in bladder cancer cell lines**

We hypothesised that p38 MAPK phosphorylation in response to EGFR and/or HER2 inhibition could represent an escape mechanism to EGFR/HER2 directed therapy and represent a therapeutic target in bladder cancer cells. We first tested chemical inhibition of p38 MAPK with the pyridinyl imidazole compound SB203580 and found an anti-proliferative effect in UM-UC-3, RT112 and T24 cell lines with mean IC$_{50}$ values of 76.78 µM, 20.85 µM and 79.12 µM respectively (Fig 3.). Next we tested the effect, in UM-UC-3, T24 and RT112 cells, of dual EGFR/HER2 inhibition using lapatinib in combination with p38 MAPK inhibition with SB203580. Cell growth inhibition when these drugs were combined was greater than the effect with either drug alone (Fig. 4A) in all cell lines tested and these differences were statistically significant (p<0.0001). Similar effects were seen when combining EGFR inhibition with erlotinib and SB203580 in all cell lines analysed (Fig. 4B).
Next we undertook fractional effect analyses to formally test for a synergistic interaction between lapatinib and SB203580 in RT112 cells (Fig. 4C). Of the dose combinations tested, if both drugs were each combined at 0.75 times their respective IC$_{50}$ values or greater, then we achieved ~100% cell growth inhibition. The calculated combination index (CI) values, where values <1 indicate ‘synergy’, at IC$_{50}$, IC$_{75}$ and IC$_{90}$ were 0.66, 0.59 and 0.53 respectively. Similar interactions with combination therapy were seen in T24 cells (Fig. 4D). The calculated CI values at ED50, ED75 and ED90 were 0.64, 0.53 and 0.45 respectively.
Discussion

Chemotherapy remains the standard of care for the systemic therapy of muscle invasive bladder cancer but outcomes are modest. We have yet to see any molecularly targeted therapeutic strategies introduced for this disease and so a significant unmet clinical need remains. EGFR and HER2 are amongst the more promising therapeutic targets currently under clinical development in bladder cancer. However it remains unclear what the most appropriate selection strategy or the potential mechanisms of resistance to this approach might be. We took chemical inhibition of EGFR or EGFR/HER2, using agents currently in clinical evaluation for bladder cancer, as a starting point for a screen/validation approach to detect downstream signalling targets of potential therapeutic importance. Using this approach, we have identified p38 MAPK as a consistently activated downstream signalling node and were able to validate this across cell lines and with multiple approaches to EGFR and/or HER2 inhibition.

p38 MAPK belongs to a family which also includes ERK1/2 (extracellular signal-regulated kinases 1 and 2) and JNKs (c-Jun-N-terminal kinases). p38 MAPK exists as four isoforms (α, β, γ and δ) encoded by different genes. p38α is expressed at high levels in most cell types whereas p38β is expressed at lower levels and expression of the other two members, p38γ and p38δ, is tissue-specific. Extracellular signals activate the MAPK cascade to phosphorylate p38α in threonine and tyrosine residues, resulting in a kinase activating conformational change. Activated p38α phosphorylates multiple cellular targets, including DNA and RNA-binding proteins involved in the regulation of gene expression, transcription factors (e.g., ATF2, MEF2A, SAP1, CHOP, Elk-1, STAT1, STAT4 and p53) serine and
threonine kinases (e.g., GSK-3β, MK2, MK3, MSK1 and MSK2) and regulatory proteins involved in cell cycle regulation (e.g., cyclin D1, cyclin D3 and p57kip2) apoptosis (e.g., Bax and BimEL) and cell survival (e.g., caspase-3 and caspase-8). p38 MAPK impacts on diverse cellular functions including immune and inflammatory responses, the cell cycle, cellular proliferation, apoptosis and acts as a tumour suppressor.

The potential role of p38 MAPK in bladder cancer is poorly defined to date and has received little investigation. In a study of 91 primary bladder cancer samples p38 MAPK expression was associated with higher tumour grade and detrusor muscle invasion. In preclinical models, p38 MAPK was shown to regulate invasion of bladder cancer cells by modulating matrix metalloproteinases 2 and 9. Additionally, p38 MAPK activation may confer resistance to apoptosis and a survival advantage to bladder cancer cells.

Our screening approach to determine the impact of EGFR and/or HER2 chemical inhibition on downstream signalling detected consistent p38 MAPK phosphorylation in RT112 and T24 bladder cancer cells. We were then able to validate these findings by siRNA depletion of EGFR or HER2. This is in accordance with findings in glioblastoma multiforme cells after treating with the dual EGFR and HER2 inhibitor GW2974. Further investigation through combination treatment experiments including fractional effect assays showed that dual EGFR/HER2 inhibition with lapatinib combined with the p38 MAPK inhibitor SB203580 had a synergistic effect on cell proliferation inhibition in UM-UC-3, RT112 and T24 cells. A possible explanation for synergism would be that lapatinib inhibits the activation of Ras-MAPK.
and PI3K/Akt pathways, leading to cell cycle arrest and apoptosis, but at the same time, p38 MAPK may be activated as a survival mechanism to this inhibition which is susceptible to chemical inhibition.

p38 MAPK is a pharmaceutical target for inhibition in non-malignant diseases such as asthma, rheumatoid arthritis, chronic obstructive pulmonary disease or inflammatory bowel disease. Earlier clinical experience with available agents suffered from target specificity limitations and off target toxicity however newer agents in clinical development may allow this to be addressed by improved target selectivity. Clinical evaluation of p38 MAPK inhibition in cancer might therefore be practical and has begun, for example in myelodysplastic syndromes. Our data provide a rationale now for clinical evaluation of both single agent p38 MAPK inhibition in bladder cancer and also potentially for its combination with EGFR/HER2 inhibition. Initial studies are in development. Further pre-clinical development of this approach in urothelial cancer is also warranted including with in-vivo assessment with xenograft approaches.
Conclusion

We have identified and validated p38 MAPK as a downstream signalling node that undergoes consistent phosphorylation in response to either EGFR or dual EGFR/HER2 inhibition using agents in current clinical evaluation in bladder cancer. Inhibition of p38 MAPK led to inhibition of cell proliferation in bladder cancer models and this effect synergised with dual EGFR/HER2 inhibition. This work detects p38 MAPK as a potential therapeutic target in bladder cancer and that warrants further development.
References


Figure 1. (A) EGFR, HER2, HER3 and HER4 protein expression in a panel of bladder cancer cell lines. Cell lines were grown to near confluence and analysed by Western blot. β-actin was used as a sample loading control. (B) ERK1/2 phosphorylation in response to erlotinib in RT112 bladder cancer cells. RT112 cells were treated with erlotinib at 5x the IC$_{50}$ dose (33µM) for cell proliferation for the indicated time points followed by Western blot for phospho-ERK1/2 (Thr202/Tyr204) and total expression of ERK1/2. Representative of two separate experiments. Bar charts showing activation in phosphokinase array experiments in RT112 cells of those phospho-protein targets that reached pre-determined criteria for a statistically significant change after either (C) erlotinib or (D) lapatinib treatment compared to DMSO solvent control. The analysis shown here is undertaken by the ‘raw data’ (pixel density) approach. Error bars represent the standard deviation of duplicates. (UT, untreated; C, DMSO solvent control; Erl, erlotinib)

Figure 2. Changes in phosphorylation of p38 MAPK in response to reduced signalling through EGFR and/or HER2 analysed by Western blot. (A and B) RT112 cells were treated with erlotinib or lapatinib respectively for the indicated durations, or (C, D) T24 bladder cancer cells were treated with erlotinib or lapatinib for 2 hours, at doses of 2.5x and 5x the IC$_{50}$ for cell proliferation. (E) EGFR or HER2 were depleted by siRNA knockdown in RT112 cells and analysed after incubation for 48 hours. (p-p38, phosphorylated p38 MAPK (Thr180/Tyr182); p38, total p38 MAPK; UT, untreated; D, DMSO solvent control; C, control siRNA). β-actin was used as a sample loading control.
Figure 3. Proliferation of bladder cancer cells following treatment SB203580. Dose-response curves in cell proliferation assays in RT112, UM-UC-3 and T24 bladder cancer cell lines treated with SB203580 for 3 days. IC\textsubscript{50} values calculated for SB203580 are indicated for each cell line.

Figure 4. Proliferation of bladder cancer cells following treatment SB203580, lapatinib or erlotinib or in combination. (A) RT112, T24 and UM-UC-3 cells were treated with lapatinib, SB203580 or both in combination (each drug at its IC\textsubscript{50} concentration for each cell line respectively) or DMSO (solvent control) as indicated for 3 days. (B) RT112, T24 and UM-UC-3 cells were treated with erlotinib and SB203580 or both in combination (each drug at its IC\textsubscript{50} concentration for each cell line respectively) or DMSO (solvent control) as indicated for 3 days. (C and D) Combination index studies: (C) RT112 or (D) T24 cells were treated with lapatinib or SB203580, with each at the indicated multiples of the respective IC\textsubscript{50} values for proliferation in each cell line or both in combination, or DMSO (solvent control). All results are expressed as the percentage metabolic activity in cell proliferation assays compared to DMSO treated cells. Each data point represents the mean of (A and B) 3 or (C to D) 2 independent experiments determinations ± standard deviation. Statistical analysis in (A) and (B) was performed 2 way ANOVA (**** p<0.0001).
Supplementary Figure Legends

**Supplementary Figure 1.** Schematic overview of the screening and validation pathway used to determine kinase phosphorylation patterns in bladder cancer cells following EGFR and/or HER2 signalling inhibition.

**Supplementary Figure 2.** Image of the 1st human phosphokinase array experiment of erlotinib treated RT112 bladder cancer cells. RT112 cells were treated with erlotinib at 5x the IC\textsubscript{50} dose (33µM) for cell proliferation for 1 hour. Proteins that had statistically significant changes in their phosphorylation status compared to DMSO solvent control exposed samples are indicated.

**Supplementary Figure 3.** Data for the 1st human phosphokinase array experiment of erlotinib treated cells analysed according to three approaches as described in Methods (pixel density, % of positive control, % of global mean). Statistical analysis was performed using Student’s t-test. Statistically significant changes are highlighted in pink p<0.01 or yellow p<0.05. * Shows those targets for which statistically significant changes (p<0.05) were found by all three analysis methods.

**Supplementary Figure 4.** Bar charts showing activation in phosphokinase array experiments in RT112 cells of those phospho-protein targets that reached pre-determined criteria for a statistically significant change after either (a, b) erlotinib or (c, d) lapatinib treatment compared to DMSO solvent control. The analyses shown here were undertaken with respect to (a, c) the % of the positive control or (b, d) the
% of the global mean as indicated. Error bars represent the standard deviation of duplicates.

**Supplementary Figure 5.** Image of the 2nd human phosphokinase array experiment of lapatinib treated RT112 bladder cancer cells. RT112 cells were treated with lapatinib at 5x the IC_{50} dose (30.5µM) for cell proliferation for 1 hour. Only the 10 proteins that showed a consistent and statistically significant change in their phosphorylation status in the initial erlotinib screen (1st phosphokinase array experiment, Figure 2a, supplementary Figures 3 and 4) were considered (according to the pre-defined screen/validation plan, Supplementary Figure 1). Of these, proteins that had statistically significant changes in their phosphorylation status compared to DMSO solvent control exposed samples are indicated.

**Supplementary Figure 6.** Data for the 2nd human phosphokinase array experiment of lapatinib treated cells analysed according to three approaches as described in Methods (pixel density, % of positive control, % of global mean). Only the 10 proteins that showed a consistent and statistically significant change in their phosphorylation status in the initial erlotinib screen (1st phosphokinase array experiment, Figure 2a, supplementary Figures 3 and 4) were considered (according to the pre-defined screen/validation plan, Supplementary Figure 1). Statistical analysis was performed using Student’s t-test. Statistically significant changes are highlighted in pink p<0.01 or yellow p<0.05. * Shows those targets for which statistically significant changes (p<0.05) were found by all three analysis methods.