Targeting focal adhesion kinase in ER+/HER2+ breast cancer improves trastuzumab response

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Abstract

The HER2 transmembrane receptor is a well-characterised predictive marker for trastuzumab benefit and may be associated with decreased benefit from endocrine therapy use. Despite the clinical effectiveness of anti-HER2 agents in such cases, resistance represents a significant limiting factor. Focal adhesion kinase (FAK) plays an important role in HER2 signalling, mediating downstream Akt activation in addition to HER2 cross talk with other growth factor receptors. In this study, we investigated the therapeutic potential of FAK in oestrogen receptor-positive (ER+)/HER2+ breast cancer using the novel FAK-specific inhibitor PF4554878 ('PF878'). The activation of the FAK/HER2 signalling pathway was assessed in ER+/HER2- (MCF7 and T47D) and ER+/HER2+ (BT-474 and MDAMB361) breast cancer cells in the presence or absence of PF878 and PF878 ± trastuzumab. The effects of PF878 on cell growth as a monotherapy and in combination with trastuzumab were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Coulter counting with isobologram analysis to determine synergy/additive effects. FAK activation (at Y861 but not at Y397) was highest in ER+/HER2+ cells, which also demonstrated the greatest sensitivity to PF878. As a monotherapy, PF878 prevented heregulin-induced MDA361 cell migration, but had no significant effect on cell growth. The treatment of ER+/HER2+ cells with PF878 and trastuzumab in combination resulted in the synergistic inhibition of cell proliferation. Underlying this was an abrogation of Akt activity and increased poly(ADP-ribose) polymerase cleavage, effects that were greatest in trastuzumab-refractory MDA361 cells. Collectively, these data support a role for FAK in ER+/HER2+ breast cancer, where its targeting has the potential to improve trastuzumab response. This is particularly important in the context of ER+/HER2+, trastuzumab-refractory disease, where FAK inhibition may present an important strategy to restore trastuzumab sensitivity.

Key Words

- ▶ focal adhesion kinase
- ▶ human epidermal growth factor receptor 2
- ▶ HER2
- ▶ luminal B breast cancer
- ▶ trastuzumab
- ▶ breast cancer

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Introduction

Molecular and biological profiling has identified a subset of oestrogen receptor-positive (ER+) luminal breast cancers that co-expresses the epidermal growth factor receptor 2 (ERBB2/'HER2') protein (Guin *et al.* 2012).

HER2 expression in ER+ breast cancers characterises a dominant form of the 'luminal B' molecular subtype and, compared with ER+/HER2- (luminal A) tumours, these tumours tend to exhibit reduced disease-free survival

(Ishitobi *et al.* 2013) or increased recurrence rates (Millar *et al.* 2009) and an approximately twofold increased adjusted risk of breast cancer mortality (Haque *et al.* 2012).

The presence of HER2 in ER+ breast cancers directs for treatment using agents targeted against the HER2 receptor. Trastuzumab (Herceptin, Genentech, South San Francisco, CA, USA), a humanised MAB directed against the extracellular domain of HER2 (Carter *et al.* 1992), has been shown to selectively exert anti-tumour effects in cancer models and patients with HER2-enriched breast cancer (Vogel *et al.* 2002, Romond *et al.* 2005). Despite the clinical effectiveness of trastuzumab in the treatment of HER2+ tumours, intrinsic or secondary resistance to trastuzumab represents a significant hurdle (Arribas *et al.* 2011), highlighting the importance of developing new therapies for this disease.

Focal adhesion kinase (FAK) is an intracellular kinase localised to focal adhesions and undergoes integrin-dependent tyrosine phosphorylation. FAK controls a number of cellular functions such as cell migration and invasion through its ability to regulate cytoskeletal function. More recently, FAK has been shown to regulate epithelial-to-mesenchymal transitions through the modulation of cell–cell junction integrity (Cicchini *et al.* 2008). Consequently, FAK activation has been shown to contribute to the malignant phenotype of breast cancer cells and is associated with invasive breast tumours (Lin *et al.* 2005).

FAK represents a link between integrin and receptor tyrosine kinases, including the erbB family, acting as a scaffold that interacts with and integrates both these elements to promote their clustering and activity within the membrane. FAK additionally serves as a key effector for erbB receptors, with the EGF being unable to phosphorylate the EGFR and promote migration in cells lacking FAK (Sieg *et al.* 2000), while heregulin/HER2 stimulation results in altered FAK phosphorylation and modification of focal contacts and cell-matrix adhesion (Vadlamudi *et al.* 2003). Moreover, FAK is key to the transduction of HER2 signalling in HER2-overexpressing MCF10A breast cells (Wang *et al.* 2005) and in MUC4-overexpressing ovarian cancer cells, which have elevated HER2 activity (Ponnusamy *et al.* 2008). FAK has been reported to be of prognostic significance in lung and oesophageal tumours (Itoh *et al.* 2004), and in breast cancer, levels of FAK (PTK2) expression correlate strongly with poor tumour differentiation and are significantly associated with HER2 overexpression (Schmitz *et al.* 2005). Interestingly, in tumours expressing HER2, the levels of FAK phosphorylation also appear to be significantly greater.

Given that intrinsic hyperactivation (Berns *et al.* 2007) and/or trastuzumab-induced activation of alternative

signalling pathways (Lee-Hoeflich *et al.* 2008) have been shown to mediate trastuzumab resistance and that FAK acts as a downstream effector of HER2 signalling, we investigated whether FAK contributes to the limitation of trastuzumab response in ER+/HER2+ breast cancer. We showed that the inhibition of FAK activity in cell models of ER+/HER2+ breast cancer augments their response to trastuzumab and is accompanied by an enhanced suppression of Akt signalling and increased apoptosis. Our data point to the potential of FAK as a novel therapeutic target in ER+/HER2+ breast cancer through which response to the existing HER2-targeted agents might be improved.

Materials and methods

Reagents

The FAK-selective inhibitor PF4554878 ('PF878' (Infante *et al.* 2012)) was provided by Pfizer (Sandwich, Kent, UK). Stock solutions of PF878 were dissolved in DMSO prior to dilution in tissue culture medium at the concentrations indicated. Antibodies used were as follows: anti-FAK (total and Y397), Src kinase (Y419) and Akt (Ser 473), which were obtained from Invitrogen, and anti-HER2 (Y1121 and total) and anti-ERK1/2 (S202/T204 and total), which were obtained from Cell Signalling Technology (New England Biolabs, Herts, UK). All other reagents were obtained from Sigma, unless otherwise stated.

Cell lines and cell culture

Two human breast carcinoma cell lines that constitutively overexpress HER2 because of the amplification of the *HER2* gene (BT-474 and MDA-MB-361 (Hynes *et al.* 1989)) and two human breast carcinoma cells that lack HER2 overexpression (MCF7 and T47D) were obtained from the American Type Culture Collection (ATCC, Middlesex, UK). All the four cell lines were routinely cultured in RPMI medium (Invitrogen) supplemented with 5% FCS, 10 IU/ml penicillin, 10 µg/ml streptomycin, 2.5 µg/ml fungizone and 200 mM glutamine. In all the experiments, the tissue culture medium (± treatments as indicated) was replaced every 3 days, unless otherwise stated.

Cell proliferation assays

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay Changes in cell number in response to treatment exposure were determined by measuring mitochondrial conversion of

the formazan reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) as follows: the cells were seeded into 96-well plates at 6×10^3 cells/well. After 24 h, the medium in the wells was replaced with fresh medium containing treatment agents as indicated. The plates were incubated at 37 °C for the times indicated, with the medium being changed every 3 days. Following this, the medium was aspirated from each well, replaced with fresh RPMI medium containing MTT reagent (0.5 mg/ml) and incubated at 37 °C for 4 h. The MTT solution was gently aspirated from the wells prior to the addition of a solubilising reagent (1% Triton X-100), and the plates were left at 4 °C overnight. The absorbance of the resulting solution was then read using a spectrophotometer ($\lambda 560$ nm).

Coulter counting assay For long-term (≥ 10 days) growth experiments to study the combination effect of PF878 and trastuzumab on cell proliferation, Coulter counting was used as follows: following harvesting of cell lines by trypsinisation, viable cells were seeded at an initial density of 3×10^4 /well in 24-well plates on day 0. On day 1, triplicate wells were trypsinised and the cells were counted using a Coulter Counter (Coulter Electronics Ltd., Luton, UK). In the remaining wells, the culture medium was aspirated and replaced with a medium containing treatment agents as indicated. Culture plates were then incubated for the times indicated prior to trypsinisation of the appropriate wells and counting of the cells using a Coulter Counter.

Western blotting

Log-phase cell cultures were lysed in 50 mM Tris, pH 7.5, 5 mM EGTA, 150 mM NaCl and 1% Triton X-100 containing protease inhibitors, 2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 20 μ M phenylarsine, 10 mM sodium molybdate, 10 μ g/ml leupeptin and 8 μ g/ml aprotinin, and proteins were resolved by SDS-PAGE using 8% gels. The proteins were immobilised on nitrocellulose membranes and subsequently probed with antibodies that recognise the active forms of FAK and additional antibodies as stated in the Results section. Repeat immunoprobings were performed using pan-antibodies to determine the total levels of these proteins and GAPDH as a loading control. Western blots were then scanned to obtain data for semi-quantitation with normalisation against GAPDH. Each

experiment was performed at least three times with representative gels reported.

Measurement of cell migration

The cells were harvested and held in suspension in the presence of PF878 for 60 min prior to seeding onto fibronectin-coated polycarbonate, microporous membranes (8 μ m pore size) at 5×10^4 cells/membrane ($\pm$ heregulin (MDA-361 cells) or serum (BT-474 cells) as a chemoattractant added to the lower chamber) and allowed to migrate to the underside of the membranes for a period of 24 h. Migratory cells were fixed, stained with 0.5% crystal violet and counted.

Ki67 proliferation antigen staining

Ki67 antigen was assessed in HER2+ BT-474 and MDA-361 cells following treatment with trastuzumab $\pm$ PF878 using MIB-1 anti-Ki67 antibodies (Coulter Electronics Ltd.). Briefly, the cells were grown and treated in petri dishes containing coverslips as indicated and then washed and fixed in formal saline. Primary antibodies (MIB-1) were applied to the coverslips after blocking with PBS/Tween, and incubation was carried out for 60 min. After washing with PBS, the secondary antibodies (Mouse Envision, DAKO UK Ltd., Ely, Cambridgeshire, UK) were applied to the coverslips for 75 min. After further washing with PBS, the chromogen ('SigmaFast' DAB, Sigma) was added to the cells for 10 min, after which the coverslips were rinsed in distilled water. The samples were counterstained with 20% haematoxylin for 3 min and mounted for examination by light microscopy. Control coverslips (no primary antibodies) were checked for non-specific binding before assessing staining intensity in the test samples. The percentage of Ki67-positive cells was calculated after counting at least 1000 tumour cells.

Isobologram analysis

An isobologram analysis was conducted as described by Menendez *et al.* (2007). To examine the interaction between trastuzumab and PF878, the cells were exposed to increasing concentrations of PF878 (0–5 μ M) and a range of concentrations (0–10 μ M) of trastuzumab to empirically determine the concentration of both agents necessary to achieve 30% inhibition (IC_{30}). The concentration of each agent that inhibited growth by 30% (IC_{30}) was calculated and plotted on the separate axes of a graph; a straight line

that joined these two points was then drawn, which represented the line of additivity (Steel & Peckham 1979), i.e. zero interaction between two agents. Subsequently, growth assays were carried out using both agents in combination (PF878 (0–5 μ M) and trastuzumab (0–10 μ M)), and the data were examined to determine the concentrations of agents in combination that decreased cell proliferation by 30%. These were then plotted on the graph. Where these experimental isoeffect points fall below the line of additivity, the combination effect of the two drugs is considered to be supra-additive or synergistic, whereas antagonism occurs if the point lies above it.

Apoptosis analysis

The cells were treated with PF878 (1 μ M), trastuzumab (10 nM) or both agents in combination for either 2 h or 7 days, after which the cells were lysed and processed for western blotting as described above. Apoptosis was determined by immunoprobining using an antibody that recognises the cleaved form of poly(ADP-ribose) polymerase (PARP).

Statistical analysis

Data were analysed using two-tailed Student's *t*-test (cell migration and proliferation using PF878 as a single agent). One-way ANOVA with *post hoc* analysis was used in order to examine whether the inclusion of PF878 significantly affected the observations obtained with trastuzumab alone. Significance was assumed at $P < 0.05$.

Results

FAK activity is elevated in ER+/HER2+ vs ER+/HER2– breast cancer cell lines

The analysis of FAK expression and activity across a panel of four cell lines reflecting ER+/HER2– (MCF7 and T47D) and ER+/HER2+ (BT-474 and MDA-361) breast cancer subtypes revealed that the levels of FAK *per se* were not changed between these cell lines (Fig. 1A). However, ER+/HER2+ cells displayed a non-significant trend for increased FAK activity (phosphorylation at Y861 but not at Y397). Interestingly, the extent of FAK phosphorylation at Y861 did not appear to correlate with the degree of intrinsic HER2 activity within the HER2-enriched cell lines, as the highest levels of FAK Y861 activity were observed in MDA-361 cells, which had the lowest HER2 activity within this cell subtype.

ER+/HER2+ cell lines are more sensitive to PF878 vs ER+/HER2– cells with respect to the inhibition of FAK phosphorylation at Y397

The effects of the FAK-specific inhibitor PF878 on FAK phosphorylation at Y397 were subsequently examined; these data revealed that PF878 was able to suppress FAK Y397 activity in both ER+/HER2– and ER+/HER2+ cell lines at sub-micromolar doses (Fig. 2). PF878 did not affect FAK phosphorylation at Y861 in T47D or BT-474 cells, but treatment did result in a partial loss of FAK Y861 activity in MCF7 and MDA-361 cells. The IC₅₀ values for FAK Y397 inhibition varied according to HER2 status, with both HER2-enriched cell lines demonstrating lower IC₅₀ values vs HER2– cells (Table 1; $P < 0.05$ for BT-474 vs MCF7 and T47D). Within the HER2+ cell line group, BT-474 cells appeared to be more sensitive to PF878 with respect to the inhibition of FAK Y397 phosphorylation vs MDA-361 cells.

Inhibition of FAK does not result in the suppression of FAK-related signalling pathway intermediates

We next investigated whether the suppression of FAK activity by PF878 resulted in the inhibition of intracellular proteins implicated in FAK and/or HER2-mediated signalling. Studies were carried out using 24-h treatment samples to reveal sustained responses rather than transient ones to FAK inhibition and thus potentially reveal signalling pathways targeted by PF878 responsible for cell proliferation. No reduction in the levels of any of the FAK-related signalling molecules investigated was observed in the ER+/HER2– MCF7 and T47D cells in response to PF878 with the exception of Akt (in MCF7 cells, Fig. 3A) and, to a much lesser extent, Src (in T47D cells, Fig. 3B).

The treatment of ER+/HER2+ BT-474 cells with PF878 again did not result in a significant suppression of FAK-related signalling, except for paxillin activity (Fig. 3C). In contrast to this, HER2 phosphorylation at Y1248 was observed to diminish in MDA-361 cells in a dose-dependent manner in response to PF878 (Fig. 3D), an effect that was not observed in BT-474 cells. Accompanying this was a reduction in the activity of Akt, Src and ERK1/2 (Fig. 3D).

Inhibition of FAK suppresses breast cancer cell migration but not proliferation

Given the potential role of FAK in the mediation of cellular behaviour downstream of growth factor receptor and

Figure 1

Expression and activity of FAK in ER+/HER2- and ER+/HER2+ breast cancer cells. (A) Cellular lysates from ER+/HER2- (MCF7 and T47D) and ER+/HER2+ (BT-474 and MDA-361) breast cancer cells grown under routine cell culture conditions as described in the Materials and methods section were immunoprobed for FAK, HER2 and signalling elements

common to these pathways. (B) Densitometry showing the ratio of active:total p-FAK (Y397 and Y861) in all the four cell lines, normalised to GAPDH ($n=3$), indicating that ER+/HER2+ cell lines displayed increased FAK activation at Y861.

integrin stimulation, we investigated whether the inhibition of FAK resulted in the suppression of cell proliferation. The inhibition of FAK using a range of doses of PF878 (0–1 μ M) that encompassed the IC_{50} for FAK phosphorylation at Y397 (Table 1) did not significantly affect the proliferation of any of the cell lines tested (Fig. 4A). Since FAK is downstream of integrins, we further investigated the hypothesis that FAK inhibition might result in greater effects in the presence of matrix components. However, the ability of PF878 to suppress the growth of BT-474 and MDA-361 cells growing on matrix (fibronectin)-coated surfaces was not significantly different from the results obtained using uncoated cell culture plates (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Since FAK is well established as a key regulator of cell migration, we sought to further determine whether FAK inhibition affected the migration of these cell lines. All the

four cell lines exhibited very little intrinsic migratory capacity in Boyden chamber assays (data not shown), suggesting that any observed differences in endogenous FAK between these cells (Fig. 1) did not affect their intrinsic migratory nature. To further investigate the role of HER2 in migratory responses, we treated the ER+/HER2+ BT-474 and MDA-361 cells with heregulin, a known activator of HER2-mediated signalling, and investigated the importance of FAK in this context. Heregulin stimulation greatly augmented the migratory capacity of MDA-361 cells, an effect that was antagonised by PF878 (Fig. 4B). Interestingly, heregulin treatment did not alter the migratory capacity of BT-474 cells over a range of concentrations tested (0–100 ng/ml), suggesting that HER2 signalling may not contribute to cell migration in these cells (data not shown). Despite a lack of response to heregulin, it has been previously shown that BT-474 cells can migrate in response to 20% serum (Worzfeld *et al.* 2012). Using serum as

Figure 2

HER2+ cell lines are more sensitive to PF878 vs HER2– cells. All four cell lines were treated with increasing concentrations of the FAK inhibitor PF878 for 60 min and immunoprobed for FAK (total and activated (Y397 and Y861)).

a chemoattractant in our assay, we were subsequently able to show that serum-induced migration of these cells could be significantly attenuated by PF878-mediated inhibition of FAK activity (Fig. 4C).

Combined inhibition of FAK and HER2 in ER+/HER2+ breast cancer cells is synergistic towards the suppression of proliferation

FAK is reported to function as an effector of erbB signalling, and our data (Fig. 2) suggested that ER+, HER2-enriched cells are more sensitive to FAK inhibition vs ER+/HER2– cells. Although the inhibition of FAK activity alone did not affect the proliferation of ER+/HER2+ cells, we sought to investigate whether FAK inhibition was more effective when combined with the targeting of the HER2 receptor itself using trastuzumab.

The initial analysis of cell growth over a period of 10 days revealed that BT-474 cells were intrinsically sensitive to the growth suppressive effects of trastuzumab in contrast to MDA-361 cells, where trastuzumab treatment did not greatly affect cell proliferation (Fig. 5A). Subsequent treatment of these cell lines with trastuzumab in the presence of PF878 (1 μM) revealed that the inclusion

of PF878 resulted in a greater level of growth suppression than that observed when either was used as a single agent (Fig. 5B, * $P < 0.05$). This effect was particularly prevalent in MDA-361 cells, which appeared to be intrinsically resistant to trastuzumab alone (Fig. 5A). Further analysis of cell proliferation utilised immunocytochemical staining of the proliferation marker Ki67. These data again revealed that the number of actively proliferating cells was greatly reduced in cells treated with PF878 and trastuzumab in combination, an effect again more prevalent in MDA-361 cells (Fig. 5C).

To determine whether trastuzumab and PF878 were additive or synergistic, an isobologram analysis was carried out. First, the IC_{30} values for both agents alone were plotted (Fig. 6). After this, the concentrations of agents that, when combined, achieved the same level of growth suppression (i.e. 30%) were added to the graph. The treatment of BT-474 and MDA-361 cells with PF878 and trastuzumab in combination was synergistic in suppressing the proliferation of these cells.

PF878 and trastuzumab in combination suppress signalling activity in ER+/HER2+ breast cancer cells and result in PARP cleavage

To explore potential mechanisms underlying the suggested synergistic inhibitory effect on ER+/HER2+ cell proliferation arising from co-targeting the HER2 receptor and FAK, western blotting was carried out for cells treated with PF878, trastuzumab or both agents in combination to reveal changes in key intracellular signalling components. Combination treatment resulted in a greater suppression of the phosphorylation of p-FAK (Y397), Akt (pS473) and MAPK (pS202/Thr204) in both cell lines after 24 h of treatment (Fig. 7A). In addition, PF878 appeared to prevent the increase in HER2 phosphorylation

Table 1 IC_{50} values for FAK inhibition (Y397) in ER+ breast cancer cells according to HER2 status

Cell line	Breast cancer molecular subtype	IC_{50} (μM), FAK inhibition (pY397)
MCF7	ER+, Her2–	0.25 ± 0.22
T47D	ER+, Her2–	0.26 ± 0.39
BT-474	ER+, Her2+	0.05 ± 0.03
MDA-361	ER+, Her2+	0.17 ± 0.12

Densitometric analysis of the immunoblots of cells treated with PF878 was carried out and the IC_{50} values of PF878 were calculated (μM $\pm$ s.d.; data are the mean of at least three independent replicates). PF878 was more effective at inhibiting FAK phosphorylation at Y397 in ER+/HER2+ cell lines vs their HER2 counterparts.

Figure 3

Inhibition of FAK activity suppresses endogenous FAK/HER2-related signalling in MDA-361 cells. All the four cell lines were treated with increasing concentrations of PF878 (0–1 μ M) for 60 min, following which the cells were lysed and lysates were immunoprobed for the proteins

indicated. While there were limited effects of PF878 on FAK-related proteins in MCF7 (A), T47D (B) and BT-474 (C) cells, PF878 treatment of MDA-361 cells resulted in the inhibition of HER2, Akt, Src and ERK1/2 activity (D).

observed after trastuzumab treatment. While no effect was observed in cell-cycle progression (data not shown), analysis of PARP cleavage as a surrogate marker for apoptosis revealed that the combination treatment using PF878 and trastuzumab elevated the amount of the 23 kDa PARP fragment detectable in the cell lysates (Fig. 7B).

Discussion

Breast cancer is known to represent a heterogeneous group of diseases possessing a wide spectrum of clinical, pathological and molecular characteristics with ER+ tumours accounting for around 70% of all cases diagnosed. A subset of ER+ tumours (around 10%) is also enriched with the HER2 receptor (Bauer et al. 2010). Prior to the advent of anti-HER2 agents such as trastuzumab, patients with HER2+ disease had a poorer 5-year overall

survival (Nguyen et al. 2008, Park et al. 2012). Interestingly, although the recent HERA trial points to a similar trastuzumab response irrespective of hormone receptor status (Untch et al. 2008), there is evidence that neoadjuvant trastuzumab may be less effective in ER+/HER2+ vs ER–/HER2+ tumours (Bhargava et al. 2011), suggesting that the presence of the ER in HER2+ tumours may be an important consideration. Conversely, the presence of HER2 in ER+ tumours may also be a limiting factor for endocrine therapy, particularly since ER+/HER2+ tumours are likely to be treated with both endocrine and trastuzumab treatments. Moreover, while trastuzumab has shown clinical effectiveness in the treatment of HER2+ tumours (both ER+ and ER–), its use is limited by resistance. Key to the development of improved therapies is the identification of molecular elements the targeting of which may have the potential

Figure 4
Inhibition of FAK activity does not affect ER+/HER2+ breast cancer cell proliferation but does suppress heregulin- and serum-induced migration. (A) The growth of MCF7, T47D, BT-474 and MDA-361 cells in response to PF878 (0–1 μM) over a period of 7 days was assessed using the MTT assay. Data are mean cell proliferation values $\pm$ s.d. ($n=3$). (B) The effects of PF878 (1 μM) on heregulin-stimulated MDA-361 migration or (C) serum-induced migration of BT-474 cells were assessed using fibronectin-coated Boyden chambers. Data represent the mean numbers of migratory cells/field ($n=3$). While FAK inhibition did not affect cell proliferation, PF878 was effective at preventing heregulin-induced migration of MDA-361 cells and serum-induced migration of BT-474 cells. MCF7 and T47D cells were not migratory either unstimulated or in response to heregulin (data not shown). * $P<0.05$ vs untreated cells and ** $P<0.05$ vs heregulin- or FCS-stimulated cells.

to augment the cellular response to anti-HER2 agents, particularly in the context of ER+/HER2+ tumours.

FAK is activated by HER2 (Nahta 2012) and can integrate signalling downstream of HER2 and other receptors resulting in the activation of the PI3K/Akt pathway (Nahta 2012). Both HER2 crosstalk and

augmented PI3K/Akt signalling have been implicated in HER2 therapy resistance (Nahta 2012, Zhu et al. 2012), and FAK, through its involvement with HER2, is emerging as an important player in trastuzumab resistance (Wang et al. 2009, Yang et al. 2010). In this study, we set out to investigate the therapeutic potential of FAK in the context of ER+/HER2+ breast cancer.

Our initial observations revealed that there was no apparent correlation between endogenous HER2 status and FAK expression or its phosphorylation at Y397. However, the levels of FAK (Y861) appeared to be increased in the HER2+ cells. Although some reports have suggested that elevated FAK expression is associated with HER2 overexpression (Lark et al. 2005, Schmitz et al. 2005), our data on FAK Y861 are in accordance with recent studies that have demonstrated higher levels of FAK Y861 expression to be associated with poor prognostic indicators including the overexpression and activation of HER-2 (Vadlamudi et al. 2002).

Importantly, both ER+/HER2+ cell lines demonstrated increased sensitivity to the FAK inhibitor vs ER+/HER2– cells; BT-474 cells, which had the highest degree of HER2 phosphorylation, demonstrated the greatest sensitivity to this compound. However, despite the effective suppression of FAK Y397, PF878 alone did not affect the proliferation of any of the cell lines. While PF878 concentrations >5 μM did elicit growth-inhibitory effects (data not shown), it is likely that these are due to off-target, FAK-independent effects, as has been reported to occur in other cell lines exposed to related FAK inhibitory compounds (Slack-Davis et al. 2007). Corresponding with the lack of significant growth inhibition, all cell lines showed a limited sensitivity to PF878 monotherapy in terms of the activation of other key growth-regulatory kinases (Akt, Src and MAPK). An exception to this was the MDA-361 cell line, in which PF878 suppressed Akt, Src, MAPK and HER2 phosphorylation. Although FAK inhibition may trigger an as-yet unidentified compensatory mechanism to sustain growth in the presence of reduced Akt and MAPK activity, a more obvious explanation for the observed discrepancy between the effects of PF878 on signalling and growth in these cells is that the residual signalling activity observed in the presence of PF878 is enough to sustain growth.

Coupled with a lack of evidence for growth inhibition, PF878 monotherapy had only a modest effect on the apoptotic profile of the cell lines tested here, as indicated by the minimal changes in PARP cleavage (Fig. 7). This was an initially surprising observation, given that FAK has been implicated as a pro-survival kinase and is able to

Figure 5

Targeting of FAK and HER2 together results in an improved suppression of HER2+ breast cancer cell proliferation vs monotherapy. (A) The ability of trastuzumab to antagonise the proliferation of HER2+ breast cancer cells was determined using the MTT assay. While BT-474 cells were growth inhibited by targeting the HER2 receptor, little effect was observed in MDA-361 cells. The anti-proliferative effects of PF878 and trastuzumab in combination on both cell lines were subsequently explored using Coulter

counting (B) and Ki67 immunocytochemistry; (C) the accompanying graph shows the mean proliferative cell counts (i.e. Ki67+) per field of view. These data revealed that the inhibition of FAK activity augmented trastuzumab-mediated growth suppression, an effect that was greatest in the trastuzumab-resistant MDA-361 cells. * $P < 0.05$ vs single-agent treatments.

Figure 6

PF878 and trastuzumab are synergistic in suppressing ER+/HER2+ breast cancer cell proliferation. The nature of the interaction between PF878 and trastuzumab was evaluated using the isobologram technique, a dose-oriented geometric method of assessing drug interactions. Using this approach, the concentrations of PF878 producing a desired effect (e.g. 30% inhibition of cell growth) were plotted in the vertical axis and the concentrations of trastuzumab producing the same growth-inhibitory effect were plotted on the horizontal axis. A straight line connecting these two points represents zero interaction between the two agents (i.e. additivity). Points were subsequently plotted for the concentrations of the two agents in combination that reduce cell proliferation by 30%. Data points above the diagonal line of the additive effects in the isobole suggest antagonism, whereas points below the line suggest synergism. PF878 and trastuzumab in combination were synergistic at inhibiting the proliferation of both BT-474 (A) and MDA-361 (B) cells at all the drug concentrations tested.

down-regulate caspase expression (Kurenova et al. 2004). FAK-overexpressing cells also display resistance to apoptotic stimuli, while FAK inhibition has been shown to result in increased apoptosis (Smith et al. 2005). However, our data are supported by other reports that have failed to detect any clear change in apoptosis following siRNA-mediated FAK knockdown (Han et al. 2004). Although these apparently conflicting data may simply reflect

differences in the role of FAK in different tumour types, it is interesting to note that many studies in which FAK has been shown to suppress apoptosis have been carried out in 3D culture systems. This in turn points to the importance that the microenvironment has in FAK activity, where, for example, integrin-mediated FAK activation may promote pro-survival signalling. Such a hypothesis is supported by recent reports that pharmacological inhibition of FAK phosphorylation at Y397 using the FAK inhibitor PND-1186 has no effect in 2D cell culture, but increases apoptosis in 3D culture (Tanjoni et al. 2010) and *in vivo* (Walsh et al. 2010).

In contrast to proliferative responses, the role of FAK in cell migration is widely established (reviewed in Mitra et al. (2005)), and many studies including the present study report that the inhibition of FAK activity suppresses breast cancer migration and invasion *in vitro* (Hiscox et al. 2011) and metastasis *in vivo* (Tanjoni et al. 2010). Interestingly, in contrast to MDA-361 cells, BT-474 cells were not responsive to the migration-promoting effects of heregulin. This may imply differential HER2 signalling between these cell types and may also explain the differential sensitivity of BT-474 and MDA-361 to trastuzumab. A further, and perhaps more significant explanation, is that these cell lines differ with respect to progesterone receptor (PR (PGR)) expression (S Hiscox, unpublished observations, 2012). This is important since ER+ tumours that lack PR expression are known to be associated with a significantly poorer prognosis and a higher relapse frequency vs ER+/PR+ tumours (Parise et al. 2009, Canello et al. 2012). Although the exact molecular mechanisms underlying the potential importance of the PR in this context are unclear, it is interesting to note that only MDA-361 cells were able to migrate in response to heregulin stimulation, which may have bearing on HER2 crosstalk with hormone receptors in these cells. These observations may allude to an increased aggressive behaviour in an *in vivo* context, where such cells would be exposed to growth factors within the tumour microenvironment.

Our data suggest a potentially important role for FAK in ER+/HER2+ tumours that exhibit reduced sensitivity to anti-HER2 agents, since PF878-mediated inhibition of FAK in trastuzumab-refractory MDA-361 cells resulted in a partial restoration of trastuzumab response, which may be linked to the increased suppression of Akt observed following combination treatment. Despite trastuzumab itself inhibiting Akt activity in both MDA-361 and BT-474 cells, only the latter were growth inhibited, suggesting that Akt is not part of the dominant growth-regulatory

Figure 7

Combined targeting of FAK and HER2 in ER+/HER2+ breast cancer results in the suppression of intracellular signalling and PARP cleavage. BT-474 and MDA-361 cells were treated with PF878 (1 μ M), trastuzumab (1 nM) or a combination of both the agents for 24 h, and protein expression and activity were assessed by western blotting. (A) Co-targeting of FAK and

HER2 resulted in a greater suppression of FAK activity (Y397) and Akt and MAPK phosphorylation in both cell lines. (B) In lysates from cells subjected to both treatments in combination, there was also a greater amount of PARP cleavage products detectable.

pathway in MDA-316 cells. However, there is also the possibility that HER2 heterodimerisation patterns in these cells might be different from those in trastuzumab-sensitive BT-474 cells; this in turn might provide differential inputs into Akt regulation in these cell models, a hypothesis supported by Ghosh *et al.* (2011), who demonstrated that HER2 heterodimerisation can be associated with a lower response to trastuzumab. Alternatively, the residual Akt signalling (which is still substantial) observed in MDA-361 cells even after trastuzumab treatment may be enough to maintain cell proliferation, as is the case for PF878 monotherapy. Thus, combination treatment strategies such as PF878 and trastuzumab that better target the Akt pathway in Her2+ cells may hold promise for the treatment of such tumours, particularly in the context of limited trastuzumab sensitivity. Indeed, other reports have also shown

that the combined targeting of the HER2 receptor is better than targeting using a single agent in terms of growth inhibition primarily because the levels of the suppression of the Akt signalling pathway are greater (Andre *et al.* 2008, Yao *et al.* 2009).

Interestingly, trastuzumab was observed to induce HER2 phosphorylation in both BT-474 and MDA-361 cells. Despite previous hypotheses that herceptin-based targeting of the HER2 receptor would inhibit HER2 signalling, recent studies have shown this not to be the case (Junttila *et al.* 2009) and have implicated ADAM17-mediated release of erbB ligands, arising through herceptin-mediated PKB inhibition, as a potential mechanism to facilitate HER2 activation (Gijzen *et al.* 2010a,b). Since our experiments were carried out in the presence of serum (required to support long-term cell growth for the proliferation assays), this may represent a confounding

issue, although the endogenous signalling activity of HER2 and FAK and the levels of their downstream associated molecules were only modestly reduced in the absence of serum. Importantly, the effects of trastuzumab and PF878 under serum-reduced conditions were similar in terms of FAK and HER2 signalling. Our data further suggest that trastuzumab-augmented HER2 activity may involve FAK, since PF878 is able to suppress this phenomenon. Indeed, the failure of trastuzumab to suppress HER2 phosphorylation may represent one explanation as to the occurrence of acquired resistance to trastuzumab monotherapy and/or the presence of *de novo* insensitivity. In such settings, co-targeting other molecular elements such as FAK to suppress HER2 activity, as we demonstrated in BT-474 cells, may provide benefits and overcome acquired resistance as has been suggested through combining trastuzumab with ADAM17 inhibitors (Gijzen *et al.* 2010b). While targeting FAK and HER2 together in trastuzumab-responsive BT-474 cells resulted in the suppression of HER2 activity, perhaps a more beneficial context is that of MDA-361 cells, where our data imply that co-targeting treatment strategies may also present an effective approach to the treatment of tumours with *de novo* trastuzumab resistance.

The use of FAK inhibitors in combination treatment regimens is further supported by recent studies showing that dual FAK and EGFR inhibition enhances apoptosis in breast cancer (Golubovskaya *et al.* 2002) and radio-sensitivity in head and neck squamous-cell carcinomas (Eke & Cordes 2011), the latter involving the suppression of the Akt and MEK pathways (Hehlhans *et al.* 2012). In pancreatic cancer models, pharmacological inhibition of both FAK and IGF1R decreases cell survival *in vitro* and leads to tumour regression *in vivo* (Zheng *et al.* 2010). Our data that PF878 and trastuzumab were synergistic in suppressing the proliferation of MDA-361 cells suggest that targeting FAK may also represent an important chemosensitisation strategy in resistant disease. This approach is supported by studies that have reported sensitisation to 5-fluorouracil (5-FU) and a partial reversal of 5-FU resistance when FAK is suppressed (Chen *et al.* 2009). Moreover, TAE226, a pharmacological inhibitor of FAK phosphorylation (Y397 and Y861), augments docetaxel sensitivity in taxane-resistant ovarian cancer cells (Halder *et al.* 2007). In such contexts, FAK may represent a convergence point for multiple signalling pathways that may contribute to resistance, highlighting its potential importance as a therapeutic target. Given that PF878 improved trastuzumab response in ER+/HER2+ cell, we further investigated whether FAK inhibition improved the

treatment regimen of tamoxifen and trastuzumab in combination. While this dual treatment regimen was effective in trastuzumab-sensitive BT-474 cells, it did not offer significant benefits over tamoxifen alone in trastuzumab-insensitive MDA-361 cells. Importantly, inclusion of PF878 alongside tamoxifen and trastuzumab in combination resulted in a greater suppression of growth in both cell lines (Supplementary Figure 2, see section on supplementary data given at the end of this article), further implicating FAK as a potential therapeutic target in ER+/HER2+ tumours.

In conclusion, our data reported herein support a role for FAK in ER+/HER2+ breast cancer, where its targeting has the potential to improve trastuzumab response. This is particularly important in the context of trastuzumab-refractory disease, where FAK inhibition may present an important strategy to restore trastuzumab sensitivity.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0019>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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