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Inhibition of focal adhesion kinase suppresses the adverse phenotype of endocrine-resistant breast cancer cells and improves endocrine response in endocrine-sensitive cells

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Abstract

Acquired resistance to endocrine therapy in breast cancer is a major clinical problem. Previous reports have demonstrated that cell models of acquired endocrine resistance have altered cell-matrix adhesion and a highly migratory phenotype, features which may impact on tumour spread in vivo. Focal adhesion kinase (FAK) is an intracellular kinase that regulates signalling pathways central to cell adhesion, migration and survival and its expression is frequently deregulated in breast cancer. In this study, we have used the novel FAK inhibitor PF573228 to address the role of FAK in the development of the adverse characteristics that accompany acquired endocrine resistance. Whilst total FAK expression was similar between endocrine-sensitive and endocrine-resistant MCF7 cells, FAK phosphorylation status (Y397 or Y861) was altered in resistance. PF573228 promoted a dose-dependent inhibition of FAK phosphorylation at Y397 but did not affect other FAK activation sites (pY407, pY576 and pY861). Endocrine-resistant cells were more sensitive to these inhibitory effects versus MCF7 (mean IC₅₀ for FAK pY397 inhibition: 0.43 μM, 0.05 μM and 0.13 μM for MCF7, TamR and FasR cells respectively). Inhibition of FAK pY397 was associated with a reduction in TamR and FasR adhesion to, and migration over, matrix components. PF573228 as a single agent (0-1 μM) did not affect the growth of MCF7 cells or their endocrine-resistant counterparts. However, treatment of endocrine-sensitive cells with PF573228 and tamoxifen combined resulted in greater suppression of proliferation versus single agent treatment. Together these data suggest the importance of FAK in the process of endocrine resistance, particularly in the development of an aggressive, migratory cell phenotype and demonstrate the potential to improve endocrine response through combination treatment.

Key Words: Focal adhesion kinase, endocrine resistance, migration, adhesion, breast cancer, tamoxifen, fulvestrant

Running title: FAK and endocrine-resistant breast cancer

Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that resides at focal adhesions, sites of integrin-mediated cell attachment to extracellular matrix components [1]. FAK is central to signal transduction initiated both through integrin clustering [2] and following activation of different classes of cell-surface receptors including G protein-coupled receptors [3], the uPAR [4] and receptor tyrosine kinases such as the EGFR [5] and c-Met [6]. Activation of signalling pathways in which FAK plays a central role have been shown to be important in cell survival [7], proliferation, adhesion, migration and invasion [8] suggesting that FAK-mediated signalling may play a key role in tumour progression and metastasis.

The importance of FAK in such events has been demonstrated both *in vitro*, where expression of dominant-negative FAK mutants prevent tumour cell spreading and migration, and *in vivo*, where FAK inhibition prevents metastases to the lung of mammary cancer cells [9]. Clinically, FAK levels are frequently elevated in tumour compared to normal tissue [10] and are reportedly higher in metastases compared to primary cancers [11]. Overexpression of FAK in tumour tissue is associated with a poor prognosis in a number of tumour types including breast cancer [12, 13]; activation of FAK in breast cancer has been shown to correlate with malignant transformation [14].

Steroid hormones play a central role in the growth and development of oestrogen receptor positive (ER+) breast cancers; as such, endocrine therapies which seek to perturb the steroid hormone environment of the tumour cells can promote extensive remissions in established disease and provide significant benefits in patient survival [15]. However despite this, many patients progress during therapy due to acquired endocrine resistance [16]; in these patients, relapse on such therapies clinically presents as local and/or regional recurrences, frequently with distant metastases and the outlook for these patients is poor [17].

Recent *in vitro* studies using cell models of acquired tamoxifen and fulvestrant resistance had revealed that acquisition of resistance to such agents is accompanied by altered growth factor signalling [18] and the development of an aggressive, invasive phenotype [19, 20] in which the FAK-associated kinase, Src, may play a central role [21]. However, despite the efficacy of Src inhibitors in suppressing these adverse characteristics of endocrine-resistant breast cancer cells, in cells lacking FAK, Src

inhibitors do not provide added benefit in terms of suppression of cell migration, suggesting that the effects of Src on cell migration are mediated through FAK [22].

In this study, we have used the novel FAK inhibitor, PF573228, to explore the role of FAK in acquired endocrine resistance and demonstrate that pharmacological inhibition of FAK may provide a suitable means to suppress the aggressive phenotype that accompanies endocrine resistance in vitro. Moreover, targeting FAK may improve endocrine response in endocrine-sensitive breast cancer cells.

Materials and methods

Reagents

The novel FAK inhibitor, PF573228 ('PF228') [23], was a gift from Pfizer. Antibodies used were as follows: anti-FAK (total, pY397, pY407, pY576, pY861), Src kinase (pY418) and Akt (pS473) were from Invitrogen (Paisley, UK); anti-EGFR (pY1045 and total) and anti-ERK1/2 (pS202/T204 and total) were from Cell Signalling Technology (New England Biolabs, Herts, UK). The specificity of these antibodies against the phosphorylation sites indicated has been previously reported [22, 24-27]. All other reagents were from Sigma (Poole, UK) unless otherwise stated.

Cell culture

Endocrine-sensitive MCF7 and T47D breast cancer cells were routinely cultured in RPMI medium (Invitrogen, Paisley, UK) supplemented with 5% foetal calf serum (FCS), 10IU/ml penicillin, 10 μ g/ml streptomycin, 2.5 μ g/ml fungizone and 200mM glutamine). Tamoxifen and fulvestrant-resistant variants of MCF7 cells were derived by prolonged culture of MCF7 cells in the presence of these agents in experimental medium (phenol red-free RPMI containing 5% charcoal-stripped FCS, glutamine and antibiotics as above) as described previously [18, 28]. Tamoxifen-resistant ('TamR') and fulvestrant-resistant ('FasR') cells were subsequently maintained in experimental medium containing 100nM 4-hydroxy tamoxifen or 100nM fulvestrant. In all experiments the tissue culture medium (\pm treatments as indicated) was replaced every three days unless otherwise stated. Stock solutions of PF228 were dissolved in DMSO prior to diluting in tissue culture medium. Corresponding DMSO controls had no effect on the parameters analysed.

Western blotting

Log-phase cell cultures were lysed in 50 mM Tris, pH 7.5, 5 mM EGTA, 150 mM NaCl and 1% Triton X100) containing protease inhibitors (2 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 20 μ M phenylarsine, 10 mM sodium molybdate, 10 μ g/ml leupeptin and 8 μ g/ml aprotinin) and proteins resolved by SDS-PAGE using 8% gels. Proteins were immobilised on nitrocellulose membranes and subsequently probed with antibodies that recognised active forms of FAK and additional antibodies as stated in the text. Repeat

immunoprobings were performed using pan antibodies to determine total levels of these proteins and GAPDH as a loading control. Western blots were then scanned to provide data for semi-quantitation with normalization against GAPDH. Each experiment was performed at least three times with representative gels shown.

Measurement of cell growth

Cells were seeded into 96-well plates at 2×10^3 cells/well. After 24 hours, the medium in the wells was replaced with fresh medium containing PF228 at a range of concentrations. In some cases, tamoxifen (10^{-7} M) or fulvestrant (10^{-7} M) was included. Plates were incubated at 37°C for the times indicated, with medium changes every 3 days. The effects of these treatments on cell growth was determined by measuring the cell number in each well as follows: medium was aspirated from each well and replaced with fresh RPMI medium containing a 1:10 dilution of WST-1 reagent (Roche). Plates were incubated at 37°C for 2 hours then read in a spectrophotometer (λ 560nm).

Cell-matrix adhesion assays

Cells, harvested and pre-treated for 60 minutes with PF228 (0-5 μ M), were seeded at 1.5×10^4 cells/well into uncoated 96 well plates in the presence of PF228, or into plates pre-coated with fibronectin or laminin (10 μ g/ml). After a further 40 minutes incubation, unattached cells were removed by gentle washing before the addition of 100 μ l WST-1 reagent in cell culture media to determine the numbers of adherent cells within each well. After a further 2 hours incubation in the presence of the WST reagent, plates were read in a spectrophotometer (λ 560nm).

Measurement of cellular migration and morphology

Cells were harvested and held in suspension in the presence of PF228 for 60 minutes prior to seeding onto fibronectin-coated polycarbonate, microporous membranes (8 μ m pore size) at 5×10^4 cells/membrane and allowed to migrate to the underside of the membrane for a period of 24 hours. Migratory cells were fixed, stained with 0.5% crystal violet and counted.

To determine effects of PF228 on cell morphology, cells were treated as above but seeded into dishes rather than polycarbonate inserts and allowed to reach log phase

growth before treatment with PF228 for 24 hours. Cells were then fixed in 3.7% formaldehyde and photographed under bright field illumination using differential interference contrast filters.

Statistical analysis

Statistical comparison between PF228-treated and untreated samples was performed using t-test of the data with SPSS version 14. Significance was observed at $p < 0.05$.

Results

FAK is differentially activated in endocrine-resistant breast cancer cells

We have previously demonstrated that the development of endocrine resistance in breast cancer cells is accompanied by an increased migratory capacity in vitro [19, 29]. Given that FAK is a key regulator of such behaviour, we first determined whether the expression and/or activation status of FAK differed in endocrine-sensitive MCF7 cells versus two acquired endocrine-resistant MCF7 derivatives. Although no change in level of total-FAK expression was detected between the cell lines, Western blotting using a panel of antibodies that recognised FAK phosphorylated at Y397, Y407, Y576 and Y861 revealed increased phosphorylation of FAK (pY397) in tamoxifen-resistant cells whilst FAK (pY861) levels were greatly elevated in both tamoxifen and fulvestrant-resistant cells compared to their endocrine-sensitive counterparts (Figure 1). FAK phosphorylated at pY925 was not detectable in any of these cells.

Endocrine resistant breast cancer cells are more sensitive to FAK inhibition versus their endocrine sensitive counterparts

To address the role of FAK in these cells, we employed the pharmacological FAK inhibitor, PF573228 ('PF228'), a compound that targets FAK catalytic activity through competitive inhibition of ATP binding [23]. Cells were first treated with increasing concentrations of PF228 for 1 hour and FAK expression and activity determined by Western blotting. PF228 promoted a dose-dependent inhibition of FAK phosphorylation at Y397 in all cell lines, whilst total FAK levels were unaffected (figure 2A). Semi-quantitative analysis using densitometry suggested that the tamoxifen-resistant cell line was significantly more sensitive to the inhibitory effects of PF228 on FAK Y397 phosphorylation compared with their endocrine-sensitive counterparts (figure 2B). Whilst FasR cells also exhibited an increased sensitivity to PF228 compared to MCF7 cells, this did not reach significance ($p=0.15$). In contrast to the effects of PF228 on FAK pY397, no significant changes in phosphorylation of FAK at other residues tested (pY407, pY576 or pY861) were detectable in any of these cells (figure 2C).

Both Src and MAPK can play a central role in signalling pathways mediated by FAK. Thus we investigated whether inhibition of FAK phosphorylation impacted on the activation status of Src and MAPK. Our data revealed that inhibition of FAK pY397

was not accompanied by any changes in either MAPK or Src activity in any of the cell lines tested (Figure 2D).

Effects of FAK inhibition on the growth of endocrine-sensitive and endocrine-resistant breast cancer cells

To investigate the functional relevance of increased FAK activity in endocrine resistant breast cancer cells, we tested whether FAK inhibition affected the growth of the cell lines. Treatment of these cells with PF228 (0-1 μ M) for 7 days did not affect the growth of either MCF7 cells or their tamoxifen and fulvestrant-resistant counterparts (Figure 3A).

To confirm that FAK activity remained suppressed following prolonged exposure to the inhibitor over this time period, Western blotting was performed on cells treated with PF228 for 7 days. FAK pY397 remained suppressed at this time point whereas total FAK levels were not changed (figure 3B).

Inhibition of FAK activity suppresses cell-matrix attachment, alters cell morphology and inhibits cell migration

Both TamR and FasR cells were seen to possess a greater affinity to uncoated and matrix-coated surfaces compared to their wild-type counterparts (Figure 4A). Inhibition of FAK activity using PF228 reduced these adhesive interactions in TamR and FasR cells (Figure 4B). Since TamR and FasR cells display a highly migratory phenotype compared to their poorly-migratory MCF7 counterparts [19, 29], we addressed the role of FAK in this process. Inhibition of FAK activity corresponded to a significant inhibition of both TamR and FasR cell migration over fibronectin (Figure 4C). MCF7 cells, which are non-migratory under control conditions, were not affected by PF228 (data not shown). Interestingly, both TamR and FasR cells were seen to display a high degree of membrane ruffling and elevated levels of lamellipodia and filopodia (figure 4D, arrowed) compared to their MCF7 counterparts, a characteristic that was greatly reduced (to levels similar to that observed in MCF7 cells) following treatment with PF228 (figure 4D).

Inhibition of FAK improves response to endocrine agent in endocrine-sensitive breast cancer cells

Previous reports have suggested that combining inhibitors of kinases that have a pro-invasive role with endocrine agents may be beneficial in suppressing growth of endocrine-sensitive breast cancer cells [25, 30] [31]. In light of this, we assessed the effects of combining PF228 with tamoxifen on the growth of ER-positive, endocrine-sensitive MCF7 cells. Inclusion of PF228 at concentrations of 1 μ M alongside tamoxifen increased inhibition of cell growth compared with monotherapy (Figure 5A). To investigate whether this effect was specific for MCF7 cells, similar assays were performed with ER+ T47D cells. Treatment of T47D cells with PF228 and tamoxifen combined again resulted in a greater suppression of cell growth versus single agent treatment (Figure 5B).

Inhibition of FAK activity partially restores sensitivity to tamoxifen in tamoxifen-resistant MCF7 cells

We next wished to determine whether inhibition of FAK was able to alter the response of endocrine-resistant breast cancer cells to endocrine agent. MCF7 models of acquired tamoxifen and fulvestrant resistant were cultured in the presence or absence of tamoxifen or fulvestrant respectively and in the presence or absence of PF228 (figure 6). Whilst tamoxifen exerted a modest stimulatory effect on the growth of TamR cells, in the absence of FAK activity tamoxifen exerted an inhibitory effect on TamR cell growth (figure 6A). In contrast to this, no change in FasR cell sensitivity towards fulvestrant was seen following FAK inhibition (figure 6B).

Discussion

FAK is known to be overexpressed in a variety of tumour types including breast cancer where it is associated with the development of malignant tumour characteristics such as increased migration and invasion. In light of this, we have examined here whether FAK plays a key role in the development of the aggressive phenotype reported to accompany the acquisition of resistance to endocrine therapies in breast cancer cells [19, 29]. Although no changes in total-FAK protein expression was observed between the cells, tamoxifen-resistant cells had much higher levels of FAK phosphorylated at Y397, an observation not apparent in fulvestrant-resistant cells. Despite this, however, both cell models of acquired resistance appeared more sensitive to the effects of PF573228 than their endocrine-sensitive MCF7 counterparts with TamR cells reaching significance (IC_{50} values for inhibition of FAK pY397 were 0.43 μ M (MCF7), 0.05 μ M (TamR) and 0.13 μ M (FasR)).

The small molecule inhibitor, PF573228, targets the kinase-dependent autophosphorylation site in FAK (Y397), however, the effect of this compound on FAK phosphorylation at other sites has not yet been reported. This is important since FAK-regulated adhesion signalling is regulated through phosphorylation within the FAK molecule at both Y397 and several other key residues (e.g. Y407, Y576 and pY861). Importantly, changes in the phosphorylation status of FAK at Y397 are known to affect the activity of FAK at these sites; for example, phosphorylation of FAK at Y397 creates a high affinity binding site for the SH2 domain of Src family kinases and thereby the formation of the FAK-Src signalling complex [32, 33], an event suggested to both enhance Src phosphorylation and precede phosphorylation of FAK at further sites [34]. Thus inhibition of FAK phosphorylation at Y397 may result in reduced phosphorylation of FAK at other residues with or without a concomitant reduction in Src activity. We therefore aimed to establish whether PF228 affected the activity of FAK at other sites by Western blotting. Our data clearly demonstrate that PF228 had no inhibitory effect on the phosphorylation of FAK at Y407, Y576 or Y861, nor did it affect the activity of Src kinase. One explanation for these observations could be that although PF228 may prevent Src from binding to FAK, no change in Src phosphorylation is seen due to the high number of other signalling components in these cells likely to activate Src. For example, we and others have previously demonstrated enhanced growth factor signalling in endocrine resistant cell models [18, 19, 35, 36] thus providing additional mechanisms by which Src can be

activated. In addition, although FAK activation at Y861 might be expected to be reduced following inhibition of FAK at Y397 as a consequence of the failure to recruit and activate Src, increased phosphorylation of FAK at Y861 has been demonstrated in cells expressing kinase-defective Src mutants [37] suggesting that FAK Y861 can be activated independently of Src. Our attempts to investigate Y925, a more reliable indicator of Src-dependent phosphorylation within FAK [37] were unsuccessful since we were unable to detect endogenous activity of FAK at this residue.

The role of FAK as a regulator of cell migration is well documented [38, 39]; given that FAK autophosphorylation at Y397 is critical for focal adhesion and focal complex assembly and disassembly and plays a crucial role in tumour cell migration, it is not surprising that inhibition of FAK through multiple techniques all result in suppression of cell migration [40, 41]. We observed that PF228 inhibited FAK phosphorylation at Y397 in all three cell types tested and inhibited the migratory phenotype of TamR and FasR cells. No effect was observed in MCF7 cells which are only weakly migratory. Thus our data further supports previous reports that pharmacological inhibition of FAK is effective in suppression of migration [23].

Previous data from our own group and others has demonstrated that acquired tamoxifen resistance is accompanied by a reduction in intercellular adhesion, enhanced membrane activity (increased membrane ruffling, filopodia and lamellipodia formation) and a gain in the migratory capacity of the cells [20, 42, 43]. Importantly, our data here suggests that this also applies to fulvestrant resistance, with FasR cells displaying a significantly elevated migratory nature and filopodia/lamellipodia production compared to their MCF7 counterparts. Underlying the regulatory function of FAK on cell migration may be the suggested ability of FAK to modulate the activity of Rho/Rac family members known to play a key role in cytoskeletal re-organisation [44]; recent studies by Jia et al [45] further highlight the potential of FAK to interact with such signalling pathways important for cytoskeletal remodelling and cellular migration. Interestingly, our data here demonstrate that inhibition of FAK by PF228 greatly reduces filopodia and lamellipodia formation further suggesting the importance of FAK in cytoskeletal reorganisation events. Moreover, FAK signalling has been reported to be required for the induction of epithelial-to-mesenchymal transition and an accompanying delocalization of membrane-bound E-cadherin [46]. Although not directly studied here, our previous

observations provide anecdotal evidence of a role for FAK in the development of an adverse cellular phenotype that accompanies endocrine resistance in that E-cadherin appears to be miss-localised in these resistant cells (S. Hiscox, A. Borley, J. Gee, unpublished observations).

The ability of PF228 to reverse the aggressive phenotype seen in endocrine-resistant breast cancer cells suggests a prominent role for FAK Y397 in this process and further suggests that FAK represents a novel target for therapeutic intervention through which tumour cell spread may be limited. Indeed, several compounds have been described with activity against FAK that may have potential application as anti-metastatic agents in vivo [22, 47, 48]. However, these compounds were not designed with FAK as a specific target and their effects may also arise through targeting of other intercellular kinases. PF228, however, represents a novel approach to targeting FAK in that it has been developed as a specific inhibitor for FAK alone with its selectivity over other kinases previously demonstrated in cell-free kinase assays [23]. Indeed, our data suggests that its selectivity for FAK holds true for intact cells, where PF228 does not affect the activity of other key kinases (Src, MAPK) implicated in adhesion signalling at concentrations of PF228 needed to achieve maximal inhibition of FAK Y397. These observations point to the use of PF228 as an important pharmacological tool that can be used to elucidate the specific role of FAK in cancer, circumventing the confounding issues of the contribution of other adhesion-regulated kinases such as Src.

Our data did not reveal a direct association between loss of FAK activity at Y397 and suppression of cell growth since none of the cell lines tested were growth inhibited by PF228 at concentrations that significantly suppressed FAK Y397 activity.

Interestingly, when concentrations of PF228 were increased to 5 μ M, both TamR and FasR cells were modestly growth inhibited whilst MCF7 cells were not (calculated IC₅₀ values for growth inhibition were MCF7, >5 μ M; TamR, 3.5 μ M; FasR, 4.0 μ M). Previous studies have demonstrated that concentrations of PF228 \geq 10 μ M are able to inhibit the growth of FAK-deficient fibroblast cell lines [23], suggesting off-target effects at high concentrations. In view of this and the reported activity of PF228 against other kinase targets [23], concentrations of 5 μ M may well inhibit a number of substrates in wild type cell lines. However, our wild-type MCF7 cells were not growth inhibited at 5 μ M PF228 as we originally expected, whilst our resistant cells were modestly inhibited. Whilst we cannot rule out the possible contribution of off-target

effects for the observed PF228-mediated inhibition of endocrine-resistant cell growth, our data may suggest an increased reliance, albeit a moderate one, on FAK signalling in such cells in terms of cellular growth responses. Indeed, a dependence on adhesion-mediated signalling has been recently shown in resistant cells as with FAK regulating crosstalk between integrins and growth factor receptor signalling pathways to sustain growth [49, 50]. Such a hypothesis is supported by studies that demonstrate that inhibition of adhesion-mediated signalling augments sensitivity to tamoxifen in vitro [51]. Importantly, phosphorylation of FAK may be regulated by the ER; whereas estradiol has been demonstrated to suppress FAK activity in breast cancer cells [52], endocrine agents themselves may induce activation of adhesion-related kinases including FAK [51], promoting adhesion-dependent survival signaling which may play a critical role in endocrine insensitivity. Such observations suggest that targeting FAK in conjunction with endocrine therapy would present a means to circumvent such phenomena. Subsequently, we additionally report here that treatment of endocrine-sensitive cells with PF228 and endocrine agent combined appears to produce a modest cooperative effect in terms of growth inhibition. Previously, we and others have demonstrated that the combined use of oestrogen receptor antagonists with potent anti-invasive agents, e.g. Src kinase inhibitors, is more effective at suppressing breast cancer cell growth than their use as single agents [25] [53]. The effectiveness of such treatment strategies likely relies on the importance of cross talk between the ER and growth factor pathways as a mechanism through which cells can escape the growth inhibitory action of anti-oestrogens; targeting of common downstream ‘convergence nodes’ in these pathways thus represents a potential strategy alongside conventional endocrine therapy to enhance growth inhibition. It follows that FAK inhibitor-based treatment regimens may also provide an effective strategy to suppress the development of endocrine insensitivity, as appears to be the case with anti-Src agents [54]. Importantly, our data also demonstrates that inhibition of FAK activity in acquired tamoxifen-resistant cells appears to partially restore their response to tamoxifen although this effects was not observed in fulvestrant-resistant cells. The latter may be explained by the fact that our models of fulvestrant resistance, derived by long-term exposure to fulvestrant, are generally negative for the estrogen receptor, with only ~2% of cells being weakly or very weakly stained using H222 antiserum [28], and thus are unable to respond to endocrine agents. However, the observations in ER-positive TamR cells are intriguing and suggest that modulation of

FAK activity in ER-positive forms of acquired endocrine resistance may provide a means to restore endocrine sensitivity through an as yet unidentified mechanism. In this study, we have highlighted the role of FAK as a key mediator of the adverse phenotype that accompanies acquired endocrine resistance in breast cancer. Importantly, FAK may represent a novel target in breast cancer since inhibition of FAK is able to reverse this behaviour and may provide additional benefit on growth suppression when combined with endocrine agent in endocrine-sensitive and ER-positive, endocrine-resistant cells.

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Legends to figures

Figure 1: Differential activation of FAK in endocrine-resistant breast cancer cells.

Endocrine-sensitive MCF7 cells and their tamoxifen- (TamR) and fulvestrant (FasR)-resistant counterparts were analysed for the presence and activation status of FAK by Western blotting. Whilst levels of FAK were similar between the cells, phosphorylation of FAK at Y397 and Y861 was increased in the resistant cells.

Figure 2: Increased sensitivity of endocrine-resistant breast cancer cells to FAK inhibitor, PF573228

Cells were treated with the FAK inhibitor, PF228 for 60 minutes prior to analysis of FAK expression and activity by Western blotting. (A) Treatment of MCF7, TamR and FasR cells with PF228 promoted a dose-dependent decrease in FAK activation at Y397 whilst levels of total-FAK were not affected. Densitometry of these data (B) revealed that resistant cells were significantly more sensitive to these effects of PF228 versus MCF7 counterparts (values are mean $IC_{50} \pm$ s.d.). PF228 did not affect the phosphorylation of FAK at Y407, Y576 or Y861 (C) nor the activity of Src and ERK1/2 (D).

Figure 3: Inhibition of FAK activity suppressed endocrine-resistant cell growth.

Cells were incubated in the presence of PF228 for 7 days following which cell growth was determined using WST-1 assay. (A) FAK inhibition did not affect MCF7 cell growth and resulted in only modest growth suppression of both TamR and FasR cells. (B) Western blotting of FAK expression and activity after 7 days treatment revealed inhibition of FAK397 but no change in FAK expression.

* $p < 0.01$ versus MCF7 cells (n=4)

Figure 4: Inhibition of FAK activity alters affinity for matrix adhesion, suppresses cell migration and reduces membrane activity.

The effects of FAK inhibition on the ability of cells to adhere to uncoated and matrix (fibronectin and laminin)-coated surfaces was determined as described in the text. (A) Both TamR and FasR cells demonstrated and enhanced ability to adhere to uncoated and matrix-coated surfaces. (B) Subsequently, inhibition of FAK significantly

suppressed TamR and FasR cell adhesion to these surfaces. (C) Inhibition of FAK also promoted a reduction in cell migration over fibronectin. (D) DIC microscopy revealed formation of large numbers of filopodia and lamellipodia (arrowed) at the membrane of resistant cells, characteristic of actively-migrating cells. Treatment with PF228 reversed this phenomena, producing tightly-packed cell colonies with little or no membrane activity akin to the MCF7 counterparts

A, B: * $p < 0.05$ versus MCF7 cells (n=3); C: * $p < 0.05$ versus untreated cells (n=4)

Figure 5: Combined FAK and ER inhibition impairs ER+ breast cancer cell growth.

MCF7 (A) and T47D (B) cell growth in response to tamoxifen, PF228 (1 μ M) or both agents in combination was assessed using WST-1 growth assays. Combining PF228 with endocrine therapy produced a greater level of inhibition of cell growth compared with each treatment as a single agent.

* $p < 0.05$ versus untreated cells (n=3); ** $p < 0.05$ versus single agent treatment

Figure 6: Inhibition of FAK activity partially restores response to tamoxifen in tamoxifen-resistant MCF7 cells

Acquired-tamoxifen (TamR) and fulvestrant (FasR)-resistant MCF7 cells were cultured for 6 days in the presence or absence of 100nM 4-hydroxy tamoxifen or fulvestrant respectively and in the presence or absence of PF228 (1 μ M). PF228 appeared to partially restore sensitivity to tamoxifen in TamR cells (A). No effects were observed in FasR cells (B). * $p < 0.005$ vs. tamoxifen alone (n=3)

Figure

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Figure 1

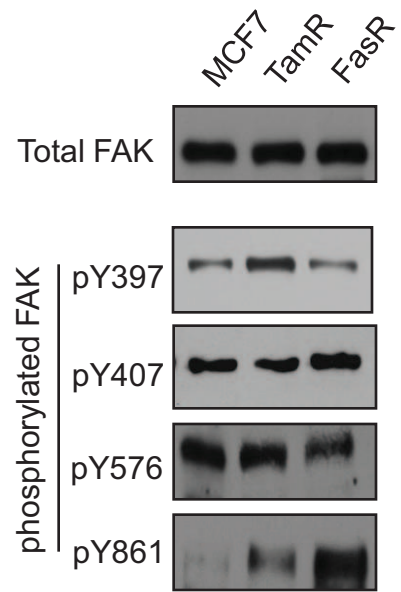


Figure 2

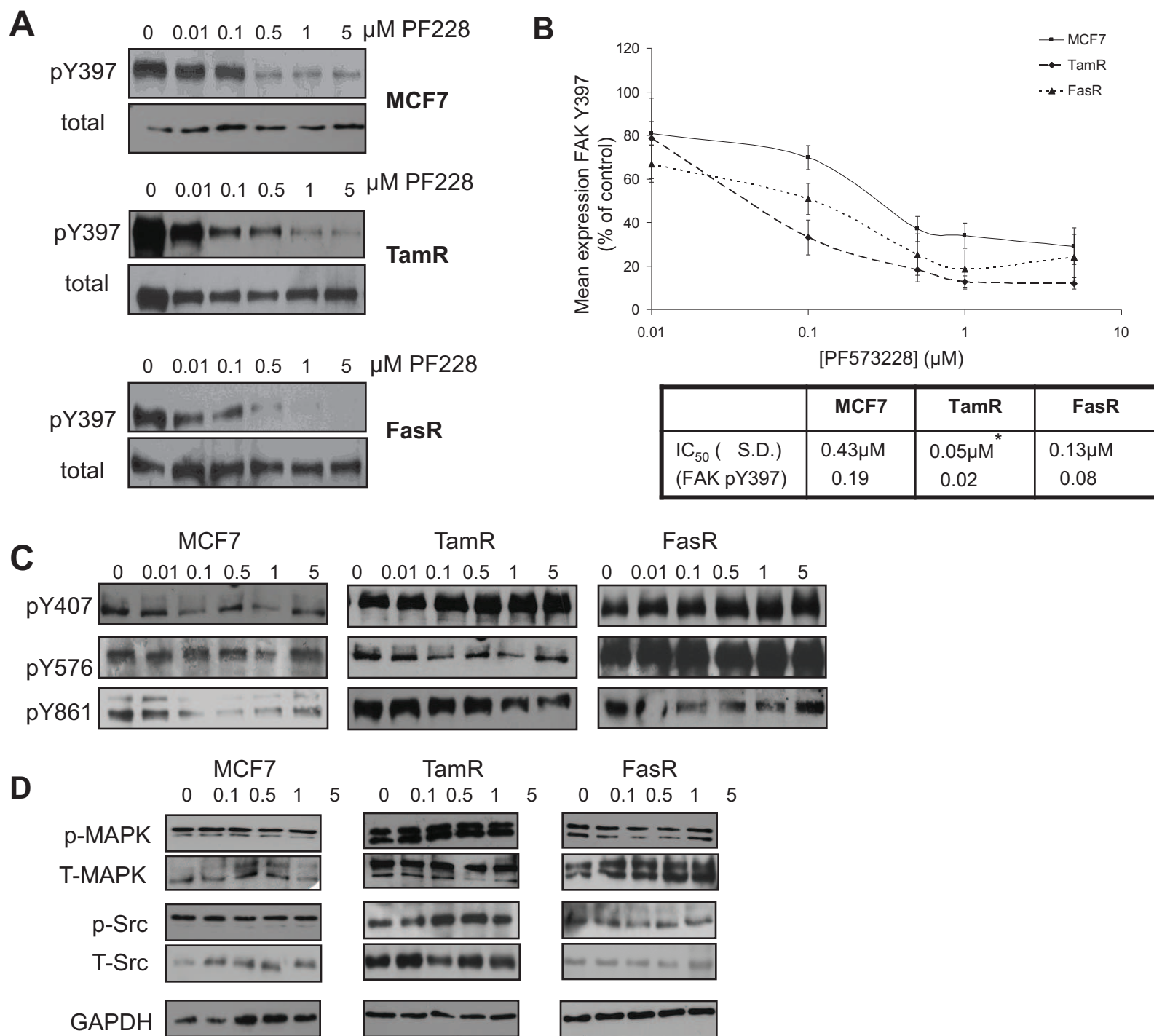
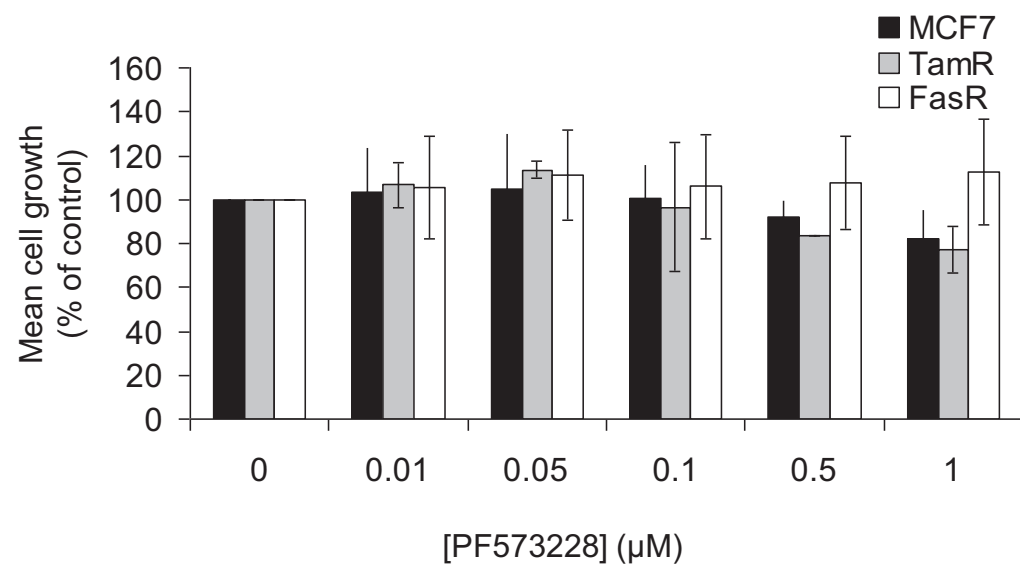


Figure 3

A



B

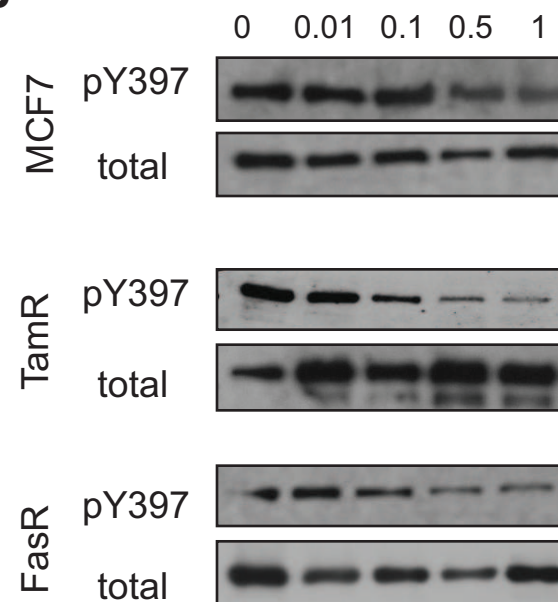
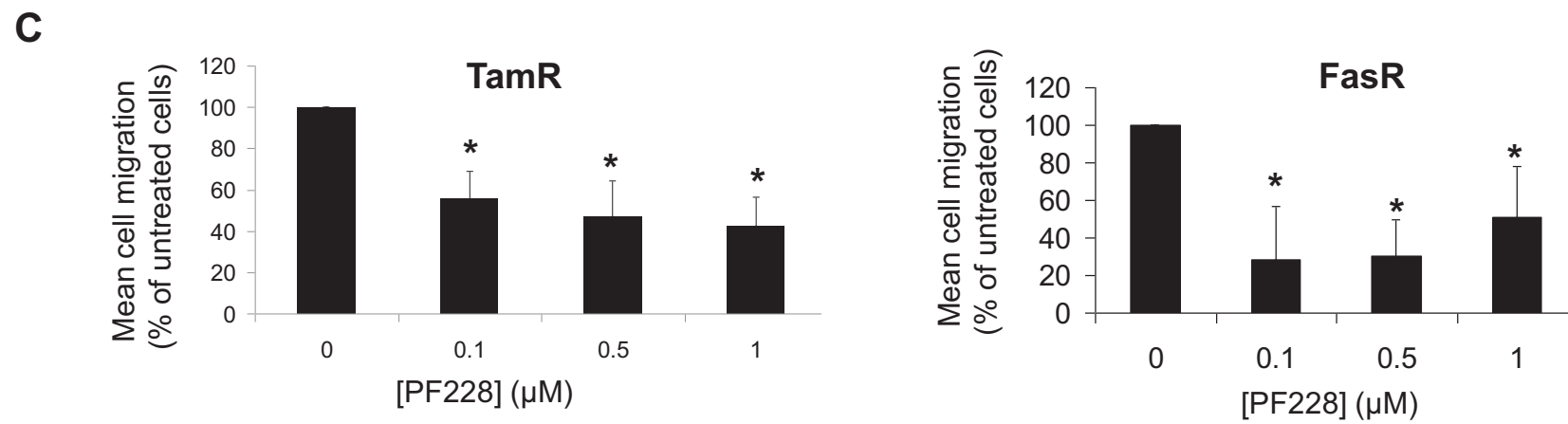
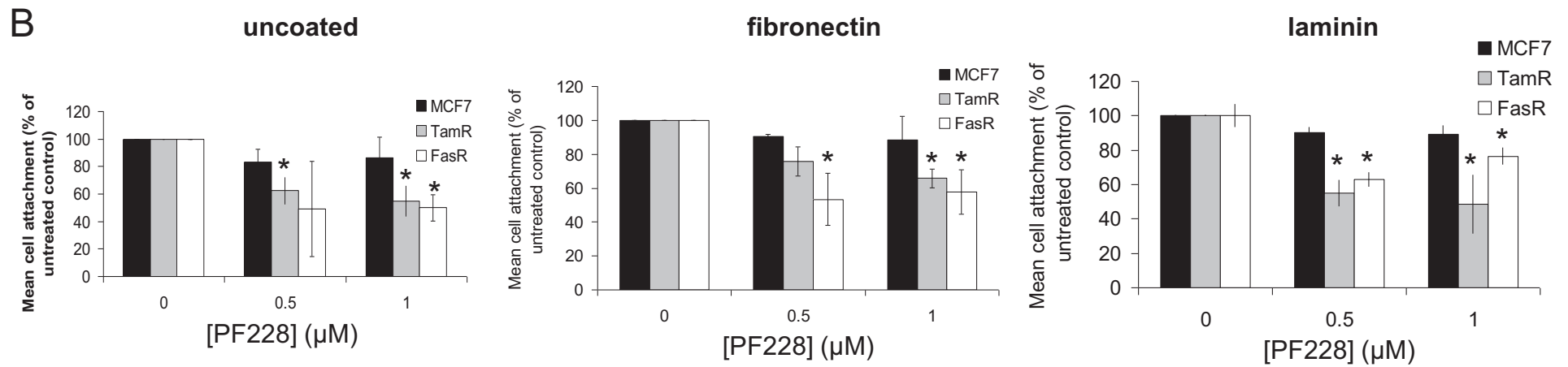
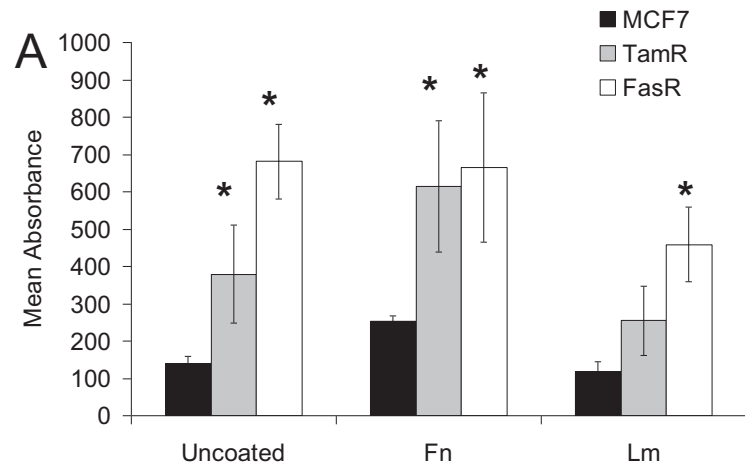
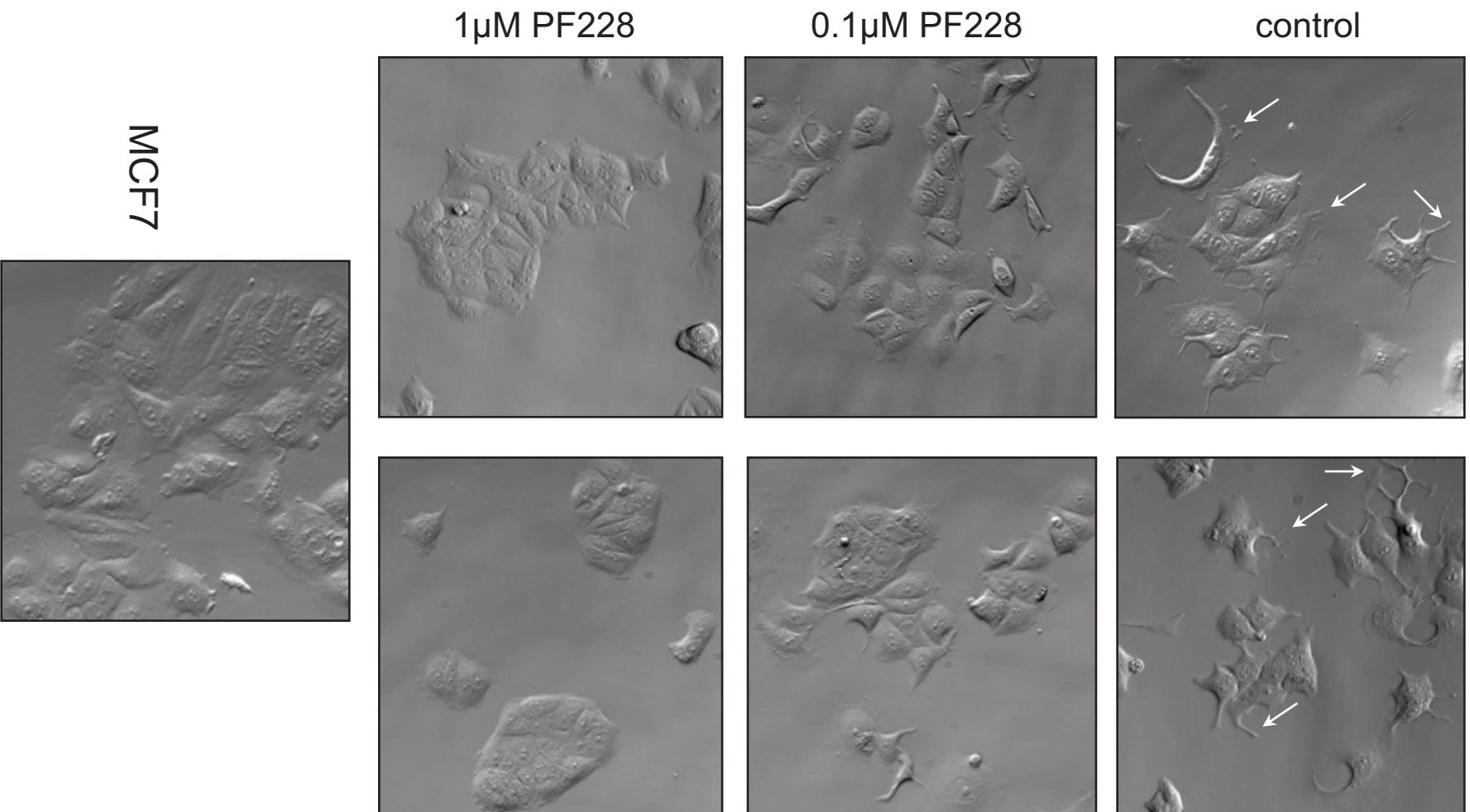


Figure 4A-C





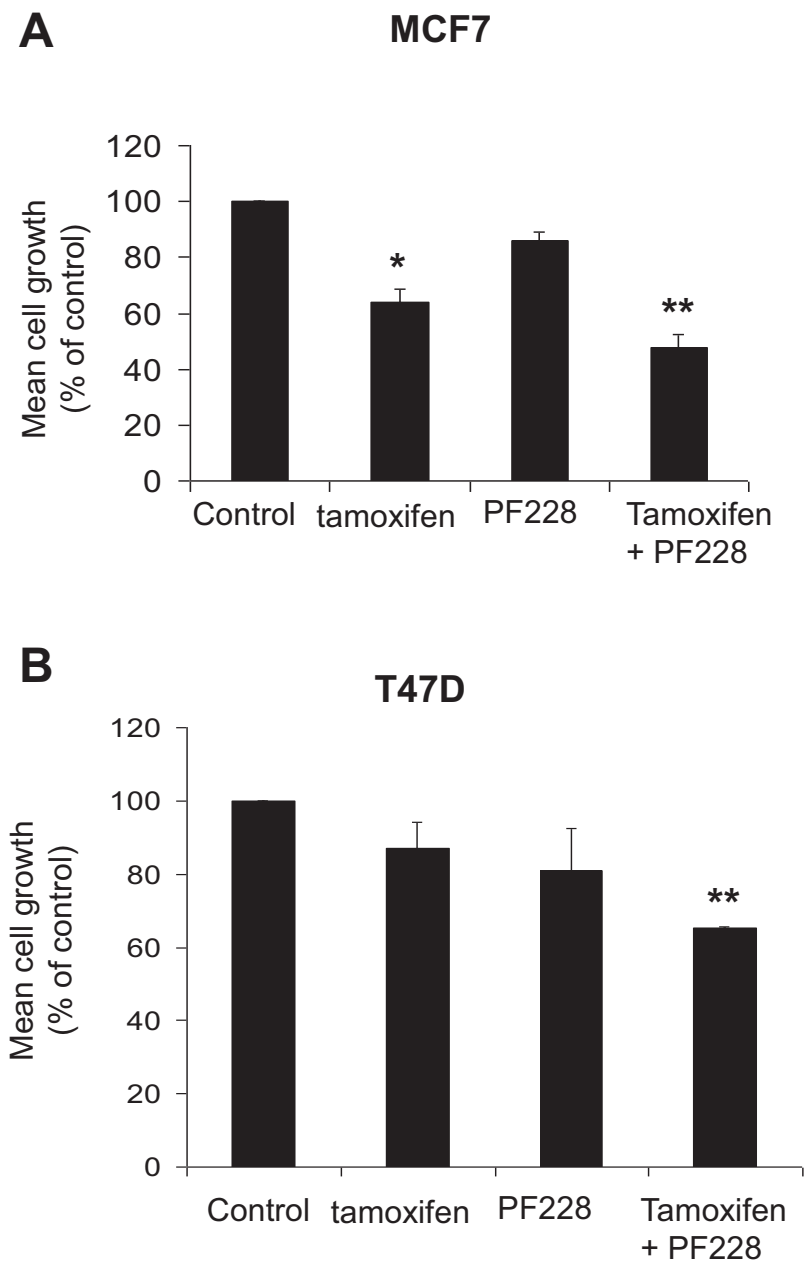


Figure
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Figure 6

