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A chemical screen in diverse breast cancer cell lines reveals genetic enhancers and suppressors of sensitivity to PI3K isoform-selective inhibition

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The PI3K (phosphoinositide 3-kinase) pathway regulates cell proliferation, survival and migration and is consequently of great interest for targeted cancer therapy. Using a panel of small-molecule PI3K isoform-selective inhibitors in a diverse set of breast cancer cell lines, we have demonstrated that the biochemical and biological responses were highly variable and dependent on the genetic alterations present. p110α inhibitors were generally effective in inhibiting the phosphorylation of PKB (protein kinase B)/Akt and S6, two downstream components of PI3K signalling, in most cell lines examined. In contrast, p110β-selective inhibitors only reduced PKB/Akt phosphorylation in PTEN (phosphatase and tensin homologue deleted on chromosome 10) mutant cell lines, and was associated with a lesser decrease in S6 phosphorylation. PI3K inhibitors reduced cell viability by causing cell-cycle arrest in the G_1 phase, with multi-targeted inhibitors causing the most potent effects. Cells expressing mutant Ras were resistant to the cell-cycle effects of PI3K inhibition, which could be reversed using inhibitors of Ras signalling pathways. Taken together, our data indicate that these compounds, alone or in suitable combinations, may be useful as breast cancer therapeutics, when used in appropriate genetic contexts.

Key words: breast cancer, genetic alteration, phosphoinositide 3-kinase inhibitor (PI3K inhibitor), phosphorylation, protein kinase B (PKB), S6.

INTRODUCTION

The PI3K (phosphoinositide 3-kinase) pathway has crucial roles in many cellular processes, including cell survival, proliferation and migration, driven by many downstream signalling pathways [1]. PI3K consists of a catalytic subunit (termed p110) and a regulatory subunit (p85, p101 or p55, depending on the enzyme) that acts to integrate stimulatory signals and direct the catalytic subunit to appropriate cellular locations. Four distinct class I p110 isoforms (α, β, γ and δ) generate PtdIns(3,4,5)P_3, which recruits downstream effectors to the sites of generation. Key to this process is the recruitment of PDK1 (3-phosphoinositide-dependent kinase 1) which plays a critical role in mediating the activation of AGC kinases, including PKB (protein kinase B)/Akt and $p70^{S6K}$ (p70 ribosomal protein S6 kinase). In turn, these kinases are known to play essential roles in cell proliferation, survival and growth [2].

Breast tumours can be defined by their morphological features, as well as their distinct patterns of underlying genetic alterations [3]. In particular, the involvement of the PI3K pathway in breast cancer is implicated from a variety of genetic changes which include EGFR [EGF (epidermal growth factor) receptor] amplification, HER2/neu amplification [4], PTEN (phosphatase and tensin homologue deleted on chromosome 10) mutation/loss [5] and PI3K p110α mutations [6]. These p110α mutations cause increased PI3K activity [6,7] are oncogenic [7,8] and are prevalent at high frequency in breast cancers [9–11]. The presence of p110α mutations in breast tumours was found to be associated with ER (oestrogen receptor) and progesterone receptor expression, and inversely correlated with PTEN expression, but did not affect prognosis or survival [12]. Phosphoinositide signals in breast cancer therefore point to therapeutic opportunities. Since the genetic diversity of commonly used breast cancer cell lines reflects many of the alterations found in breast cancer, these cell lines represent an ideal system to assess the potential for therapeutic agents which act on the PI3K pathway [13,14].

The paradigms for clinical use of protein kinase inhibitors suggest that their effectiveness will be dictated by the specificity profile of a given compound and the genetic context in which it is being applied. This is well illustrated when considering the dramatic impact that imatinib (Gleevec®) has had on recent cancer

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ER, oestrogen receptor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FMK, fluoromethylketone; GSK3, glycogen synthase kinase 3; HEK, human embryonic kidney; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; mTOR, mammalian target of rapamycin; $p70^{S6K}$, p70 ribosomal protein S6 kinase; $p90^{rsk}$, p90 ribosomal S6 kinase; PDGFR, platelet-derived growth factor receptor; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase, PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; siRNA, short interfering RNA; WT, wild-type.

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treatment. Although imatinib was first approved for chronic myelogenous leukaemia patients expressing an activated form of Abl, the knowledge that it also inhibits PDGFR (platelet-derived growth factor receptor) and c-kit led to successful treatment of gastrointestinal stomach cancers and hypereosinophilic syndrome. This latter indication is especially revealing as it was not known previously that these patients harboured alterations in PDGFR, kit or Abl, but their dramatic responses to this agent led to sequencing efforts showing activating translocations of the PDGFR gene [15]. Therefore detailed knowledge of the target (and off-target) specificities of new molecular therapies is critical to our understanding of potential side effects, as well as to predicting which tumours will be most likely to respond to these agents.

Historically, the availability of p110 inhibitors has been largely limited to a single natural product, wortmannin, and the synthetic chromone, LY294002, which broadly act on the majority of PI3Ks along with other related kinases such as mTOR (mammalian target of rapamycin), and are toxic when administered systemically *in vivo* [16]. Potential therapies have therefore been slow because selective compounds have been lacking. Recently, however, a number of new classes of isoform-selective PI3K inhibitors have been reported [17–20]. The most selective molecule, IC87114, exhibits >100-fold selectivity for PI3Kδ compared with all other PI3K family members, allowing for precise analysis of this isoform in neutrophil migration [21,22] and oxidase activation [23]. This compound has also revealed important roles for p110δ in breast cancer cell chemotaxis [24], and in myeloid leukaemia [25]. The next most selective molecule is TGX-221, which inhibits PI3Kβ with high specificity, allowing for the analysis of the role of this isoform in thrombosis [26]. Compounds with true selectivity for p110α have not thus far been reported, although the toolkit of compounds available has pinpointed a critical role for p110α in insulin signalling [17], also confirmed by genetic approaches [27].

The availability of isoform-selective PI3K inhibitors allows fundamental questions regarding the role of individual p110 isoforms in the control of cell biology to be addressed, including the following. (i) In cells co-expressing p110α and p110β, are distinct signalling functions regulated by each isoform? (ii) In cells expressing all four isoforms, can selective inhibitors reveal unique sensitivities under distinct growth conditions or in a genotype-specific manner? (iii) Is the loss of PTEN- compared with p110α-activating mutations equivalent or different in terms of creating inhibitor sensitivities? (iv) Between the two most closely related p110 isoforms (α and β), why have only p110α-activating mutations been identified in human cancers?

We have generated and characterized a panel of the most potent reported inhibitors with respect to biochemical activity against 18 PI3Ks and protein kinases, as described previously [17]. With this set of PI3K inhibitors, we can target virtually any member of the PI3K class I family, as well as selected members of other PI3K-related kinases, such as DNA-PK (DNA-dependent protein kinase) or mTOR. Our goal is to utilize this panel of inhibitors in a family-wide approach to probe the role of PI3K family members in regulating breast tumour cell proliferation. These compounds comprise a wide variety of chemotypes with varying cross-selectivities among the p110 isoforms. The advantage of this approach is that compound-specific pharmacology, which often masks the real targets of lead compounds, is somewhat ameliorated because of the presence of multiple chemotypes with similar biochemical targets. Any compounds that exhibit different biological responses, but display apparent equivalent biochemical specificity can be identified rapidly. Thus each inhibitor in the panel becomes a drug candidate itself and a control for other molecules in the panel. The compounds used in the present study include p110δ-selective PIK-23, the p110β-selective compounds TGX-286 and PIK-108, and multi-targeted PI3K inhibitors PIK-75, PI-103, PIK-85, PIK-90 and PIK-124. For full *in vitro* activity characterization along with chemical structures of these compounds, see [17] and Table 2.

A suppressor-enhancer chemical genetic screen was performed in order to look for differential sensitivity between genetically diverse breast cancers with a view to identify new therapeutic opportunities for breast cancer. Our results display a surprising heterogeneity of responses to different compounds in a cell-line-dependent manner. We conclude that the effectiveness of these drugs as targeted therapies will probably be determined by the cellular genetic alterations, as well as differences in drug metabolism and/or adaptation properties of individual tumours. The diversity of genetic changes leading to different types of breast cancers means that molecular markers of therapeutic sensitivity will be necessary to best match up therapies with genetic backgrounds. Importantly, we have demonstrated that, although p110β activity is not required for PI3K signalling in normal and WT (wild-type) PTEN-expressing cancer cells, PTEN loss sensitizes cells to p110β inhibitors. In addition, breast cancer cell lines expressing mutant Ras are resistant to all PI3K inhibitors tested, which can be reversed using a combination approach of PI3K inhibitors and MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] inhibitors.

EXPERIMENTAL

Materials

Rabbit polyclonal phospho-PKB (Ser^{473}) was generated as described previously [28]. Phospho-S6 (Ser^{235}/Ser^{236}) and phospho-GSK3 (glycogen synthase kinase 3) (Ser^{21}/Ser^{9}) were from Cell Signaling Technology. Anti-β-actin antibody was from Sigma and anti-PTEN antibody was from Cascade Biosciences. LY294002, UO126 and rapamycin were obtained from Calbiochem. Hoechst dye, Alexa Fluor® 488-conjugated rabbit secondary antibody was from Molecular Probes. All other reagents were from Sigma unless stated otherwise.

Cell lines, cell culture and transfection

Cell culture reagents were purchased from Invitrogen. BT474, BT549, BT20, Hs578t and T47D cells were maintained in RMPI 1640 with 10% (v/v) FBS (fetal bovine serum). MDA-MB-468, MDA-MB-231, MCF-7 and SKBr3 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with 10% (v/v) FBS. MCF-10A cells were cultured in medium composed of 50% DMEM and 50% Ham's F12 supplemented with 20 ng/ml EGF, 100 ng/ml cholera toxin, 10.08 μg/ml insulin, 500 ng/ml hydrocortisone and 5% FBS. All cell lines were obtained from the UCSF (University of California, San Francisco) inter-SPORE (Specialized Program of Research Excellence) collection as deposited with the A.T.C.C. Transfection of PTEN siRNA (short interfering RNA) was performed using Oligofectamine™ reagent and either PTEN siRNA-1 (target sequence, AAGGCGTATACAGGAACAATA) or PTEN siRNA-1 (validated PTEN siRNA from Qiagen; catalogue number SI00301504). Two rounds of transfection of siRNA at 100 nM were performed followed by treatment with either PIK-108 or PI-103 at 1 μM for 1 h before harvesting. To create BT-20 cells stably expressing K-RasG12D, they were initially infected with retroviruses expressing the ecotropic receptor and selected in

Table 1 Breast cell lines studied, cell types and known genetic alterations

Cell line	Cell type	ER	Ras	*PTEN*	*HER2*	*PIK3CA*
BT474	Epithelial	Positive			Amplified	K111N
MCF-7	Epithelial	Positive				E545K
SKBr3	Epithelial	Positive			Amplified	WT
T47D	Epithelial	Positive				H1047R
BT20	Trans/basal	Negative				P539R/H1047D
MCF-10A	Trans/basal	Positive				WT
MDA-MB-468	Trans/basal	Negative		Δ44bp exon 3		WT
Hs578t	Mesenchymal	Positive	H-RasG12D			WT
MDA-MB-231	Mesenchymal	Negative	K-RasG13D			WT
BT549	Mesenchymal	Negative		W274stop		WT

medium containing 5 μg/ml blastocidin. Following selection for 2 weeks, these cells were subsequently infected with retroviruses containing K-RasG12D, or empty vector, and maintained in 1 mg/ml G418 for 4 weeks to obtain pools of cells expressing activated Ras.

Western blotting

Cells were seeded on six-well plates at 80 % confluence before the addition of p110 inhibitors and other compounds. Cells were harvested by scraping into 150 μl of lysis buffer [1 % Nonidet P40, 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (dithiothreitol), 1 mM $NaVO_4$ and Complete™ protease inhibitor cocktail (one tablet in 20 ml) (Roche)]. Protein samples were quantified using the Bio-Rad protein assay. Typically, 10 μg of protein per sample was separated by SDS/PAGE (10 % gels), immunoblotted with indicated antibodies and visualized by ECL® (enhanced chemiluminescence) on film (GE Healthcare). Western blot analysis was performed using NIH Image™.

Immunofluorescence

Cells were seeded at 80 % confluence on 96-well plates. Compounds were added as indicated in the Figure legends, and cells were then washed and fixed in 4 % (w/v) paraformaldehyde for 15 min. Washes and incubations were performed in PBS unless stated otherwise. Cells were permeablized with 0.2 % (v/v) Triton X-100 for 5 min, washed and incubated in 1 % (w/v) BSA for 20 min. Cells were incubated with primary antibody containing 1 % (w/v) BSA for 1 h. Cells were then washed and incubated with fluorescent dye-conjugated secondary antibody for 1 h. Quantification of phospho-S6 immunoflourescence was performed using the Cellomics ArrayScan® HCS (High Content Screening) reader system and determined by the compartmental analysis bioapplication from Cellomics. Cell number was determined by counting nuclei stained with Hoechst.

FACS analysis

Cells were seeded on six-well plates at 80 % confluence before the addition of compounds as indicated in the Figure legends. Adherent cells were collected by trypsinization and pooled with medium containing non-adherent cells, washed with PBS and subsequently fixed in 70 % ethanol. A further wash with PBS containing 5 % (v/v) FBS was followed by resuspension in PBS containing propidium iodide (10 μg/ml) and RNase A (0.1 mg/ml). Analysis of 10 000 cells was performed on FACScan using Cell Quest software.

RESULTS

A panel of breast cancer cell lines was selected to represent various genetic alterations seen in primary breast tumours such as PTEN mutation/loss, EGFR overexpression, ErbB2 overexpression, *BRCA1* mutation, ER expression and Ras mutation. These cell lines, BT474, SKBr3, BT20, T47D, MCF-7, MDA-MB-468, BT549, MDA-MB-231 and Hs578t, are summarized in Table 1. MCF-10A cells were used for comparative purposes as a non-tumorigenic karyotypically normal cell line. Affymetrix gene expression profiling performed on these cell lines show that p110α, p110β and p110δ all show similar mRNA expression levels, whereas p110γ, which is known to be more restricted in its tissue distribution [29], is expressed at low to undetectable levels [29a].

Breast cancer cell lines display distinct biochemical sensitivities to p110 isoform-selective inhibitors

Of the eight compounds in our study, five were chosen for detailed analysis because of their target representation and promising activity in preliminary experiments. These include the potent p110α inhibitors PIK-75 and PI-103, the relatively selective p110β inhibitors TGX-286 and PIK-108, and PIK-85, which inhibits p110α, p110γ and p110δ, and p110β less potently. The selectivity of these compounds as assessed by *in vitro* lipid kinase assays is shown in Table 2. Compounds were added to each of the cell lines at concentrations of 0.5, 1 and 10 μM for 1 h to assess inhibition of PI3K signalling using phospho-specific antibodies against PKB/Akt (Ser^{473}) and S6 (Ser^{235}/Ser^{236}). Ser^{473} is generally associated with activated forms of PKB/Akt and is known to be phosphorylated in a PI3K-dependent manner. However, as this site is phosphorylated directly by mTORC2 (mTOR complex 2) [30], PI3K inhibitors that also directly inhibit mTOR will have an effect on this site. Ser^{235} and Ser^{236} are generally phosphorylated by $p70^{S6K}$, although under some circumstances they can be phosphorylated by $p90^{rsk}$ (p90 ribosomal S6 kinase) [31,32].

A surprising degree of heterogeneity in biochemical responses to p110 inhibitors was observed across the different cell lines examined. In general, p110α inhibitors were the most effective at inhibiting phosphorylation of these target proteins (Figure 1A). PIK-75 and PI-103 are the most potent p110α inhibitors, followed by PIK-85. In seven of the ten cell lines examined, the inhibition of PKB/Akt and S6 phosphorylation was consistent with p110α driving the activity of this pathway, with PIK-75 and PI-103 being equipotent at inhibiting the phosphorylation of these proteins, PIK-85 being less potent, and TGX-286 and PIK-108 being ineffective. This includes the cell line chosen for its normal karyotype and non-transformed morphology (MCF-10A), as well

Table 2 ***In vitro*** **IC_{50} data for the isoform-selective small-molecule inhibitors determined in the presence of 10 μM ATP**

Most of these data are taken from [17]. ND, not determined. ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; DNA-PK, DNA-dependent protein kinase; hsVPS34, *Homo sapiens* vacuolar protein sorting 34; mTORC, mTOR complex; PI4K, phosphoinositide 4-kinase; PIKK, phosphoinositide kinase-related kinase; PIPK, phosphoinositide phosphate kinase; PI4P5K, phosphoinositide 4-phosphate 5-kinase; PI5P4K, phosphoinositide 5-phosphate 4-kinase.

	IC_{50} value (μM)							
Compound . . .	PIK-23	TGX-286	PIK-75	PIK-85	PIK-90	PIK-108	PI-103	PIK124
PI3Ks								
p110α/p85α	>200	4.5	0.0058	0.044	0.011	2.6	0.0082	0.023
p110β/p85α	42	0.12	1.3	0.8	0.35	0.057	0.088	1.1
p110δ/p85α	0.097	1	0.51	0.08	0.058	0.26	0.048	0.34
p110γ	50	10	0.076	0.05	0.018	4.1	0.15	0.054
PI3KC2α	>100	>100	~10	ND	0.047	~100	~1	0.14
PI3KC2β	100	~100	~1	ND	0.064	~20	0.026	0.37
PI3KC2γ	>100	ND	ND	ND	ND	ND	ND	ND
hsVPS34	~50	3.1	2.6	ND	0.83	~5	2.3	10
PI4Ks								
PI4KIIα	>100	>100	>100	>100	>100	>100	>100	>100
PI4KIIIα	>100	>100	>100	2.5	0.83	~50	>100	>100
PI4KIIIβ	>100	>100	>100	3.6	3.1	>100	>100	>100
PIKKs								
ATR	>100	>100	21	ND	15	>100	0.85	2
ATM	>100	>100	2.3	~50	0.61	35	0.92	ND
DNA-PK	>100	~50	0.0017	0.061	0.013	0.12	0.0019	1.5
mTORC1	>100	>100	1	>100	1.05	ND	0.02	ND
mTORC2	>101	ND	0.16	ND	ND	ND	0.125	ND
hSMG-1	ND	ND	ND	ND	ND	ND	ND	ND
PIPKs								
PI4P5KIα	>100	>100	>100	>100	>100	>100	>100	>100
PI4P5KIβ	>100	>100	>100	>100	>100	>100	>100	>100
PI5P4KIIβ	>100	>100	>100	>100	>100	>100	>100	>100

as cell lines BT474, SKBr3, BT20, T47D, MCF-7 and Hs578t. In contrast, MDA-MB-231 cells, which showed very low basal PI3K pathway activity, were resistant to all p110 inhibitors tested when analysing PKB/Akt phosphorylation. Surprisingly, S6 phosphorylation was still sensitive to p110α inhibitors PIK-75 and PI-103 in this cell line. MDA-MB-468 cells and BT549 cells showed a distinct profile of inhibition, with PI-103 (a p110α and p110β inhibitor) being more potent than PIK-75 (a potent p110α inhibitor, but not p110β inhibitor) at inhibiting PKB/Akt and S6 phosphorylation. Moreover, selective p110β inhibitors, such as TGX-286 and PIK-108, were able to inhibit the phosphorylation of PKB/Akt exclusively in MDA-MB-468 and BT549 cells. These cell lines harbour PTEN mutations [33], unlike the other eight cell lines in the panel which express WT PTEN. Although both PKB/Akt and GSK3 phosphorylation was inhibited by p110β inhibitors in PTEN mutant cell lines, S6 phosphorylation remained unchanged (Figure 1C). As shown for MCF-10A, SKBr3 and BT474, no inhibition of PI3K signalling as assessed by PKB/Akt phosphorylation was apparent in any cell line when using p110δ-selective compounds such as PIK-23. However, S6 phosphorylation was sometimes inhibited, suggesting a potential p110δ-dependent but PKB/Akt-independent pathway for regulating S6 phosphorylation in breast cancer cells. As expected, LY294002 was effective at inhibiting PI3K signalling in all cell lines examined (Figure 1A). The PKB phosphorylation in response to the p110 inhibitors was quantified, and the in cell IC_{50} values were calculated. These are displayed in Figure 1(C), providing a summary of the effects of the p110 inhibitors in the panel of breast cancer cell lines. Therefore PKB/Akt and S6 phosphorylation are dependent on either p110α or p110β activity, in a manner that correlates with PTEN status.

Distinct pharmacodynamic properties of p110 inhibitors in different cell lines

We next analysed the temporal responses to p110 inhibitors. The inhibition of PKB/Akt and S6 phosphorylation was sustained differentially among the cell lines studied (Figure 1B). Some cell lines (e.g. BT549) showed transient inhibition of PKB/Akt and S6 phosphorylation by PIK-75, which returned by 6 h, whereas in other cell lines, PIK-75 inhibition was sustained (e.g. T47D, MCF-10A and BT20). Similar disparate results were also seen for PI-103 (transient inhibition in MDA-MB-468, SKBr3 and MCF-7 compared with sustained inhibition in BT549 and BT20). Whether this represents differences between cell lines in drug metabolism or in feedback compensation is currently unknown. However, such disparate pharmacodynamic responses are likely to influence the biological responses to these compounds. An increase in PKB phosphorylation in response to TGX-286 is apparent from Figure 1(B). We believe that this is an artefact of antibody labelling or 'edge effect', since this finding was not replicated in results shown in Figure 1(A) or in other experiments.

The phosphorylation status of S6 was also monitored by immunoflourescence. This was performed using the Cellomics ArrayScan® HCS reader system and intensity of fluorescence was determined by the compartmental analysis bioapplication. This analysis confirmed that PI-103 and PIK-75 inhibit S6 phosphorylation in SKBr3 cells after 1 h, which was sustained with PIK-75, but transient for PI-103 (Figures 2A and 2B). p110β inhibitors TGX-286 and PIK-108 showed only a minimal inhibition of S6 phosphorylation, similar to the results seen by Western blotting in this cell line. A cursory visual inspection of the immunofluorescence data suggested that PIK-75 and PI-103 appeared to reveal an 'all or nothing' pattern of inhibition

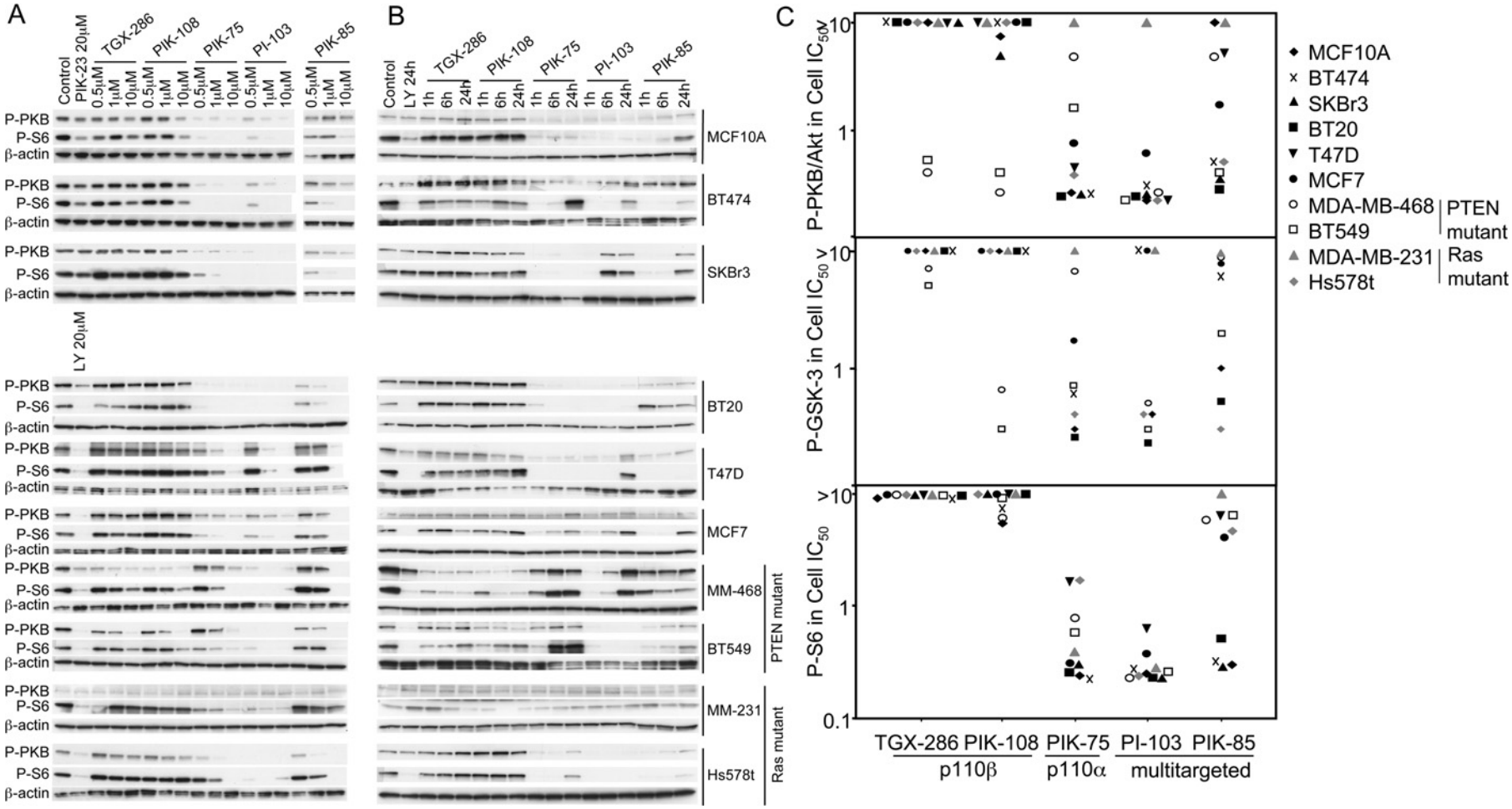

Figure 1 Dose-dependent and temporal biochemical responses of different breast cancer cell lines to increasing concentrations of isoform-selective PI3K inhibitors

(**A**) MCF-10A, BT474, SKBr3, BT20, T47D, MCF-7, MDA-MB-468, BT549, MDA-MB-231 and Hs578t cells were cultured in six-well dishes as described in the Experimental section. The indicated concentrations of TGX-286, PIK-108, PIK-75, PI-103 and PIK-85 were added to the culture medium, and 1 h later cells were harvested into lysis buffer. Proteins were separated by SDS/PAGE and Western blotted using antibodies against phospho-PKB/Akt (Ser^{473}) (P-PKB), phospho-S6 (Ser^{235}/Ser^{236}) (P-S6) and β-actin. Proteins were visualized by horseradish-peroxidase-coupled secondary antibodies and ECL®. LY, LY294002. (**B**) MCF-10A, BT474, SKBr3, BT20, T47D, MCF-7, MDA-MB-468, BT549, MDA-MB-231 and Hs578t cells were cultured in six-well dishes as described in the Experimental section. TGX-286, PIK-108, PIK-75, PI-103 or PIK-85 (each at 1 μM) was added, and the cells were harvested into lysis buffer 1, 6 or 24 h later. Proteins were separated by SDS/PAGE and analysed by Western blotting with the indicated antibodies as in (**A**). (**C**) In cell IC_{50} values (μM) for phospho-PKB/Akt (Ser^{473}) (P-PKB/Akt), phospho-GSK3 (Ser^{21}/Ser^{9}) (P-GSK-3) and phospho-S6 (Ser^{235}/Ser^{236}) (P-S6) in the different cell lines against the p110 isoform-selective inhibitors.

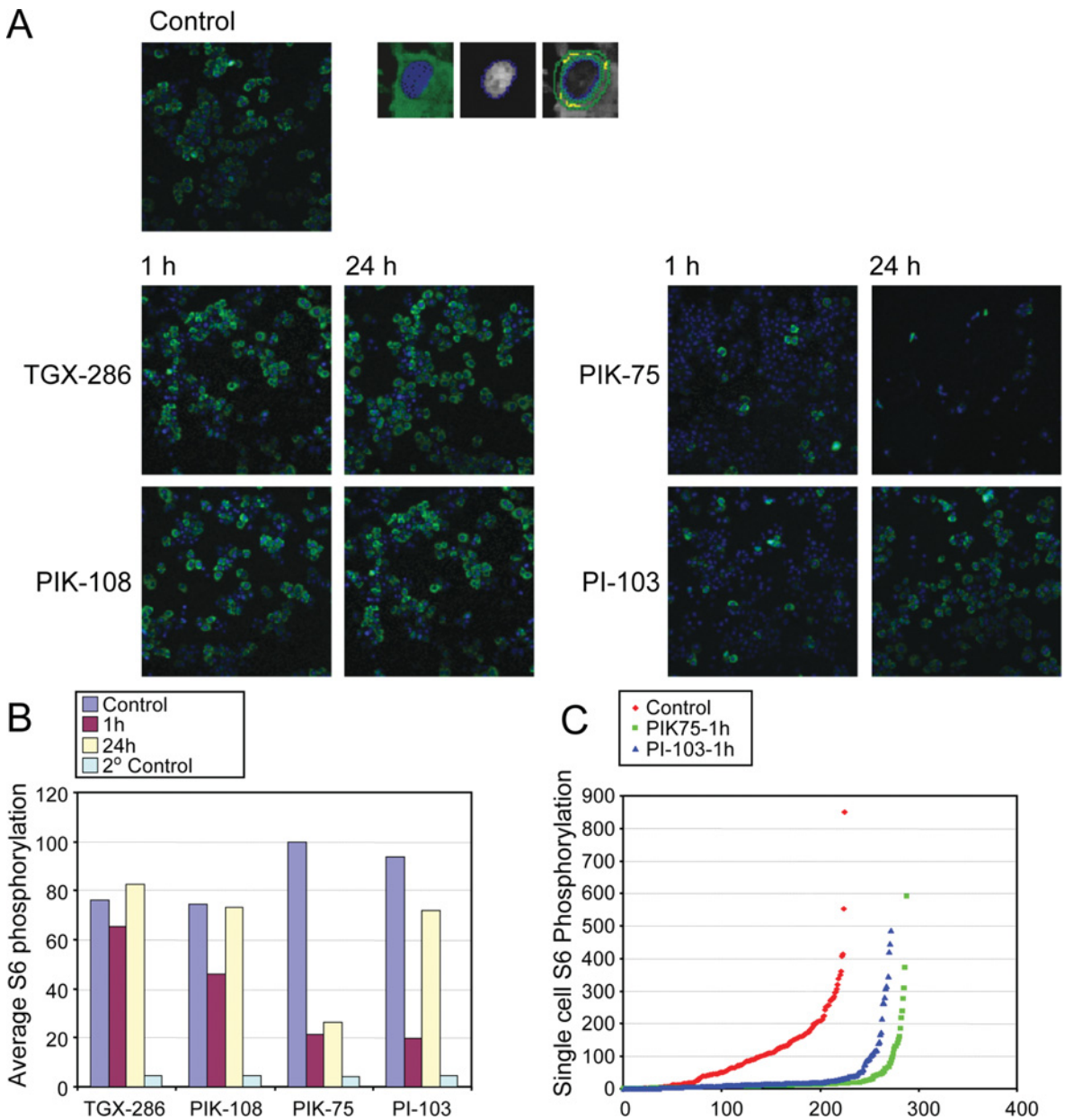

Figure 2 Recovery of breast cancer cells following treatment with isoform-selective PI3K inhibitors by immunofluorescence

(**A**) SKBr3 cells were plated in 96-well dishes and treated 18 h later with 1 μM TGX-286, PIK-108, PIK-75 or PI-103 for 1 or 24 h. Cells were fixed and permeablized as described in the Experimental section, and incubated with antibodies against phospho-S6. Fluorescently labelled secondary antibodies and Hoechst were then added to visualize phospho-S6 (green) and DNA (blue) respectively. Quantification of cytoplasmic phospho-S6 immunoflourescence was performed using the Cellomics ArrayScan® HCS reader system using the compartmental analysis bioapplication from Cellomics. Hoechst fluorescence was used to define nuclei, and a 2 μm ring was drawn around the region of Hoechst fluorescence to define cytoplasmic localization. A single image of four collected for each condition is shown. (**B**) Quantification of average fluorescence from 200 cells in the presence of the indicated inhibitors at the indicated time points, as well as the fluorescence from addition of secondary antibody alone. (**C**) Phospho-S6 fluorescence of individual cells treated with PIK-75 and PI-103 for 1 h, or no drug.

whereby the S6 phosphorylation is either fully inactivated or fully maintained, in a manner reminiscent of the switch-like behaviour seen in the ERK pathway under some conditions [34]. However, when intensity values for single cells were quantified and plotted, there was, in fact, a graded range of fluorescence values that were inhibited in a uniform manner (Figure 2C).

Breast cancer cell lines show distinct responses following p110 isoform inhibition

In order to assess the biological effects of these compounds, cell numbers were examined at various times following addition of p110 inhibitors. Most of the cell lines were inhibited by many of the compounds to a greater or lesser extent, as illustrated in Figure 3(A) for MCF-10A cells. In general, p110α-selective inhibitors had the greatest ability to reduce cell number in a dose-dependent manner (Figure 3A). However, other cell types, such as Hs578t cells, showed much greater resistance to decreases in cell number in response to these inhibitors (Figure 3A), despite their sustained biochemical inhibition in response to PI-103 and PIK-85 (Figures 1C and 3B). Following 48 h of treatment, Hs578t cells were resistant to all compounds except PIK-75, which strongly inhibited cell number (Figure 3A). Paradoxically, in this cell

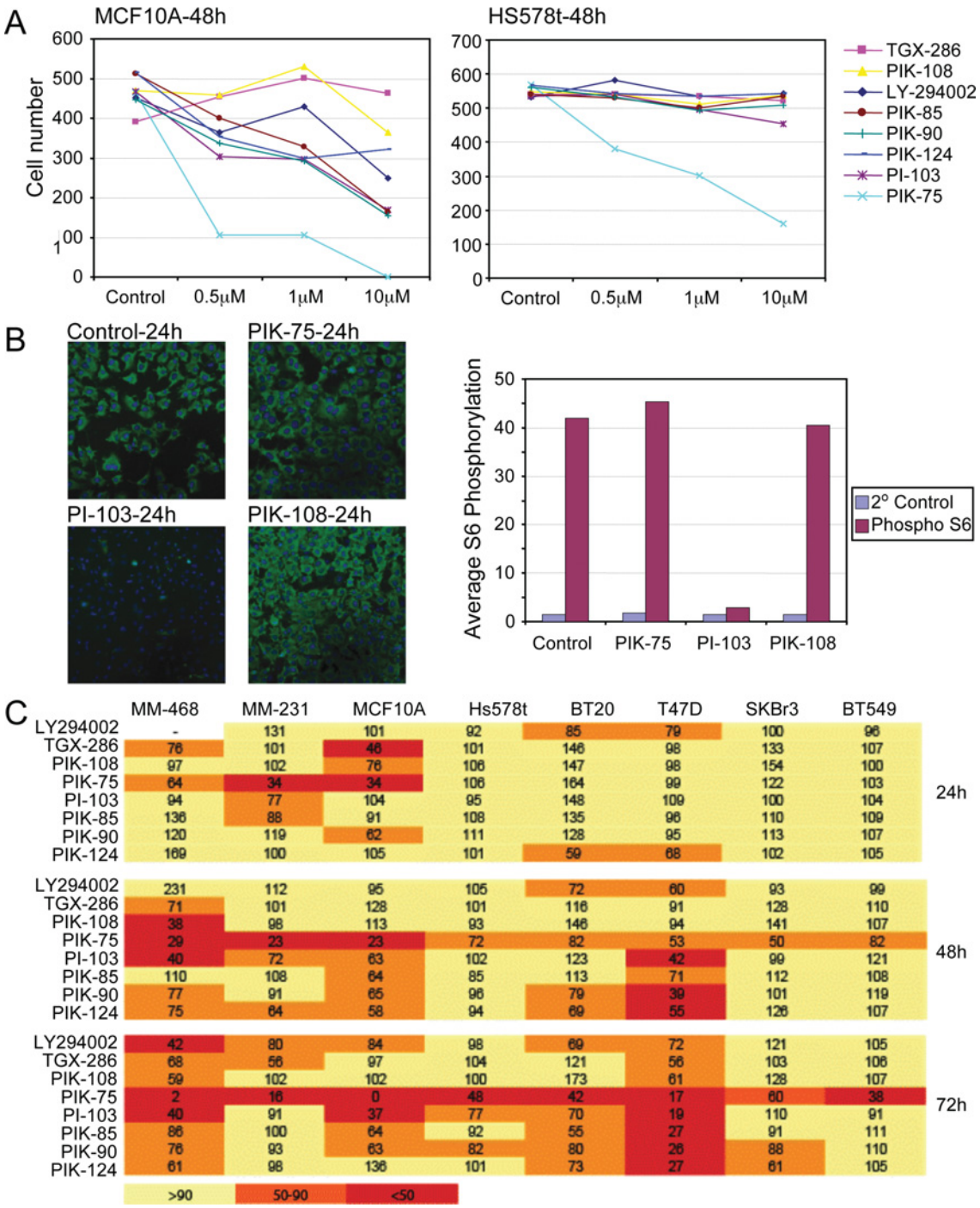

Figure 3 Effects of PI3K isoform-selective inhibitors on breast cancer cell number

(**A**) MCF-10A and Hs578t cells were plated in 96-well dishes, and the indicated concentrations of TGX-286, PIK-108, PIK-75, PI-103, PIK-85, PIK-90 or PIK124 were added to the medium the following day. Cells were incubated for a further 48 h (replacing medium with fresh compound every 24 h), then fixed and permeablized. Hoechst was added to the cells, and the number of nuclei was quantified using Cellomics ArrayScan® software. (**B**) Hs578t cells grown in 96-well dishes were treated with 1 μM PIK-75, PI-103 or PIK-108 for 24 h. Cells were fixed, permeablized and incubated with anti-phospho-S6 antibodies. After incubation with fluorescent secondary antibodies and Hoechst, S6 phosphorylation was quantified using Cellomics ArrayScan® software. Average values per cell following treatment with ZK 75, PI-103 and PIK-108 are shown. (**C**) TGX-286, PIK-108, PIK-75, PI-103, PIK-85, PIK-90 or PIK124 (each at 1 μM) was added to the indicated cell lines for 24, 48 or 72 h (replacing media with fresh compound every 24 h). Cells were fixed and permeablized, and cell numbers were quantified using Cellomics ArrayScan® software by Hoechst fluorescence. Numbers are expressed as a percentage of untreated cells, and colour-coded for degree of inhibition

line, S6 phosphorylation rapidly recovered to untreated levels following treatment with PIK-75, but remained inhibited by PI-103 up to 24 h (Figures 1C and 3B). This dissociation between PI3K inhibition and cell number suggests that PIK-75 decreased cell numbers due to effects on targets other than PI3K and is consistent with selectivity data for PIK-75 reported previously [17].

The effects of PI3K inhibitors on cell number were performed on eight of the ten cell lines at 1 μM (it was difficult to obtain consistent cell number data for MCF-7 and BT474 cells

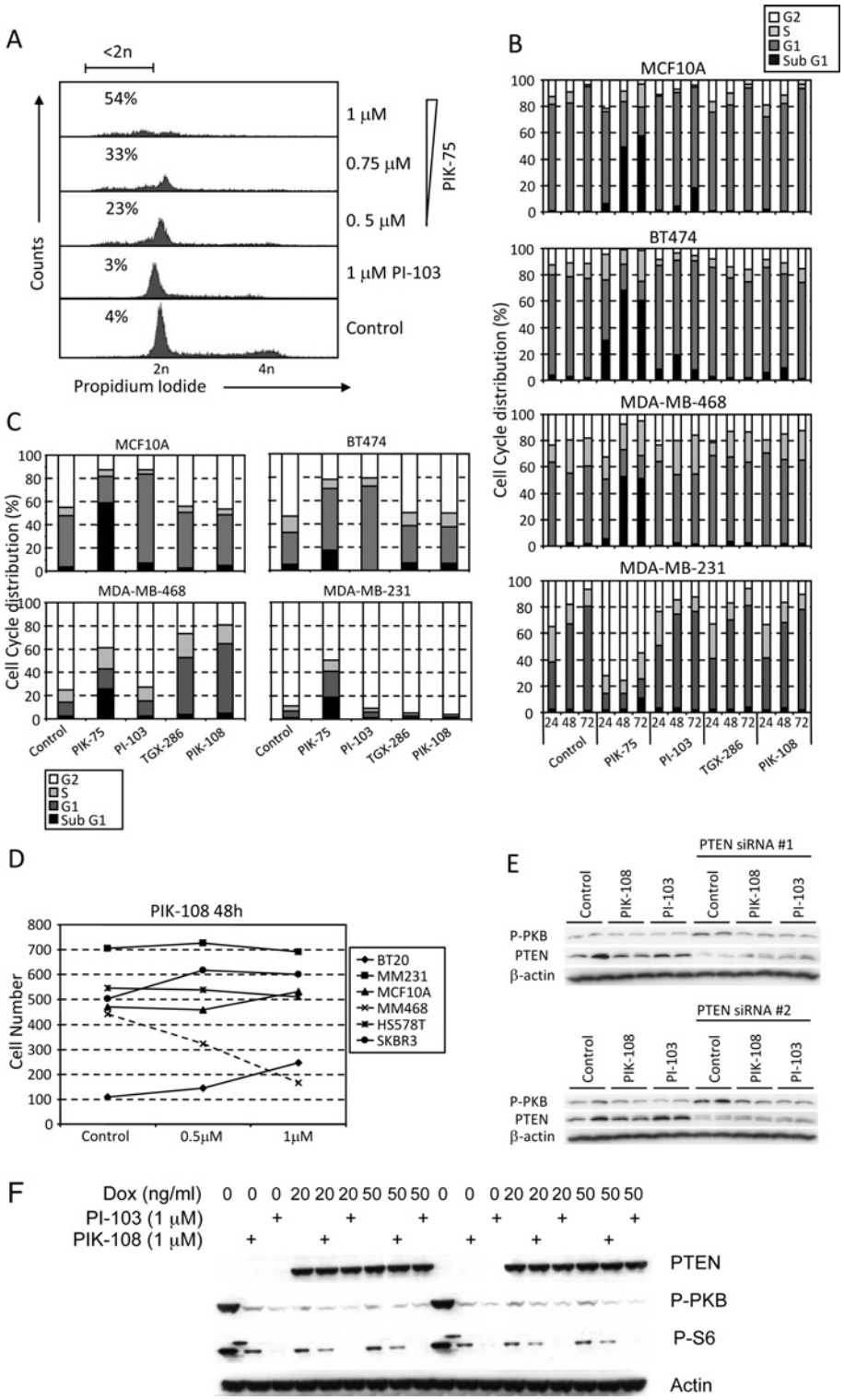

Figure 4 Effects of PI3K-isoform selective inhibitors on cell-cycle progression in breast cancer cell lines; loss of PTEN induces sensitivity to p110β inhibition

(**A**) SKBr3 cells were grown in six-well dishes and treated with the indicated concentrations of PIK-75 or PI-103. After 24 h, cells were fixed, permeablized and treated with propidium iodide (10 μg/ml) and RNase A (0.1 mg/ml). A total of 10000 cells were analysed for DNA content by flow cytometry, and the proportion of cells showing less than 2*n* DNA content (diploid) is indicated. (**B**) MCF-10A, BT474, MDA-MB-468 and MDA-MB-231 cells were grown in six-well dishes and treated with 1 μM PIK-75, PI-103, TGX-286 or PIK-108 for the indicated time periods (24, 48 and 72 h, replacing medium with fresh compound every 24 h). Cells were fixed, permeablized and treated with propidium iodide (10 μg/ml) and RNase A (0.1 mg/ml), and 10000 cells were analysed for DNA content by flow cytometry. The proportion of cells showing less that 2*n* DNA content is indicated in black, 2*n* DNA content is indicated in dark grey, 4*n* DNA (tetraploid) content is indicated in light grey, and $2n <$ DNA content $< 4n$ is indicated in white. (**C**) MCF-10A, BT474, MDA-MB-468 and MDA-MB-231 cells were grown in six-well dishes and treated with 1 μM of the indicated compounds for 24 h. The medium was then replaced with 1 μM fresh compound in the presence of nocodazole (70 ng/ml) for a further 24 h. Cells were fixed, permeablized and treated with propidium iodide (10 μg/ml) and RNase A (0.1 mg/ml), and 10000 cells were analysed for DNA content by flow cytometry. Shading for DNA content is the same as in (**B**). (**D**) The indicated cell lines were grown in 96-well dishes and treated with 0.5 or 1 μM PIK-108. After 48 h (replacing medium with fresh compound every 24 h) cells were fixed, permeablized and treated with Hoechst for analysis of cell number.

owing to cell losses incurred by the washing and fixation protocol). Whereas PIK-75 consistently showed the greatest ability to induce loss of cell number in all cells examined, the responses to other compounds were more heterogeneous. MDA-MB-468, MCF-10A, BT20 and T47D cells displayed time-dependent inhibition of cell number with several compounds, whereas MDA-MB-231, Hs578t, SKBr3 and BT549 were more resistant (Figure 3C). In some cases, the pattern of cell number inhibition displayed properties reflective of biochemical sensitivity and pharmacodynamic properties. For example, MDA-MB-468 (p110β inhibitors), MCF-10A, BT20 and T47D cell lines displayed potent and sustained biochemical inhibition of their respective p110 inhibitors (Figure 1C), whereas SKBr3 cells showed transient inhibition and MDA-MB-231 cells showed less potent biochemical inhibition (Figure 1C), and are among the most biologically resistant cell lines (Figure 3C). Exceptions to these observations include BT549 and Hs578t, which both showed strong and sustained inhibition by PI-103, yet were resistant to cell number decreases in response to this compound. Hs578t cells harbour an activating mutation in H-Ras, and will be discussed later.

p110 isoform-selective inhibitors differentially induce cell death and/or cell-cycle changes in breast cancer cell lines

Although changes in cell number analysed by automated cell counting were a useful indication of the differential effects on different cell lines, we found the results to be somewhat variable, and, in addition, they do not give any suggestion as to the underlying cause of cell number decreases. We therefore next analysed the responses to p110 inhibitors in more detail using flow cytometry. As mentioned above, PIK-75 and PI-103 are potent inhibitors of p110α and reduce PKB/Akt and S6 phosphorylation, although PIK-75 is much more efficient at reducing cell number compared with PI-103 (Figure 3). Using flow cytometry in SKBr3 cells, PIK-75 induced a significant sub-G_1 population (Figure 4A), consistent with the induction of apoptosis. Equivalent and even higher concentrations of PI-103 did not induce such an effect. These data support the concept that the potent effects of PIK-75 in reducing cell number are due to apoptosis, which occurs in a PI3K-independent manner.

We proceeded to perform further flow cytometry analyses on MCF-10A, BT474, MDA-MB-231 and MDA-MB-468 cells over 24, 48 and 72 h with PIK-75, PI-103, TGX-286 or PIK-108 (Figure 4B). Similarly to the effects seen in SKBr3 cells, PIK-75 induced a large sub-G_1 population that occurred rapidly in BT474 cells, and was more delayed in MCF-10A and MDA-MB-468 cells. In contrast, PIK-75 caused a strong accumulation of cells with a 4*n* DNA content (tetraploid) in MDA-MB-231 cells. PI-103, TGX-286 and PIK-108 did not show strong effects on DNA content in these cells under these conditions.

In order to determine any potential additional effects on the cell cycle of TGX-286, PIK-108, PIK-75 and PI-103 cells, we used nocodazole (a microtubule depolymerization agent which synchronizes cells in G_2) following addition of compound. This protocol accentuates any effects on G_1 arrest by arresting the cells at the G_2/M transition, due to destabilized microtubules. In contrast, cells arrested in G_1 by p110 inhibitors will not transition through to G_2. These studies revealed that PI-103 induced a G_1 arrest in BT474 and MCF-10A cells, although not in MDA-MB-231 cells or in MDA-MB-468 cells (Figure 4C).

The PTEN mutant cells MDA-MB-468 and BT549 were the only cell lines in the present study in which PKB/Akt phosphorylation was inhibited by the addition of the p110β-selective compounds TGX-286 and PIK-108 (Figure 1). Strikingly a G_1 arrest was uniquely observed in MDA-MB-468 cells after TGX-286 and PIK-108 treatment, supporting further the finding that these cells are sensitive to p110β inhibition (Figure 4C). Interestingly, MDA-MB-468 cells also experienced a dose-dependent reduction in cell number upon addition of these compounds, whereas five other cell lines remained broadly unaffected by TGX-286 and PIK-108 (Figure 4D). BT549 cells, however, which also exhibit biochemical sensitivity to TGX-286 and PIK-108, did not exhibit these responses to cell number, although this could be the result of lower sensitivity when compared with MDA-MB-468 cells (Figure 1, and results not shown). We tested the hypothesis that loss of PTEN may induce sensitivity to p110β inhibition by using siRNA to PTEN in the biochemically resistant line, MDA-MB-231, which induced an increase in PKB phosphorylation, as expected. Moreover, this increase was abrogated following addition of PIK-108 or PI-103 (Figure 4E). To determine whether the converse was also true, namely that expression of PTEN in a *PTEN*-null cell line would make these cells resistant to p110β inhibitors, we used MDA-MB-468 cells expressing a doxycycline-inducible PTEN. Unfortunately, under these conditions, expression of PTEN almost fully inhibited PKB/Akt Ser^{473} phosphorylation (while having relatively minor effects on phospho-S6 phosphorylation), making conclusions difficult to draw (Figure 4F).

Ras mutations are an important determinant of response to PI3K inhibitors

We next explored the observations that MDA-MB-231 cells were insensitive to cell-cycle changes in response to PI-103 addition (Figure 4C) and that Hs578t cells were insensitive to cell number inhibition across a range of PI3K inhibitors (Figure 3A), despite responding biochemically to these compounds (Figure 1). Both of these cell lines possess Ras mutations (K-Ras and H-Ras respectively) [35,36]. We therefore tested whether activation of pathways downstream of Ras were responsible for resistance to PI3K inhibitors in these cells, and whether inhibition of these pathways could co-operate with inhibition of PI3K. Analysis of DNA content using the nocodazole protocol in both of these cell lines showed a marked insensitivity to the p110α/p110β inhibitor PI-103, in contrast with the WT Ras-expressing cell lines BT474, BT20 and MCF-10A (Figure 5A). PIK-108 was also sufficient to cause a partial G_1 arrest in the PTEN mutant cell line MDA-MB-468, as seen previously. Inhibition of MEK (using U0126) caused a G_1 arrest in all cell lines examined, although the extent was variable (Figure 5A). Strikingly, in the mutant Ras cell lines, U0126 and PI-103 caused a synergistic effect on G1 arrest (Figure 5A). Furthermore, since PI-103 has been shown to have potent activity against mTOR [17], we tested the extent to which PI-103 was acting through inhibition of PI3K or mTOR. Addition of rapamycin to MDA-MB-231 or Hs578t cells was ineffective

(**E**) MDA-MB-231 cells were cultured in six-well dishes as described in the Experimental section. Two rounds of transfection of PTEN siRNA was performed as indicated on consecutive days and as described in the Experimental section. PIK-108 or PI-103 (each at 1 μM) was added to the culture medium as indicated and 1 h later cells were harvested into lysis buffer. Proteins were separated by SDS/PAGE, and Western blotted using antibodies against phospho-PKB/Akt (Ser^{473}) (P-PKB), PTEN and β-actin. Proteins were visualized by horseradish-peroxidase-coupled secondary antibodies and ECL®. (**F**) MDA-MB-468 cells expressing doxycyclin-inducible PTEN were cultured in DMEM with 10% (v/v) FBS in six-well dishes, and treated with either doxycycline (Dox) or solvent (methanol) for 24 h. PIK-108 and PI-103 were added (to 1 μM final concentration) for 1 h, and then cell lysates were harvested. Lysates were separated by SDS/PAGE, and Western blotted using the indicated antibodies. Lanes 1–9 and 10–18 represent two duplicate samples. P-PKB, phospho-PKB/Akt; P-S6, phospho-S6.

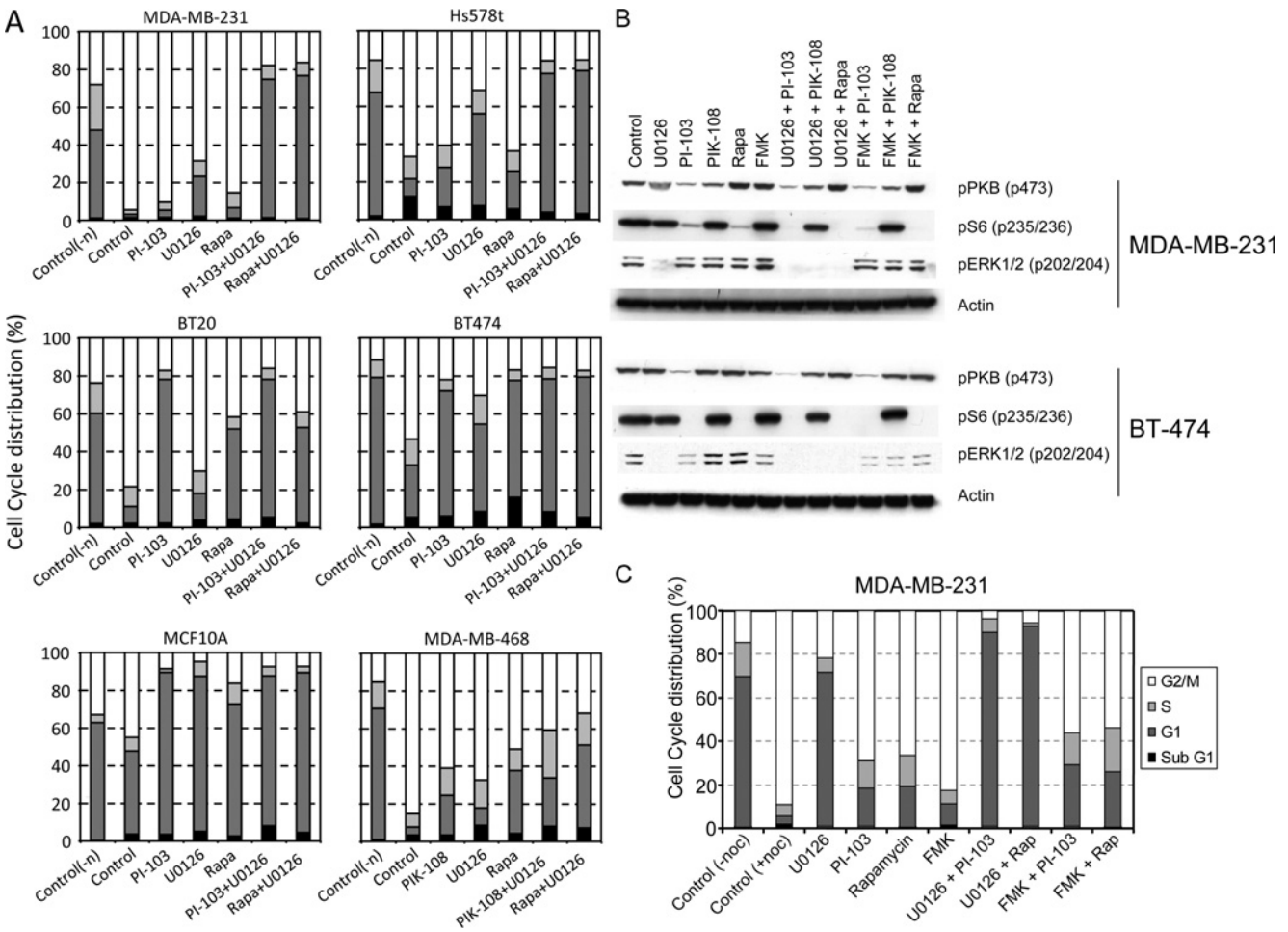

Figure 5 Effects of combined PI3K isoform-selective inhibitors and MEK inhibition on cell-cycle progression in breast cancer cell lines

(**A**) MDA-MB-231, Hs578t, BT20, BT474, MCF-10A and MDA-MB-468 cells were grown in six-well dishes and treated with the indicated compounds for 24 h (1 μM PI3K inhibitor, 10 μM U0126 and 100 nM rapamycin as indicated). Then the medium was replaced with fresh compound at the same concentration in the presence of nocodazole (70 ng/ml) for a further 24 h. Cells were fixed, permeabilized and treated with propidium iodide (10 μg/ml) and RNase A (0.1 mg/ml), and 10000 cells were analysed for DNA content by flow cytometry. Shading for DNA content is the same as in Figure 4(B). Rapa, rapamycin. (**B**) MDA-MB-231 cells and BT-474 cells were treated with the indicated compounds for 1 h and then harvested for cell lysates. Proteins were separated by SDS/PAGE, and analysed for phosphorylation by Western blotting with the indicated antibodies. pPKB (p473), phospho-PKB (Ser^{473}); pS6 (p235/236), phospho-S6 (Ser^{235}/Ser^{236}); pERK1/2 (p202/204), phospho-ERK1/2 (Thr^{202}/Tyr^{204}); Rapa, rapamycin. (**C**) MDA-MB-231 cells were treated as in (**A**), but with the inclusion of 1 μM FMK alone or in combination with 1 μM PI-103 or 100 nM rapamycin for 24 h before the addition of 70 ng/ml nocodazole for a further 24 h. FACS analysis was performed as described in Figure 4(B), and the proportion of cells in the indicated phases of the cell cycle is shown.

at causing a G_1 arrest, similarly to PI-103. In contrast, rapamycin caused a G_1 arrest (to varying degrees) in all cells expressing WT Ras. Rapamycin also synergized with U0126 in the mutant Ras-expressing cell lines, and caused a G_1 arrest identical with that seen with the combination of U0126 and PI-103. Such synergy was not apparent in BT20 or MDA-MB-468 cells. This synergy was also not apparent in BT474 or MCF-10A cells, although this is due to the fact that either compound alone was very effective in these cells. Therefore the status of Ras mutations is likely to play an important role in determining resistance to PI3K inhibition, but this resistance can be reversed using MEK inhibitors.

There was also a discrepancy in the inhibition of S6 phosphorylation in Ras mutant compared with WT Ras cell lines. Although rapamycin was sufficient to fully inhibit S6 phosphorylation in the WT Ras-expressing cell lines BT-474 (Figure 5B) and BT20 (results not shown), there was residual phosphorylation of S6 in the mutant Ras-expressing MDA-MD-231 cell line. We hypothesized that this could be due to a Ras-driven ERK/$p90^{rsk}$ contribution to S6 phosphorylation in MDA-MB-231 cells, similar to that seen in cells lacking $p70^{S6K}$ [31], and in HEK-293E (human embryonic kidney) cells expressing N-Ras Q61L or stimulated with phorbol esters [32]. Consistent with this, the combination of U0126 with PI-103 or rapamycin completely abolished S6 phosphorylation in these cells. Similarly, the selective $p90^{rsk}$ inhibitor FMK (fluoromethylketone) [37] also co-operated with U0126 or rapamycin in reducing S6 phosphorylation (Figure 5B). However, FMK was not sufficient to cause any effect on cell-cycle arrest, nor did it co-operate with PI-103 or rapamycin (Figure 5C).

To determine whether expression of mutant Ras was sufficient to cause partial resistance to the ability of PI-103 and rapamycin to inhibit S6 phosphorylation, we stably expressed K-RasG12D in BT20 cells. Although the expression levels of exogenous mutant K-Ras were quite low relative to endogenous Ras isoforms, they were sufficient to elevate ERK phosphorylation under starved conditions (Figure 6A). Expression of K-RasG12D was also sufficient to cause a

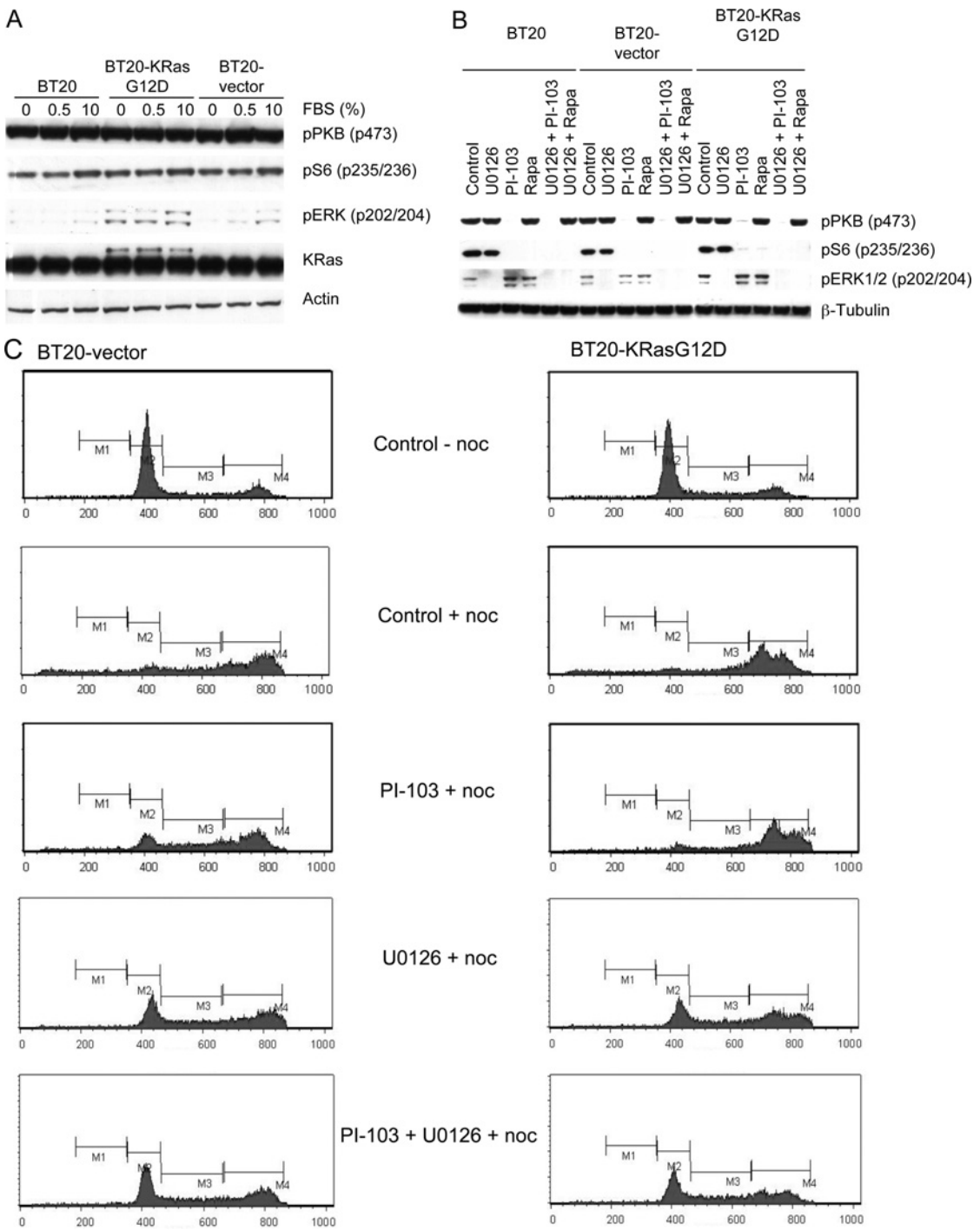

Figure 6 Expression of mutant Ras results in signalling to S6 phosphorylation and resistance to G_1 arrest by PI3K inhibitors

(**A**) BT-20 cells were infected with retroviruses expressing empty vector or K-RasG12D, and selected in neomycin for 4 weeks. Parental, vector or K-RasG12D-expressing cells were serum-starved overnight, and then stimulated with 0.5 or 10% FBS for 1 h and then harvested for cell lysates. pPKB (p473), phospho-PKB (Ser^{473}); pS6 (p235/236), phospho-S6 (Ser^{235}/Ser^{236}); pERK1/2 (p202/204), phospho-ERK1/2 (Thr^{202}/Tyr^{204}). (**B**) Cells from (**A**) were treated with the indicated compounds for 1 h and then harvested for cell lysates. Proteins were separated by SDS/PAGE and analysed for phosphorylation by Western blotting with the indicated antibodies. (**C**) The cell lines shown were treated with the indicated compounds for 24 h, and treated as described for Figure 5(A). noc, nocodazole (70 ng/ml).

minor resistance to inhibition of S6 phosphorylation following treatment with either PI-103 or rapamycin (Figure 6B). Moreover, expression of K-RasG12D reduced the G_1 arrest induced by treatment with PI-103, although an additive or synergistic interaction with U0126 was not apparent from these experiments (Figure 6C).

DISCUSSION

The data of the present study describe the biochemical and biological effects of PI3K isoform-selective small-molecule inhibitors on a panel of breast cancer cell lines. We demonstrate that these are highly effective at inhibiting the PI3K pathway, at

inhibiting cell number and inducing cell death and/or cell-cycle arrest, in a cell-type-dependent manner depending on distinct genetic characteristics.

The importance of increased PI3K signalling in breast cancer is highlighted by the finding that p110α is mutated at high frequency in this disease [9–11]. In particular, these have been shown to be activating mutations, making this isoform of great interest with regard to the usefulness of p110α-selective small molecules [6]. The p110α mutation status in the breast cancer cell lines studied in the present study has been determined, both by the Sanger Institute (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=mutations&ln=PIK3CA&sn=breast&start=1&end=1069&coords=AA:AA&neg=off&page=1) and by Saal et al. [12]. BT20, BT474, T47D and MCF-7 contain p110α mutations, and BT549, Hs578t, MDA-MB-468, MDA-MB-231 and SKBr3 express WT p110. We show that, although p110α inhibitors are generally effective at the inhibition of PI3K signalling both biochemically and biologically, there was no clear increase in sensitivity seen in cell lines expressing mutant p110α. Moreover, it was also not obvious that cells expressing mutated p110α showed increased basal phosphorylation of either PKB/Akt or S6. A possible explanation for this is that, although four of the ten cell lines showed p110α mutations, all of the cancer cell lines analysed had mutations in p110α, PTEN, Ras or amplification of ErbB2, all of which could cause elevated PI3K pathway activity. Another explanation attests to whether the presence of a particular genetic alteration automatically implies that the altered gene is a good target for therapy. Although this has been proved true for oncogenes such as bcr-Abl and kit [38], current evidence suggests that mutations in EGFR may not predict response to EGFR inhibitors in lung cancer [39,40], and B-Raf mutations in melanoma do not currently appear to predict response to sorafenib [41], a compound which inhibits B-Raf activity.

It should be noted that MCF-10A cells, which are karyotypically normal and are not transformed, were equally sensitive to p110α inhibitors, and were in fact the most sensitive to MEK inhibitors, as measured by flow cytometry. p110α inhibitors were also recently shown to inhibit glucose uptake in non-transformed adipocytes and myotubes [17]. It remains unclear whether strong effects on 'normal' cells in culture will translate into systemic toxicity in the clinic.

Loss of PTEN is well documented to be associated with a poor clinical outcome in breast cancer [42,43]. The unique sensitivity of MDA-MB-468 and BT549 cells to the p110β-selective inhibitors TGX-286 and PIK-108 is therefore of particular interest. These are the only cell lines studied which respond to these compounds when examining PKB/Akt and S6 phosphorylation. In addition, these compounds also cause a G_1 arrest in MDA-MB-468 cells. Therefore, whereas 'normal' and WT PTEN-expressing tumour cells are equally sensitive to p110α inhibitors (with the exception of cell lines expressing mutant Ras, discussed below), PTEN-mutant tumour cells may become more dependent on additional isoforms such as p110β. This correlation was extended to an isogenic system in which knockdown of PTEN in MDA-MB-231 cells using siRNA was sufficient to confer responsiveness to p110β inhibitors. It is unclear why PTEN-mutated/deleted cells show a particular isoform-dependence, although cellular localization of PTEN and particular p110 isoforms could play a role. In this regard, it has been reported that both PTEN [44] and p110β [45] can be localized to the nucleus. Although somewhat preliminary, these results may suggest a window of therapeutic opportunity in tumours expressing mutant PTEN to p110β inhibition. Interestingly, a particular reliance of p110β in prostate tumour cells has been noted previously by other investigators [46]. PC3 cells, which express a mutant form of PTEN, show a high basal level of PKB/Akt phosphorylation, which is reduced upon inhibition of p110β expression, but not p110α. p110β inhibition also resulted in inhibition of invasive growth through matrigel in these cells, in contrast with p110α inhibitors [46]. Although PTEN mutations are relatively rare in breast tumours, decreases in PTEN levels are more frequent and have been shown to have prognostic significance in this disease. Tumours that have low levels of PTEN without mutations may therefore also be candidates for p110β inhibition. In addition, preliminary experiments in prostate cancer (results not shown) and glioblastoma [47] cell lines show a similar correlation between PTEN mutations and sensitivity to p110β inhibitors. These tumours display a greater incidence of PTEN mutations and may represent a wider arena for this class of drugs.

Distinct pharmacodymamic responses were observed among the inhibitors tested and the cell lines studied. Although we have not analysed the stability of these compounds, their structures do not suggest any aqueous instability, and most are effective over a 72 h period in at least one or more cell lines. In addition, other cellular screens have shown phenotypes consistent with inhibition of their respective targets [17]. One explanation for such heterogeneous pharmacodynamic responses could be that different cell lines metabolize drugs at differing rates. An alternative explanation could be that different cell types have the ability to switch their dependence for a particular PI3K isoform after one is inhibited. These hypotheses could be tested by re-adding either the same compounds and/or different ones at later time points to see whether this is sufficient to reduce the restored PKB/Akt phosphorylation. Given that PI-103 also inhibits p110β, one might have expected this compound to also induce a G_1 arrest in MDA-MB-468 cells. However, pharmacodynamic data from Figure 2 show that, in MDA-MB-468 cells, the effects of PI-103 on PI3K inhibition are considerably more transient when compared with the effects of TGX-286 and PIK-108, which could explain this apparent discrepancy. These data illustrate further the importance of pharmacodynamics in drug responses and highlight the need for reliable pharmacodynamic markers in treated patients.

The finding that Hs578t cells are relatively resistant in terms of cell proliferation to the drugs studied, whereas PI3K signalling is inhibited, indicates that these cells can dispense with PI3K signals for cell proliferation. One explanation for this could be that these cells have activation of parallel pathways, which are able to compensate for the loss of PI3K signals. For example, Hs578t cells are known to express activated forms of the H-Ras oncogene [36]. Another resistant cell line (both biochemically and biologically) is MDA-MB-231 which is known to harbour K-Ras mutations [35]. The clinical importance of these observations is apparent from the finding that Ras mutations confer resistance to additional signal transduction inhibitors such as anti-EGFR therapies [39,48]. Importantly, in these two breast cancer cell lines examined, the resistance to PI3K inhibitors could be overcome by the simultaneous addition of a MEK inhibitor. Part of the reason for this could be the additional input to S6 Ser^{235} and Ser^{236} phosphorylation through the MEK–ERK–$p90^{rsk}$ pathway seen in the context of activated K-Ras. This observation has been seen previously in HEK-293E cells expressing activated N-Ras [32]. However, this residual phosphorylation could also be abolished using the $p90^{rsk}$ inhibitor FMK, without having any large effect on cell-cycle profile, suggesting that additional MEK-dependent pathways downstream of Ras are important for this. Therefore the rational combination of targeted therapies in the appropriate genetic contexts is likely to extend the patient populations that will respond to these agents. We have shown previously that high PKB/Akt phosphorylation predicts a poor response to EGFR

inhibitors in patients with glioblastoma [49]. These patients would therefore be candidates for combination therapy with a PI3K or PKB/Akt inhibitor. In support of this, a strong synergistic response between LY294002 and gefitinib was demonstrated previously in glioblastoma xenograft tumours [50].

Although PIK-75 was very effective at killing all cell types examined, several lines of evidence suggest that this compound is unlikely to mediate its cell death responses through inhibition of PI3K. First, PI-103 is as potent at inhibiting p110α, but does not cause such rapid and potent inhibition of cell number. Secondly, PIK-75 results in inhibition of cell number in MDA-MB-231 cells, which are not biochemically affected by this compound. Thirdly, PIK-75 causes apoptosis, in contrast with PI-103 (as shown for SKBr3 cells; Figure 3B). Fourthly, in some cell lines, PIK-75 appears to be causing arrest of the cell cycle at G_2/M. This is in contrast with the G_1 arrest as seen with PI-103, and is also more typically seen with previous studies showing inhibition of PI3K [51]. Given the potent and somewhat selective ability of PIK-75 to induce cell death, the task of finding its additional cellular target(s) remains an interesting one.

The cell lines described in the present study may well reflect the behaviour of tumour subtypes with respect to p110 isoform inhibition. Our findings that some small molecules described are able to inhibit PI3K and induce cell-cycle arrest differentially among the breast cancer cell lines studied indicates that these compounds may be able to operate within a therapeutic window. The behaviour of sensitive and resistant cell lines described here in xenograft models will support further the validity of these compounds as therapeutics. Taken together, our data reinforce the importance of PI3K in maintaining normal cell growth and suggest that p110-selective drugs may be of value as targeted therapies.

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