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**Parallel RNAi and compound screens identify the PDK1 pathway as a target for
tamoxifen sensitisation**

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Running head: Tamoxifen sensitisation by inhibition of the PDK1 pathway

Key words: Tamoxifen, RNA interference screen, chemical screen, PDK1, triciribine,
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Synopsis

Tamoxifen is the most commonly used drug to treat breast cancer and acts by blocking estrogen receptor (ER α) signalling. Although highly effective, its usefulness is limited by the development of resistance. Given this, strategies that limit resistance by sensitising cells to tamoxifen may show utility in the clinic. To gain insight into how this might be achieved, we used chemical and genetic screens to identify targets and small molecule inhibitors that cause tamoxifen sensitisation. A high-throughput genetic screen, using an RNA interference library targeting 779 kinases and related proteins, identified the PDK1 signalling pathway as a strong determinant of sensitivity to multiple ER α antagonists including tamoxifen. A chemical screen using existing drugs and known kinase inhibitors also identified inhibitors of the PDK1 pathway, including triciribine and tetrandrine. Aside from identifying novel agents and targets for tamoxifen sensitisation, this approach also provides evidence that performing chemical and genetic screens in parallel may be useful.

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Introduction

Approximately 70% of breast tumours express estrogen receptor α (ER α), and of these, most are fully dependent on estrogen signalling for their growth and can therefore be treated with anti-estrogen therapies. Tamoxifen, the most commonly used anti-estrogen therapy, has shown significant benefit in the treatment of ER α positive breast cancer [1] and works by blocking ER α signalling in the breast. This ER α antagonism leads to the inhibition of cell cycle progression and arrest at the G₁ checkpoint [2, 3]. Despite its effectiveness, the utility of tamoxifen is limited by the development of drug resistance, which occurs in all patients with metastatic disease and approximately 40% of those with early onset breast cancer who are treated with this agent as an adjuvant to surgery [4, 5].

Considerable efforts have been made to identify the determinants of tamoxifen resistance and sensitivity. Primarily, this has been performed on a gene-by-gene basis and while this has been informative, a complementary approach is to perform high throughput simultaneous analysis of many genes and pathways. This can be achieved genetically by the use of approaches such as RNA interference (RNAi) screening [6], which relies on the ability of short-interfering (si)RNA to silence gene function in a sequence-specific fashion. We have used such a high-throughput RNAi screen to identify CDK10 as a novel determinant of tamoxifen resistance [7]. In addition to genetic screens, a separate approach is to use libraries of chemical inhibitors to interrogate cellular phenotypes such as tamoxifen response. Given that chemical inhibition of proteins may sometimes produce differing phenotypes to those achieved by gene silencing or knockout [8], the use of chemical screening has the potential to identify effects not achievable by genetic screening. Moreover, if the chemical libraries used include drugs in clinical use and with known pharmacokinetic and safety profiles, these screens offer the possibility of identifying effects that can be rapidly evaluated in phase II clinical trials, eliminating much of the toxicological and pharmacokinetic assessments required for novel compounds [9]. Additionally, the targets of existing drugs are often established, potentially allowing the mechanism of action to be determined more easily.

Given the relative benefits of genetic and chemical screens, we reasoned that parallel RNAi and inhibitor screens might be a useful approach to the identification of targets and inhibitors that cause sensitisation to tamoxifen. This proved to be the case and a number of novel targets were identified. Notably, multiple components of the PDK1 pathway were identified by the RNAi screen. Results from the chemical screen validated the results of the RNAi screen, therefore providing proof of the principle that genetic and chemical screens may indeed have complementary roles in target discovery.

Experimental

Cell lines, compounds and siRNA

MCF7 cells were obtained from ATCC (USA) and maintained in phenol red free RPMI 1640 (Invitrogen), supplemented with 10% dextran charcoal treated FCS (10% (v/v)), 1nM estradiol, insulin, glutamine and antibiotics. 4OH tamoxifen and estradiol were obtained from Sigma, ICI 182780 was obtained from Tocris Bioscience UK, triciribine was obtained from BIOMOL International, Akti-1,2 was obtained from Calbiochem. MCF7 cells were transfected with SMARTpool siRNAs using Dharmafect 3 transfection reagent according to manufacturer's instructions (Dharmacon). The kinase siRNA library (siARRAY – targeting 779 known and putative human kinase genes including protein kinases, lipid kinases, adaptors and related genes) was obtained in ten 96 well plates from Dharmacon (USA). Each well in this library contained a SMARTpool of four distinct siRNA species targeting different sequences of the target transcript. The compound library encompassed the Prestwick Chemical Library® (Prestwick Chemicals) and the Screen-Well™ library (BIOMOL International).

Antibodies

Antibodies targeting the following epitopes were used: p21^{CIP1} (556430, BD Biosciences, UK), phospho AKT^{Ser473} (4058, Cell Signalling, UK), AKT (9272, Cell Signalling, UK), and β -tubulin (T4026, Sigma, UK). All secondary antibodies used for western blot analysis were HRP conjugated.

siRNA screen method

The RNAi screening methodology has been described previously [7]. In brief, MCF7 cells plated in 96 well plates were transfected 24 hours later with siRNA (final concentration 100 nM), using Dharmafect 3 (Dharmacon, USA) as per manufacturer's instructions. Twenty-four hours following transfection, cells were trypsinised and divided into four identical replica plates. At 48 hours following transfection, two replica plates were treated with 50 nM 4OH tamoxifen in media and two replica plates with 0.05% (v/v) ethanol vehicle in media. Media containing 4OH tamoxifen or vehicle was replenished after 48 and 96 hours, and cell viability was assessed after seven days of 4OH tamoxifen exposure using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA) as per manufacturer's instructions. The luminescence reading for each well on a plate was expressed relative to the median luminescence value of all wells on the plate. The screen was completed in duplicate.

For each transfection the effect on cell growth and tamoxifen sensitivity were calculated. Cell growth: The effect of each individual siRNA SMARTpool on cell growth alone was calculated by dividing mean luminescence in the two replica wells treated with ethanol vehicle by mean luminescence of the replica wells transfected with siControl, and expressed as a percentage.

Cell growth effect of siRNA (%) = [mean luminescence (2 replica wells with siRNA)/ mean luminescence (20 replica well treated siControl)] x 100.

Tamoxifen sensitivity: Sensitivity to 4OH tamoxifen for each siRNA SMARTpool was assessed by calculating the surviving fraction following 4OH tamoxifen.

Surviving fraction = log₂ mean luminescence (2 replica wells with 4OH tamoxifen) – log₂ mean luminescence (2 replica wells with ethanol vehicle).

The surviving fractions were centred on the median surviving fraction of all 80 SMARTpools from one 96 well plate transfection, the results from all ten siRNA plates combined and results expressed as a Z score [10]. For the Z score the standard deviation of the screen was estimated from the standard deviation of all siControl wells.

Validation of gene silencing by siRNA

Validation of RNAi gene silencing was measured by real time quantitative RT-PCR (qPCR). MCF7 cells were transfected with siRNA, and RNA extracted 48 hours later with Trizol and phenol/chloroform extraction followed by isopropanol precipitation. cDNA was synthesized using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) with oligo dT as per the manufacturer's instructions. Assay-on-Demand primer/probe sets were purchased from Applied Biosystems (Foster City, CA) details of which are available on request. Real-Time qPCR was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems), with endogenous control GAPDH. Standard curves were calculated for all reactions with serial dilutions of siControl transfected cells to calculate reaction efficiency. Gene expression was calculated relative to expression of GAPDH endogenous control, and adjusted relative to expression in siControl transfected cells.

Small molecule screening

MCF7 cells were plated in 96 well plates. 24 hours later cells were treated with 10 μ M BIOMOL and Prestwick compound libraries, half the plates in combination with 50 nM 4OH tamoxifen in media and half with 0.05% (v/v) ethanol vehicle in media. Media containing compounds, 4OH tamoxifen or vehicle was replenished after 48 and 96 hours, and cell viability was assessed after seven days 4OH tamoxifen exposure using CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA) as per manufacturer's instructions. The luminescence reading for each well on a plate was expressed relative to the median luminescence value of all wells on the plate. The screen was completed in duplicate. For each replicate the effect on cell growth and tamoxifen sensitivity were calculated.

Cell growth: The effect of each individual compound on cell growth alone was calculated by dividing luminescence in the well treated with ethanol vehicle by luminescence of the replica wells treated with DMSO control, and expressed as a percentage.

Cell growth effect of compound (%) = [luminescence (well with compound) / mean luminescence (10 replica wells treated DMSO control)] x 100.

Tamoxifen sensitivity: Sensitivity to 4OH tamoxifen for each compound was assessed by calculating the surviving fraction following 4OH tamoxifen.

Surviving fraction = \log_2 luminescence (well treated with 4OH tamoxifen) - \log_2 luminescence (wells treated with ethanol vehicle).

The surviving fractions were centred on the median surviving fraction of all 80 compounds from each plate and results expressed as a Z score [10]. For the Z score the standard deviation of the screen was estimated from the standard deviation of all DMSO control wells.

Cell viability assays to measure drug sensitivity

MCF7 cells were transfected with siRNA using Dharmafect 3 as per the manufacturer's instructions, divided 24 hours later into 96 well plates and exposed to various doses of drug from 48 hours post transfection. Cell viability was assessed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA) at nine days post transfection and surviving fraction for each dose of drug assessed by dividing the luminescence value of drug treated wells by the luminescence value of vehicle treated wells.

The median effect/combo index (CI) analysis [11] was used to determine synergy of combination exposures to inhibitors. Cell cultures were treated with each agent individually at its IC₅₀ concentration and at fixed multiples (two and four times) and fractions (0.50, 0.25 and 0.125) of the IC₅₀ concentrations. The inhibitors were also combined in these same dose-fixed ratios to determine CI. Synergy was defined as any CI value below 1. Experiments were performed in triplicate, and each experiment yielded CI values for each survival fraction. Experiments that yielded an average CI value of less

than 1 were repeated at least three times to allow for determination of confidence intervals for the CI values obtained.

Protein analysis

MCF7 cells were transfected with siRNA using Dharmafect 3 as per the manufacturer's instructions. After 24 hours the media was refreshed with no estradiol, 1 nM estradiol, or 1 nM estradiol with 100 nM 4OH tamoxifen. Protein lysates were prepared after 48 hours using RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 0.1% (v/v) DOC, 1% (v/v) TritonX-100, 50 mM NaF, 1 mM sodium vanadate and protease inhibitors). 100 µg of total cell lysate was loaded onto prefabricated 4-12% Bis-Tris gels (Invitrogen), with full range rainbow molecular weight marker (GE Healthcare, UK) as a size reference, and resolved by electrophoresis. Proteins were transferred to nitrocellulose membrane (Bio-Rad, USA), blocked and probed with primary antibody diluted 1 in 1000 in 1 x TBS-T with 5% (w/v) BSA overnight at 4°C. Secondary antibodies were diluted 1 in 5000 in 1 x TBS-T with 5% (w/v) marvel and incubated for one hour at room temperature. Protein bands were visualised using ECL (GE Healthcare, UK) and MR or XAR film (Kodak).

Fluorescence Activated Cell Scanning (FACS) analysis

MCF7 cells were transfected with siRNA using Dharmafect 3 as per the manufacturer's instructions. After 24 hours the media was refreshed with 1 nM estradiol, or 1 nM estradiol with 100 nM 4OH tamoxifen. After 48 hours the cells were fixed in 70% (v/v) ice cold ethanol and stained with 4% (w/v) propidium iodide (PI) and 10% (w/v) RNase A in PBS. The sample readout was performed on the FACSCalibur (Becton Dickinson, USA) and the data was analysed using CellQuest Pro (Becton Dickinson, USA).

Results

We recently reported the results of a high throughput RNAi screen to identify determinants of tamoxifen sensitivity [7]. In this screen, we targeted 779 kinases and related proteins by RNAi and measured changes in the cellular response to tamoxifen, as assessed by cell viability. We elected to screen this protein family as kinases represent targets that are amenable to inhibitor development. Furthermore, we reasoned that this approach would increase the likelihood that agents already developed could be used to inhibit targets identified in our screen. In brief, the ER α positive, tamoxifen sensitive, human breast cancer cell line, MCF7, was transfected with short-interfering (si)RNA in a 96 well-plate format and then divided the day after transfection into four replica plates. Forty-eight hours after transfection, two replica plates were treated with 4OH tamoxifen (the active metabolite of tamoxifen) and two with vehicle (Figure 1A). The screen was repeated in duplicate and comparison of data from each screen revealed this approach to be highly reproducible. Data from the duplicate screens was combined in the final analysis (Figure 1B).

In our initial report, we focused upon RNAi effects that caused resistance to tamoxifen and established that CDK10 silencing could cause resistance, an effect most likely explained by an upregulation of MAPK signalling [7]. Here, we used the same screen data to identify tamoxifen sensitisation effects. The most sensitising siRNA SMARTpools are listed in Table 1. As a complementary approach to this RNAi identification of sensitisation effects, we also screened a library of 1200 drugs and kinase inhibitors to identify tamoxifen sensitisers. This chemical screen involved treating MCF7 cells with either 4OH tamoxifen or vehicle (EtOH) in combination with the compounds, for seven days before cell viability was assessed. The screen was repeated in duplicate and comparison of data from each screen revealed this approach to be highly reproducible. Data from the duplicate screens was combined in the final analysis (Figure 1C). Of the compounds causing sensitisation to tamoxifen, the 10 most significant results are listed

in Table 2. As a mark of the validity of the chemical screen, tamoxifen itself was identified as causing a sensitising effect.

By cross-referencing the results of the RNAi and compound screens we identified the PDK1 intracellular signalling pathway [12] as an important target for tamoxifen sensitisation. Seven of the 20 most sensitising hits identified from the RNAi screen were components of the PDK1 signalling pathway as depicted in Figure 2A. In addition, all of the top 10 most sensitising hits identified from the compound screen, with the exception of betulin, significantly inhibited the PDK1 signalling pathway, as demonstrated by inhibition of phosphorylation of the well-characterised PDK1 substrate AKT (Figure 2B). The most potent inhibitor of PDK1 signalling identified from the compound screen, triciribine, was studied further along with PDK1 siRNA. We confirmed that PDK1 siRNA as well as triciribine inhibited AKT activation, as measured by phosphorylation (Figure 2C,D; Supplementary Tables 1, 2). Furthermore, we confirmed that targeting the PDK1 pathway could sensitise to tamoxifen. This was achieved by performing cell survival assays with differing combinations of tamoxifen and an AKT inhibitor (Akti1,2) that is structurally unrelated to triciribine. To assess true synergy, the survival data was analysed using the median effect model [13] that estimates the Combination Index (CIs) of two compounds. CIs of less than 1 are indicative of synergy and those greater than one indicate antagonistic activity [13]. Tamoxifen and Akti1,2 combinations gave a mean CI of 0.41 (Figure 2E), indicating that inhibition of the PDK1 signalling pathway was synergistic with tamoxifen.

In the high-throughput RNAi screen we used pools of siRNA (SMARTpools) each consisting of four individual siRNA targeting one gene. To assess the possibility the tamoxifen sensitisation effect caused by PDK1 silencing in the high-throughput screen was the result of off-target effects [14], we confirmed gene silencing of PDK1 by the individual siRNA contained within the individual SMARTpool (Figure 3A). We also demonstrated that each of these siRNAs were able to elicit tamoxifen sensitisation. In contrast to the single dose of tamoxifen used in the parallel screens, we assessed the effect of individual PDK1 siRNAs over a range of tamoxifen doses (Figure 3B). This analysis indicated that the PDK1 siRNA effect observed in the screen was unlikely to be the result of an off target siRNA effect. We also validated the effect of triciribine over a range of tamoxifen doses (Figure 3C). This demonstrated that triciribine increased sensitivity to tamoxifen seven fold (DMSO SF₅₀ = 8.5 nM, Triciribine SF₅₀ = 1.2 nM). Finally we confirmed tamoxifen/triciribine synergy by use of the median effect model [13]. Triciribine and tamoxifen gave a mean CI of 0.39, indicative of synergy.

Estrogen signalling can be targeted by pharmacological agents other than tamoxifen and we assessed the possibility that the tamoxifen sensitisation effects observed with triciribine and PDK1 silencing were also applicable to other forms of endocrine therapy. All four siRNA targeting PDK1 sensitised cells to ICI 182780 (fulvestrant), a drug that induces ER α degradation [15] (Figure 4A). Furthermore, combinations of triciribine and ICI 182780 also showed clear synergy (Figure 4B). The action of aromatase inhibitors may be modelled by estrogen deprivation [16] and either PDK1 siRNA or triciribine sensitised cells to estrogen deprivation (Figure 4C,D). Taken together with the observations using tamoxifen and ICI 182780, this suggested that suppression of the PDK1 pathway sensitises to inhibition of ER α signalling generally, rather than to tamoxifen specifically.

To understand the role of PDK1 in tamoxifen response, we examined whether the induction of cell cycle arrest at the G₁ checkpoint caused by tamoxifen [17] could be enhanced by PDK1 silencing. In the absence of tamoxifen, transfection of PDK1 siRNA did not grossly alter the cell cycle profile of cells, compared to cells transfected with a control siRNA (compare siRNA PDK1 G₀/G₁ = 54.4 %, S = 6.0 %, G₂/M = 14.8 % and

control siRNA $G_0/G_1 = 58.5\%$, $S = 4.2$, $G_2/M = 15.7\%$, Figure 4E). Upon addition of tamoxifen, the G_0/G_1 proportion of cells was modestly increased in control siRNA transfected cells, from 58.5% to 63.7%, consistent with arrest at the G_1 checkpoint (Figure 4E,F). In PDK1 siRNA transfected cells this tamoxifen-induced increase in G_0/G_1 cells was more profound, rising from 54.4% before tamoxifen treatment to 84.7% after, suggesting that PDK1 silencing exacerbated tamoxifen-induced G_1 arrest. This increase in G_1 arrest was also mirrored by a decrease in cells in the G_2/M phase of the cell cycle (Figure 4E,F). Tamoxifen is thought to induce cell cycle arrest by causing the up-regulation of cyclin dependent kinase inhibitor proteins, including p21^{CIP1} [2, 18]. p21^{CIP1} protein levels are also known to be downregulated in an AKT phosphorylation-dependent mechanism [19, 20]. Consistent with these observations, we found that p21^{CIP1} expression was significantly enhanced after tamoxifen treatment in PDK1 silenced cells (Figure 4G), suggesting a mechanism by which down-regulation of PDK1 could enhance G_1 arrest caused by tamoxifen.

In addition to triciribine, the chemical screen also identified sertraline, tetrandrine and methotrimeprazine maleate as non-toxic putative tamoxifen sensitisers (toxicity was defined as cell viability less than 0.5). To confirm the observations of the high-throughput screen and to establish the magnitude of the effect, dose response curves were performed over a range of 4OH tamoxifen concentrations. All of the compounds significantly increased sensitivity to tamoxifen (Supplementary Figure 1), confirming the results from the tamoxifen sensitisation drug screen. The most potent tamoxifen sensitiser was tetrandrine.

Tetrandrine, (1 β)-6,6',7,12-teramethoxy-2,2'-dimethyl-berbaman, is derived from the root of *Stephania tetrandra*, and has been used for cardiovascular diseases in traditional Chinese medicine for centuries [21]. Tetrandrine has also been used in animal and human studies, showing little toxicity alongside both anti-inflammatory and anti-neoplastic activity [21, 22]. To confirm the observations of the high-throughput screen, we performed dose-response curves and demonstrated that tetrandrine did indeed sensitise cells to tamoxifen, causing a 9.9 fold increase in sensitivity (DMSO SF₅₀ = 14.8 nM, 10 μ M tetrandrine SF₅₀ = 1.46 nM Figure 5A). As for triciribine, we used the median effect model to assess tamoxifen-tetrandrine synergy, which, although moderate, was confirmed (mean CI 0.72, Figure 5B). Recent work has demonstrated that tetrandrine is able to induce G_1 arrest by AKT inhibition [23] and we also observed moderate AKT inhibition by tetrandrine (Figure 2B). Given this, and in light of our data from triciribine and PDK1 silencing, we assessed whether tetrandrine was able to exacerbate tamoxifen-induced G_1 arrest. Examination of cell cycle profiles supported this hypothesis (Figure 5C,D). In cells treated with the tetrandrine vehicle, DMSO, tamoxifen induced a modest G_1 arrest (G_1 proportion rises from 32.9% to 37.1%). Conversely tamoxifen plus tetrandrine produced a more pronounced G_1 arrest (G_1 proportion rises from 38.4% to 54.6%).

Discussion

Here, we have performed chemical and genetic screens which reveal the potential for targeting the PDK1 pathway as a means to sensitise cells to tamoxifen. PDK1 is activated by phosphoinositide-3 kinase (PI3K) and serves as a master regulator of the AGC (cAMP-dependent or cGMP-dependent protein kinases and protein kinase C) family of protein kinases [12]. Interestingly, PDK1 is highly expressed in many human cancer cell lines [24] and breast tumours [25], suggesting a role for PDK1 in breast cancer tumorigenesis. However, few studies have evaluated PDK1 as a potential target for cancer therapy. Significantly, both our compound and RNAi screens identified components of the PDK1 pathway as targets for tamoxifen sensitisation.

Consistent with this, previous studies have shown that inhibition of the PDK1 activator, PI3K, can sensitise to endocrine therapies in both *in vitro* and *in vivo* models and that activation of the PDK1 substrate, AKT, causes resistance to tamoxifen in breast cancer models and tumours [26, 27]. However, the PI3K inhibitors used, such as wortmannin or LY294002 have poor clinical potential and are unlikely to be developed for clinical use [28]. Wortmannin has a short half-life [29] and LY294002 causes dermatitis in animal models [30]. Furthermore, both act non-specifically on other PI3K family members resulting in considerable toxicity [31]. For these reasons neither compound has been used in clinical trials despite their anti-tumour activities *in vitro* and *in vivo* [32]. The identification of triciribine from the compound screen is therefore significant as triciribine has previously shown promise as a potential anticancer drug in *in vitro* models [33] and preliminary clinical trials demonstrated that low concentrations of triciribine were non-toxic [34, 35], suggesting that combining low concentrations of triciribine with tamoxifen, fulvestrant or aromatase inhibitors in the clinic may be a relatively non-toxic approach for delaying the development of resistance. In addition to triciribine, tetrandrine was also identified as a potent sensitiser to tamoxifen. Tetrandrine has been used in animal and human studies with little toxicity and has shown both anti-inflammatory and anti-neoplastic activity [21, 22]. Previous studies have also identified tetrandrine as a sensitiser to a range of cancer therapies including doxorubicin [36], vincristine and ionizing radiation [37], although the mechanisms underlying these effects remains unknown. Importantly, this is the first study to identify tetrandrine as a sensitiser to tamoxifen.

Our study verifies the importance of PI3K signalling in the modulation of tamoxifen sensitivity, and is the first to identify PDK1 as a target for tamoxifen sensitisation. We discovered that silencing of PDK1 increases sensitivity to multiple endocrine therapies including tamoxifen, fulvestrant and estrogen deprivation. The observation that inhibition of PDK1 sensitises to a range of estrogen signalling inhibitors, suggests that the combination of PDK1 inhibitors with endocrine therapy merits investigation in the clinic. Although not currently in clinical use, PDK1 specific inhibitors have recently been developed and may be suitable for this purpose in the future [38].

Here we provide an example of how parallel RNAi and chemical screens performed in mammalian cells may complement each other [6]. Given the complexity of biochemical pathways and the number of protein-protein interactions now described for each of the proteins within a cell, deconvoluting the mechanism by which one RNAi screen hit controls a phenotype can be difficult. To this end, we have demonstrated that, by the use of a parallel chemical screen, it is possible to simultaneously validate data from an RNAi screen and suggest potential mechanisms by which RNAi hits determine a given cellular phenotype. It is also possible that RNAi screens could be used to partially deconvolute hits from parallel compound screens [6]. While *in vitro* small molecule screens are generally used to identify compounds that inhibit a validated protein target, the inhibitors identified may perform poorly in living cells. One solution is to perform small molecule inhibitor screens in cells, but this approach is also limited, as it requires the cellular targets of inhibitors to be identified, which can be challenging. By performing parallel RNAi and compound screens, small molecules and gene-specific siRNA reagents can be identified that cause similar cellular phenotypes, thus simplifying target identification. This proof-of-principle suggests that a combination RNAi/chemical approach in human cell lines could streamline the development of small molecules into drugs by improving compound target deconvolution [6].

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Tables

Table 1. Results of tamoxifen sensitisation siRNA screen. List of the top 20 sensitising siRNA SMARTpools from siRNA screen displayed in Z score order with siRNA SMARTpool target gene, full name, tamoxifen sensitivity Z score, and the effect of siRNA on cell viability in vehicle alone plates.

siRNA SMARTpool	Full name	Tamoxifen sensitivity Z score	Cell viability
CIT	Citron (rho-interacting, serine/threonine kinase 21)	-8.40	0.54
PRKCZ	protein kinase C, zeta	-7.18	0.75
PDK1	3-phosphoinositide dependent protein kinase-1	-6.19	0.35
FLJ23074	Yeast Sps1/Ste20-related kinase 4 (S. cerevisiae)	-5.56	0.70
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	-4.78	0.91
ADAM9	ADAM metalloproteinase domain 9 (meltrin gamma)	-4.78	1.00
PRKCA	protein kinase C, alpha	-4.78	0.73
CALM3	calmodulin 3 (phosphorylase kinase, delta)	-4.76	0.52
PRKCL2	protein kinase N2	-4.73	1.07
GRK7	G protein-coupled receptor kinase 7	-4.59	0.63
AKT1	v-akt murine thymoma viral oncogene homolog 1	-4.57	0.63
ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1	-4.39	0.54
ILK	integrin-linked kinase	-4.36	0.51
CD3E	CD3e molecule, epsilon (CD3-TCR complex)	-4.35	0.96
ACK1	tyrosine kinase, non-receptor, 2	-4.33	0.81
PIM2	pim-2 oncogene	-4.26	0.81
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	-4.21	0.88
IRAK3	interleukin-1 receptor-associated kinase 3	-4.18	0.66
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	-4.18	0.87
PIP5K1A	phosphatidylinositol-4-phosphate 5-kinase, type I, α	-4.03	0.61

Table 2. Results of tamoxifen sensitisation compound screen. List of top 10 sensitising compounds from drug screen displayed in Z score order with library, compound name, tamoxifen sensitivity Z score, and the effect of the compound on cell viability in vehicle alone plates.

Library	Compound	Tamoxifen sensitivity Z score	Cell viability
Prestwick	Thioridazine hydrochloride	-15.68	0.28
Prestwick	Betulin	-12.58	0.21
Prestwick	Amiodarone hydrochloride	-11.73	0.23
Prestwick	Sertraline	-9.67	0.63
Prestwick	Tetrandrine	-6.87	0.88
BIOMOL	Triciribine	-5.84	0.54
Prestwick	Mometasone furoate	-4.83	0.36
Prestwick	Tamoxifen citrate	-4.30	0.26
Prestwick	Fenbendazole	-3.93	0.17
Prestwick	Methotrimeprazine maleate	-3.42	1.29

FIGURE LEGENDS

Figure 1. High throughput tamoxifen sensitisation screens. **A.** RNAi screen method. MCF7 cells plated in 96 well plates were transfected with siRNA. Each transfection plate contained 80 experimental siRNAs (SMARTpools of four different siRNA targeting the same gene (black circles)) supplemented with ten wells of non-targeting siControl (grey circles). Transfected cells were divided into four replica plates, half treated with ethanol vehicle alone and half with 4OH tamoxifen at 50 nM, the SF₈₀ of MCF7. Cell viability was assessed after seven days of 4OH tamoxifen exposure using CellTiter-Glo Luminescent Cell Viability Assay (Promega). **B.** Scatter plot of averaged Z scores from tamoxifen sensitisation siRNA screen carried out in duplicate. Black diamonds – siRNA SMARTpools targeting 779 kinase genes, Grey diamonds – siControl. **C.** Scatter plot of averaged Z scores from tamoxifen sensitisation compound screen carried out in duplicate. Black diamonds – 1200 compounds, Grey diamonds – vehicle control (DMSO).

Figure 2. Silencing of the PDK1 pathway increases sensitivity to tamoxifen. **A.** Diagram illustrating components of the PDK1 pathway identified in the top 20 most sensitising hits from the RNAi screen. **B.** All of the top 10 most sensitising hits from the chemical screen, with the exception of betulin, significantly inhibit phosphorylation of the PDK1 target protein, AKT. Following 24 hours of serum starvation, MCF7 cells were treated with 10 μ M compound or DMSO control for 30 minutes and then stimulated with 10% FBS to elicit AKT phosphorylation. Lysates were made 30 minutes following stimulation. A western blot using antibodies recognising phospho and total AKT is shown. **C.** PDK1 silenced cells have reduced AKT phosphorylation. MCF7 cells transfected 48 hours earlier with siRNA were treated with 1 nM estradiol (E2), or left untreated. Lysates were made 24 hours following treatment. A western blot using antibodies recognising phospho and total AKT is shown. **D.** Triciribine treated cells have reduced AKT phosphorylation. MCF7 cells were treated with 1nM estradiol (E2), or left untreated in combination with triciribine (Tri) or DMSO vehicle control. Lysates were made 24 hours following treatment. A western blot using antibodies recognising phospho and total AKT is shown. **E.** Combination indices of Akti-1,2 and 4OH tamoxifen treated MCF7 cells after seven days continuous exposure. Akti-1,2 is synergistic with 4OH tamoxifen (combination index < 1.0). Error bars represent the 95% confidence interval.

Figure 3. Sensitisation screen hits, PDK1 siRNA, and triciribine, increase sensitivity to tamoxifen. **A.** Left panel. Real time quantitative PCR on cDNA prepared from MCF7 cells transfected with siRNA. Individual siRNAs 1, 2, 3, 4 and the PDK1 SMARTpool significantly reduced transcript expression of PDK1 compared to siControl transfected cells. * - p<0.05 compared to siControl (Student's t-test). Error bars represent the standard error of the mean (SEM). Right panel. Western blot analysis of lysates prepared from MCF7 cells transfected with PDK1 SMARTpool siRNA or siControl. Antibodies recognising PDK1 were used with β -tubulin as a loading control. PDK1 SMARTpool siRNA significantly reduced PDK1 protein expression compared to siControl transfected cells. **B.** Cell viability assay in MCF7 cells transfected with PDK1 SMARTpool, individual PDK1 siRNAs 2, 3 and 4, or siControl and treated with 4OH tamoxifen. PDK1 silenced cells have significantly increased sensitivity to 4OH tamoxifen. siControl SF₈₅ = 360 nM, PDK1 SMARTpool SF₈₅ = 19nM; a 19 fold increase in sensitivity. Error bars represent the SEM. **C.** Cell viability assay in MCF7 cells treated with triciribine or vehicle control (DMSO) in combination with 4OH tamoxifen. Triciribine treated cells are significantly more sensitive to 4OH tamoxifen than vehicle treated cells (Student's t-test p<0.05). DMSO SF₅₀ = 8.5 nM, Triciribine SF₅₀ = 1.2 nM; a seven fold increase in sensitivity. Error bars represent the SEM. **D.** Triciribine is synergistic with 4OH tamoxifen treatment. Combination indices of triciribine and 4OH tamoxifen treated MCF7 cells after seven days continuous exposure. Triciribine is synergistic with 4OH tamoxifen (combination index < 1.0). Error bars represent the 95% confidence interval.

Figure 4. Tamoxifen sensitisation screen hits, PDK1 siRNA, and triciribine, increase sensitivity to multiple ER α inhibitors. **A.** Cell viability assay in MCF7 cells transfected with PDK1 SMARTpool, individual PDK1 siRNAs 2, 3 and 4, or siControl and treated with ICI 182780. PDK1 silenced cells have significantly increased sensitivity to ICI 182780. siControl SF₇₀ = 84 nM, PDK1 SMARTpool SF₇₀ = 8 nM; a 10.5 fold increase in sensitivity. Error bars represent the SEM. **B.** Triciribine is synergistic with ICI 182780 treatment. Combination indices of triciribine and ICI 182780 treated MCF7 cells after 7 days continuous exposure. Triciribine is synergistic with ICI 182780 (combination index < 1.0). Error bars represent the 95% confidence interval. **C.** Cell viability assay in MCF7 cells transfected with PDK1 SMARTpool, individual PDK1 siRNAs 2, 3 and 4, or siControl and deprived of estradiol. PDK1 silenced cells have significantly increased sensitivity to 4OH estrogen deprivation. * - p<0.05 compared to siControl (Student's t-test). Error bars represent the SEM. **D.** Survival fraction of triciribine and vehicle control (DMSO) treated MCF7 cells after 7 days continuous estrogen deprivation. Triciribine treated cells are significantly more sensitive to estrogen deprivation than vehicle treated cells. * - p<0.05 compared to siControl (Student's t-test). Error bars represent the SEM. **E.** Propidium iodide FACS plots. MCF7 cells were transfected with PDK1 SMARTpool siRNA or siControl and 48 hours later treated with 1 nM estrogen (E2), or 1 nM estrogen plus 100 nM 4OH tamoxifen (4OHT). 24 hours later, cell cycle profiles were assessed by propidium iodide FACS. Increased sensitivity to tamoxifen in PDK1 silenced cells is characterised by increased tamoxifen-induced G₁ arrest. **F.** Absolute differences were calculated by subtracting the % of cells in each phase of the cell cycle before 4OH tamoxifen treatment from the % of cells in each phase after 4OH tamoxifen treatment. Significantly larger differences in G₁ and G₂/M phases after 4OH tamoxifen treatment were observed in PDK1 silenced cells, indicating increased tamoxifen-induced G₁ arrest. * - p<0.05 compared to siControl (Student's t-test). Error bars represent the SEM. **G.** PDK1 SMARTpool transfected cells have significantly higher induction of p21^{CIP1} following tamoxifen treatment than siControl transfected cells. MCF7 cells transfected 48 hours earlier with siRNA were treated with 1 nM estradiol (E2), or 1 nM estradiol plus 100 nM 4OH tamoxifen (4OHT). Lysates were made 24 hours following treatment. A western blot using antibodies recognising p21^{CIP1} and β -tubulin (as a loading control) is shown.

Figure 5. Tamoxifen sensitisation by tetrandrine. **A.** Cell viability assay in MCF7 cells treated with tetrandrine or vehicle control (DMSO) in combination with 4OH tamoxifen. Tetrandrine treated cells are significantly more sensitive to 4OH tamoxifen than vehicle treated cells (Student's t-test p<0.05). **B.** Tetrandrine is synergistic with 4OH tamoxifen treatment. Combination indices of tetrandrine and 4OH tamoxifen treated MCF7 cells after seven days continuous exposure. Tetrandrine is synergistic with 4OH tamoxifen (combination index < 1.0). Error bars represent the 95% confidence interval. **C.** Propidium iodide FACS plots. MCF7 cells were treated with 10 μ M tetrandrine or DMSO vehicle in combination with 1 nM estrogen (E2), or 1 nM estrogen plus 100 nM 4OH tamoxifen (4OHT). 24 hours later, cell cycle profiles were assessed by propidium iodide FACS. Increased sensitivity to tamoxifen in tetrandrine treated cells is characterised by increased tamoxifen-induced G₁ arrest. **D.** Absolute differences were calculated by subtracting the % of cells in each phase of the cell cycle before 4OH tamoxifen treatment from the % of cells in each phase after 4OH tamoxifen treatment. Significantly larger differences in G₁ phase after 4OH tamoxifen treatment were observed in tetrandrine treated cells, indicating increased tamoxifen-induced G₁ arrest. * - p<0.05 compared to DMSO (Student's t-test). Error bars represent the SEM.

FIGURE 1

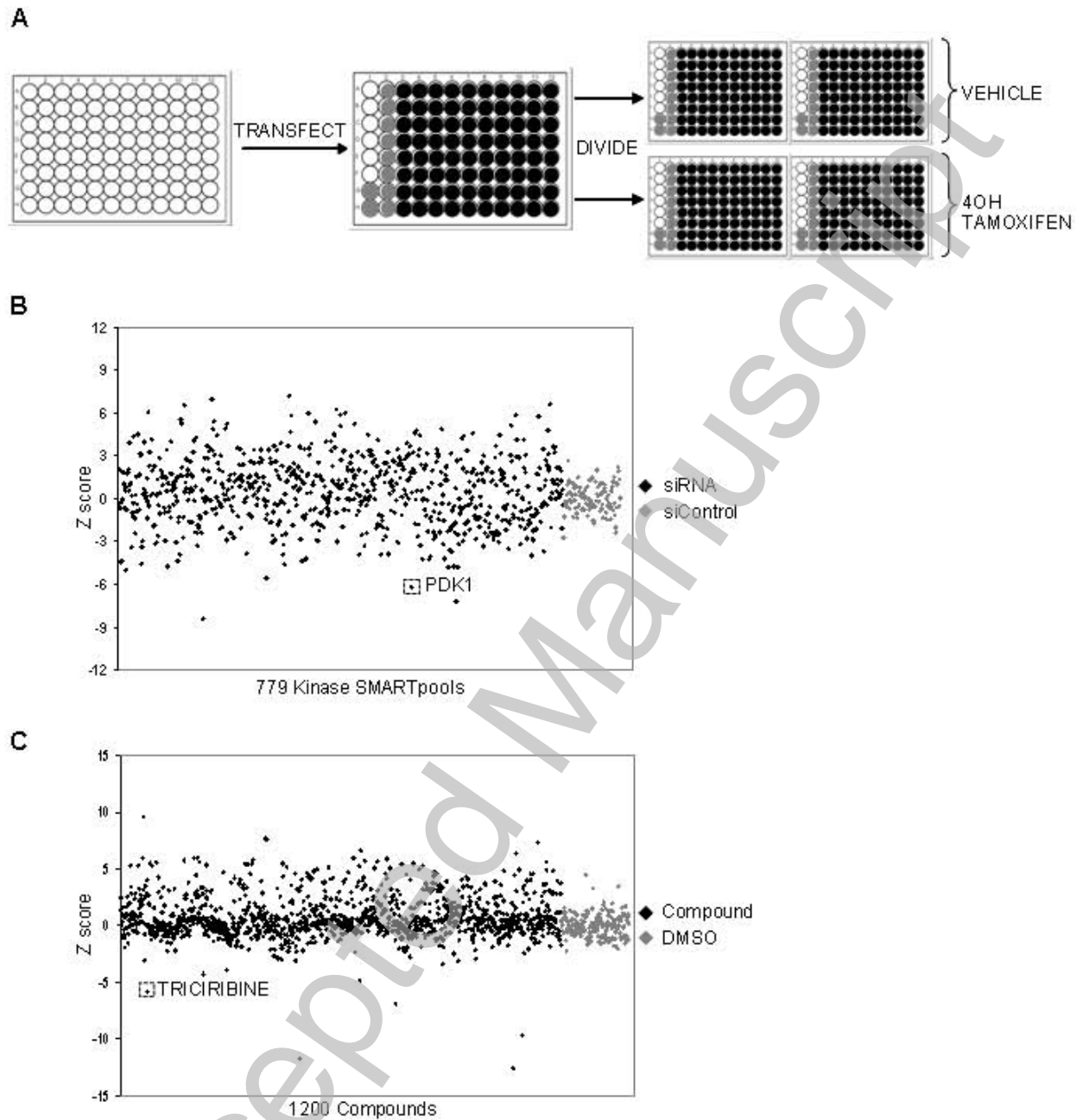
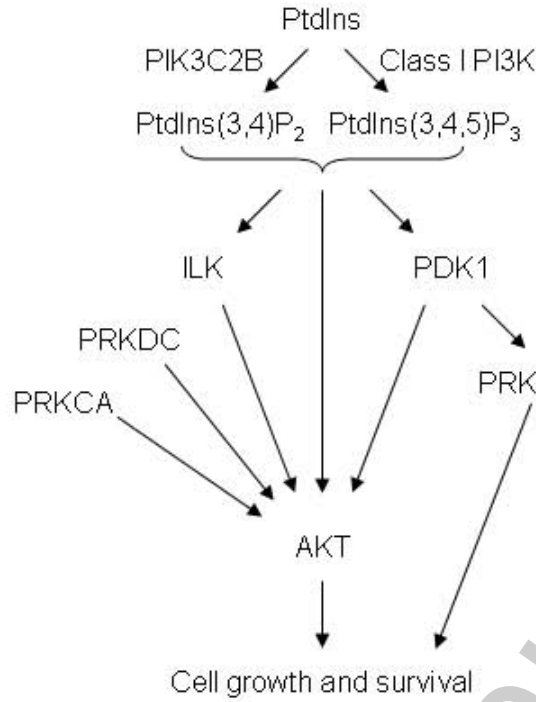
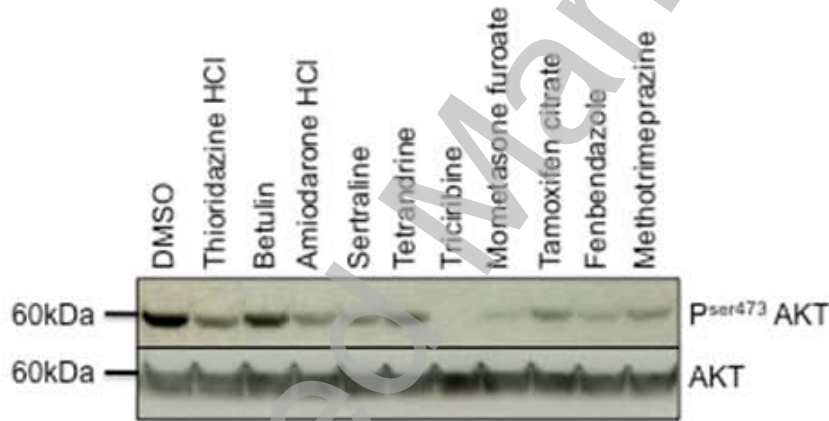


FIGURE 2

A



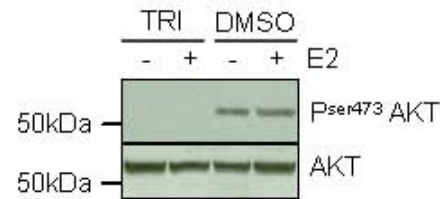
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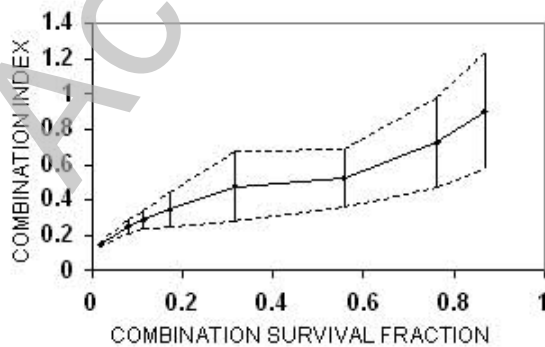


FIGURE 3

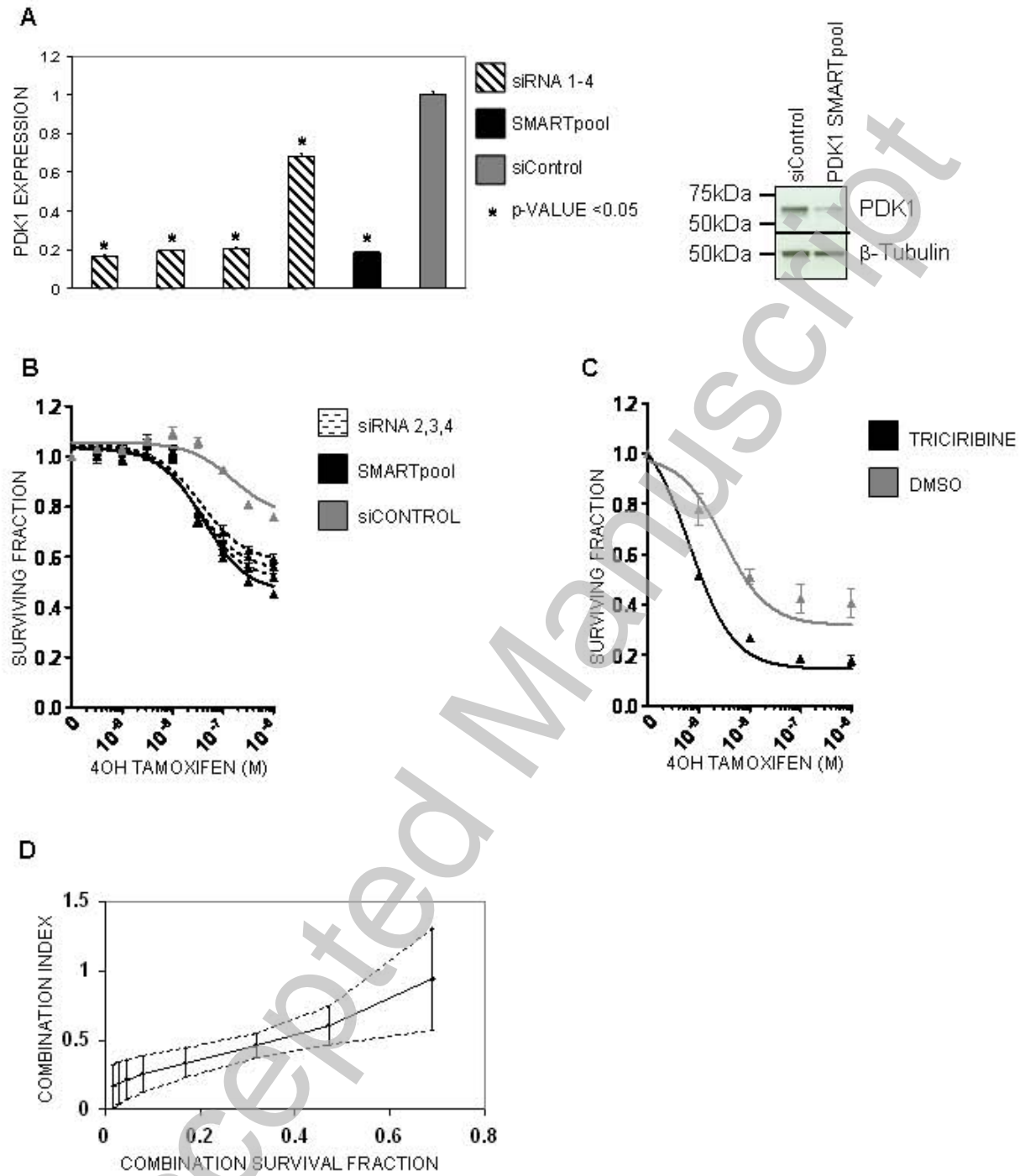


FIGURE 4

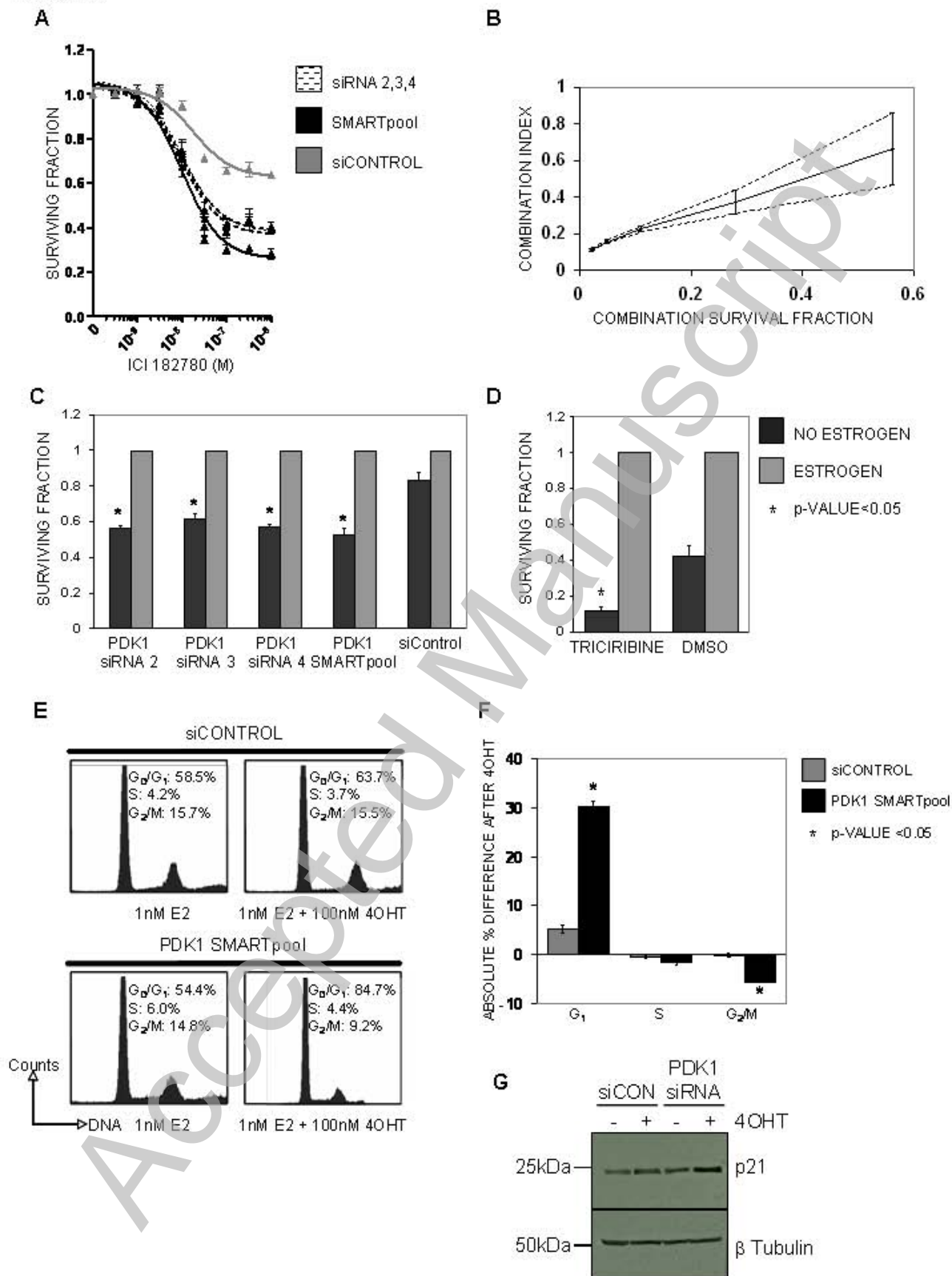


FIGURE 5

