A kinase inhibitor screen reveals MEK1/2 as a novel therapeutic target to antagonize IGF1R-mediated antiestrogen resistance in ERα-positive luminal breast cancer

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ABSTRACT

Antiestrogen resistance of breast cancer has been related to enhanced growth factor receptor expression and activation. We have previously shown that ectopic expression and subsequent activation of the insulin-like growth factor-1 receptor (IGF1R) or the epidermal growth factor receptor (EGFR) in MCF7 or T47D breast cancer cells results in antiestrogen resistance. In order to identify novel therapeutic targets to prevent this antiestrogen resistance, we performed kinase inhibitor screens with 273 different inhibitors in MCF7 cells overexpressing IGF1R or EGFR. Kinase inhibitors that antagonized antiestrogen resistance but are not directly involved in IGF1R or EGFR signaling were prioritized for further analyses. Various ALK (anaplastic lymphoma receptor tyrosine kinase) inhibitors inhibited cell proliferation in IGF1R expressing cells under normal and antiestrogen resistance conditions by preventing IGF1R activation and subsequent downstream signaling; the ALK inhibitors did not affect EGFR signaling. On the other hand, MEK (mitogen-activated protein kinase kinase) 1/2 inhibitors, including PD0325901, selumetinib, trametinib and TAK-733, selectively antagonized IGF1R signaling-mediated antiestrogen resistance but did not affect cell proliferation under normal growth conditions. RNAseq analysis revealed that MEK inhibitors PD0325901 and selumetinib drastically altered cell cycle progression and cell migration networks under IGF1R signaling-mediated antiestrogen resistance. In a group of 219 patients with metastasized ER + breast cancer, strong pMEK staining showed a significant correlation with no clinical benefit of first-line tamoxifen treatment. We propose a critical role for MEK activation in IGF1R signaling-mediated antiestrogen resistance and anticipate that dual-targeted therapy with a MEK inhibitor and antiestrogen could improve treatment outcome.

1. Introduction

Breast cancer is the most common cancer among women and about 75% of all breast tumors are estrogen receptor-α (ERα) positive (ER +). ER + breast cancers depend on ERα activation and signaling for their growth and proliferation [1]. Treatment of ER + breast cancer has drastically improved with the introduction of antiestrogen (AE) therapies that target ERα or estrogen biosynthesis. AEs have now been used to treat women with ER + breast cancer for several decades [2] and are effective in up to 60% of all patients [3]. Tamoxifen and fulvestrant are standard first line AE therapies that bind to the ERα and prevent receptor activation and stimulation of downstream targets in breast tissue. Although AE therapies work well for many patients, some tumors never respond (intrinsic resistance), while others develop resistance after long-term treatment (acquired resistance). Current estimates are that about 50% of all ER + breast cancers acquire resistance to tamoxifen or fulvestrant. Therefore, novel therapeutic targets are needed for long-term effective AE treatment of ER + breast cancer.

The mechanisms of AE resistance are complicated [4] and may include ERα mutations [5-7], that have been linked to ligand-independent constitutive activation, or enhanced sensitivity to

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estradiol; c-Src induced extranuclear ERα signaling [8]; different ERα phosphorylation patterns, resulting in enhanced estrogen induced or ligand independent ERα activation [1,9,10]; or alternative survival signaling parallel to ERα signaling [11-13]. The latter two mechanisms of AE resistance are often mediated by the activities of receptor tyrosine kinases (RTKs) and involve the increased expression and activity of e.g. epidermal growth factor receptor (EGFR), insulin-like growth factor 1 (IGF1R) and human epidermal receptor 2 (HER2) [8,11,14-17].

Ligand binding to IGF1R or EGFR leads to receptor autophosphorylation and subsequent formation of signaling complexes at the intracellular domain that activate various downstream signaling pathways. Two major pathways are the Ras/Raf/MEK/MAPK and the PI3K/PDK/Akt/mTOR signaling pathway [11-13,16]. Several MAP kinases, such as MAPK1 and MAPK3, can phosphorylate ERα, e.g. at Ser-118, thereby activating this receptor such that it no longer requires ligand binding to become active [1]. Other kinases, such as PI3K and Akt can phosphorylate ERα at other positions, e.g. at AF-1 (PI3K) and AF-2 (PI3K and Akt), also resulting in ligand-independent activation of the receptor [1].

Ligand binding of IGF1R or EGFR may also activate signaling pathways that operate independent of, and parallel to ERα signaling [11,12,18]. These Ras/Raf/MEK/MAPK and the PI3K/PDK/Akt/mTOR signaling pathways may modulate the biological programs that drive cell cycle progression, as well as other biological processes, such as cell survival and cell migration. Phospho-proteomics approaches have identified broad signaling networks next to these canonical signaling pathways, involving a multitude of kinases being activated after EGFR and/or IGF1R activation [19]. In addition, RNA knockdown screens have identified various proteins that may modulate AE resistance. For instance, Iorns et al [20] identified CDK10 as an important determinant of resistance to endocrine therapy for breast cancer in an RNAi screen in MCF7 cells. Also the PDK1 pathway and IGFBP5 were shown to modulate AE resistance in MCF7 cells [21,22]. Whole genome shRNA screening in MCF7 cells showed a compendium of genes affecting sensitivity to tamoxifen [23] and a large scale loss-of-function genetic screen in ZR-75–1 breast cancer cells showed that suppression of *USP9X* prevents proliferation arrest by tamoxifen [24]. Furthermore, depletion of *VAV3* influenced acquired AE resistance in MCF7 cell models [25]. Also RNAi knockdown of *PLK1* inhibited ER expression, estrogen-independent growth, and ER transcription in MCF7 and HCC1428 long term estrogen deprived cells [26]. In a siRNA screen, we have previously identified additional kinases that induce tamoxifen resistance in tamoxifen resistant MCF7 cells with enhanced expression of IGF1R (CHEK1, PAK2, RPS6KC1, TTK, and TXK) [27]. Although we mainly focused on PAK2 as the strongest resistance inducer, each of

these kinases could be a critical target for strategies to combat IGF1R signaling-mediated AE resistance.

Since various RTKs have been implicated in AE resistance, AEs have been combined with RTK monoclonal antibodies (mAbs) or small-molecule kinase inhibitors for treatment of AE resistant tumors. Unfortunately, targeting IGF1R or EGFR in combination with AEs has been disappointing thus far [28-33], indicating a need for more efficient drugs and/or therapeutic strategies. One promising approach is the combination of AE therapy with mTOR inhibitors and PI3K inhibitors. The rationale for such a combination is that mTOR and PI3K act downstream of activated IGF1R and EGFR. Indeed, the tamoxifen response could be restored in ER + breast cancer cells with high Akt activity when treated with mTOR inhibitors [34]. Also, combining PI3K or mTOR inhibitors and antiestrogen therapy produced synthetic lethality and triggered apoptosis [35,36]. Several clinical studies have been performed with the mTOR inhibitor everolimus, however, this led to mixed results with regards to efficacy (reviewed in [37]).

To identify novel targets to antagonize IGF1R- or EGFR-driven AE resistance, we performed kinase inhibitor screens in EGFR or IGF1R overexpressing ER + MCF7 breast cancer cell lines treated with tamoxifen [11,12]. In total we evaluated 273 different kinase inhibitors, targeting 42 individual kinases. Our screening approach revealed a number of kinase inhibitors that inhibit RTK-mediated AE resistant cell proliferation and additional functional analysis revealed a critical role of MEK1/2 in IGF1R-mediated antiestrogen resistance.

2. Materials and Methods

Chemicals, antibodies, and hormones. The kinase inhibitor library (L1200) and individual kinase inhibitors for validation were purchased from Selleck Chemicals (Houston, TX, USA). All other chemicals were from Sigma Aldrich (St. Louis, MO, USA), unless specified otherwise. Antibodies against EGFR (sc-03) and GAPDH (sc-32233) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-EGFR (Tyr1173, 4407L), IGF1R (3027S), phospho-IGF1R (Tyr1150/1151, 3024S), Akt (9272S), phospho-Akt (Ser473, 9271L), MAPK1/3 (4695S) and phospho-MAPK1/3 (Thr202/Tyr204, 9101S) were from Cell Signaling Technology (Danvers, MA, USA). The tubulin (T9062) antibody, 4-hydroxy tamoxifen (4OHT), fulvestrant, 17β-estradiol (E2), and EGF were from Sigma Aldrich (St. Louis, MO, USA). Secondary antibodies against mouse HRP tagged (115–035-003), mouse Alexa647 tagged (115–605-006) and rabbit HRP tagged (111–025-003) were from Jackson ImmunoResearch (West Grove, PA, USA). Human IGF1 (Increlex®) was from Ipsen Biopharmaceuticals Inc. (Cambridge,
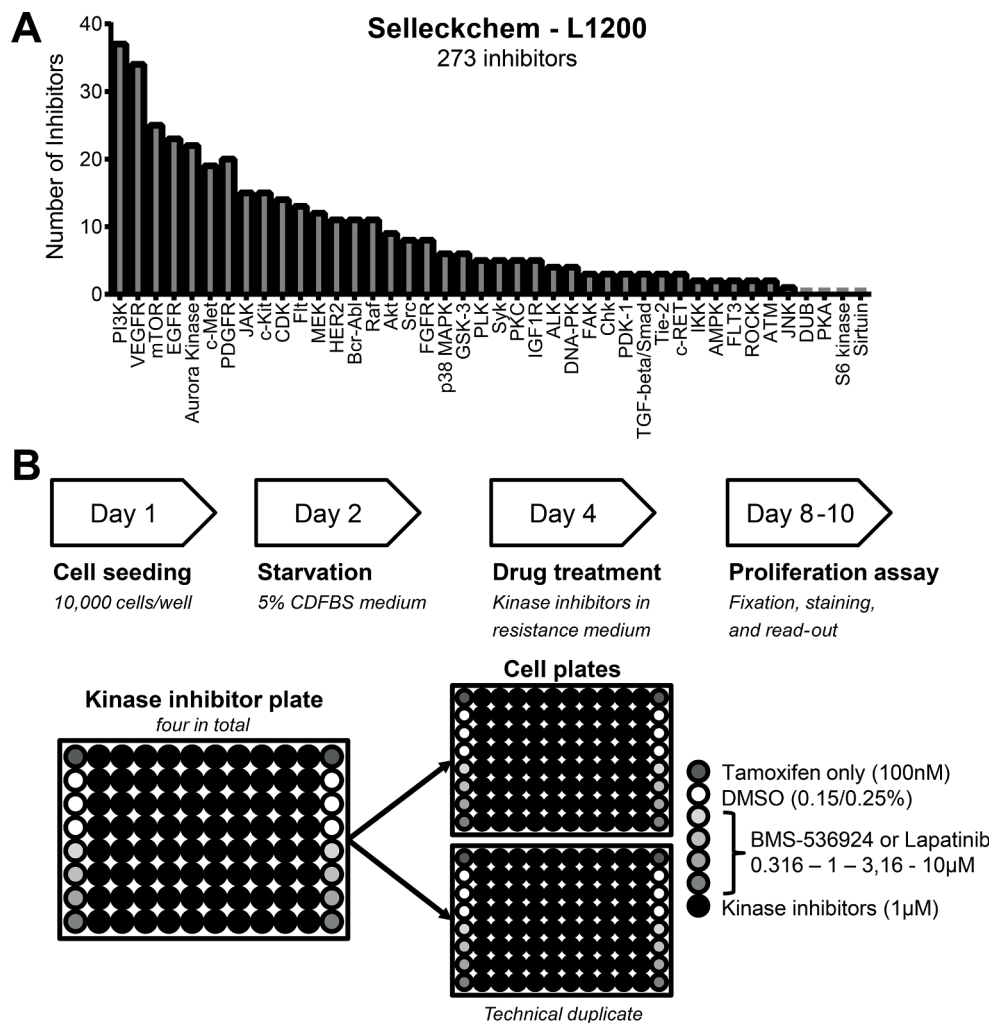
Fig. 1. Kinase inhibitor library composition and screening set-up. A) Bar graphs representing the number of inhibitors per target of the L1200 inhibitor library from SelleckChem. B) Screening set-up for the kinase inhibitor library screen, indicating proceedings per day and layout of kinase inhibitors plates.

MA, USA).

Cell culture. Parental MCF7 (MCF7/WT) cells (American Type Culture Collection; Manassas, VA, USA), MCF7/EGFR and MCF7/IGF1R cells (obtained as described before [11 12]) were cultured in RPMI 1640 L-Glutamine 25 mM HEPES (Gibco, via Thermo Fisher Scientific; Waltham, MA, USA), supplemented with 10 % v/v fetal bovine serum (FBS) (Gibco, Life Technologies; Grand Island, NY, USA) and penicillin-streptomycin (125 Units/mL and 125 µg/mL) (Invitrogen; Grand Island, NY, USA) (standard RPMI complete medium; RPMI++). Parental T47D (T47D/WT) cells (American Type Culture Collection; Manassas, VA, USA) and T47D/IGF1R cells were cultured in DMEM with D-glucose, L-Glutamine and pyruvate (Gibco, Life Technologies; Grand Island, NY, USA), supplemented with 10 v/v % FBS and penicillin-streptomycin (125 Units/mL and 125 µg/mL) (standard DMEM complete medium; DMEM++). T47D/IGF1R cells were obtained by transfection with the same vector as used for the MCF7/IGF1R cell line [12], followed by mass selection after virus transfection, and validated for overexpression of IGF1R, resistance against tamoxifen induced inhibition of cell proliferation using a sulforhodamine B (SRB) assay [38] and high IGF1R signaling after IGF exposure. All cells were cultured at 37C and 5 % carbon dioxide. For hormone and growth factor starvation, cells were maintained for 48 h in starvation medium consisting of phenol red free RPMI 1640x medium (Gibco, Life Technologies; Grand Island, NY, USA) supplemented with 5 % v/v charcoal dextran-treated fetal bovine serum (CDFBS) (Hyclone, Thermo Scientific; Waltham, MA, USA) and

penicillin-streptomycin. The MCF7 and T47D IGF1R/EGFR cell lines were used within 15 passages after their establishment.

Proliferation assay. All cells were plated at a density of 10,000 cells/well in 96-well plates (Corning; NY, USA), allowed to attach overnight and thereafter maintained in starvation medium for 48 h. Subsequently, estrogen (E2), growth factor (EGF or IGF1), and compound (4OHT and kinase inhibitor) were added, and cells were allowed to proliferate for another 96 h. Tamoxifen resistant (TamRes) culturing condition for MCF7/IGF1R and T47D/IGF1R was phenol red free RPMI 1640x medium with 5% v/v CDFBS, 1 µM 4OHT, 1 nM E2 and 100 ng/ml IGF1 [12,29]. TamRes culturing condition for MCF7/EGFR was phenol red free RPMI 1640x medium with 5% v/v CDFBS, 0.1 µM 4OHT, 0.1 nM E2 and 100 ng/ml EGF [11]. After 96 h cell number was determined by a colorimetric SRB assay [38]. This assay was previously adapted and validated by us for the cells used in this study [11,12]. In short, cells were fixed with 20 µl 50 % w/v trichloroacetic acid in 1 v/v % acetic acid for 1 h at 4 °C, washed five times with tap water, and air-dried. Thereafter cells were stained with 0.4 % w/v SRB in 1 % v/v acetic acid at room temperature for 30 min, washed five times in 1 % v/v acetic acid and air-dried overnight. Subsequently, protein-bound SRB in the wells was dissolved in 200 µl 10 mM unbuffered Tris solution (pH > 10) and absorbance was measured at 540 nm in a plate reader.

The kinase inhibitor screen was performed with 273 kinases targeting 42 cancer-related kinases as illustrated in Fig. 1. All inhibitors were tested in duplicate at a concentration of 1 µM alongside different
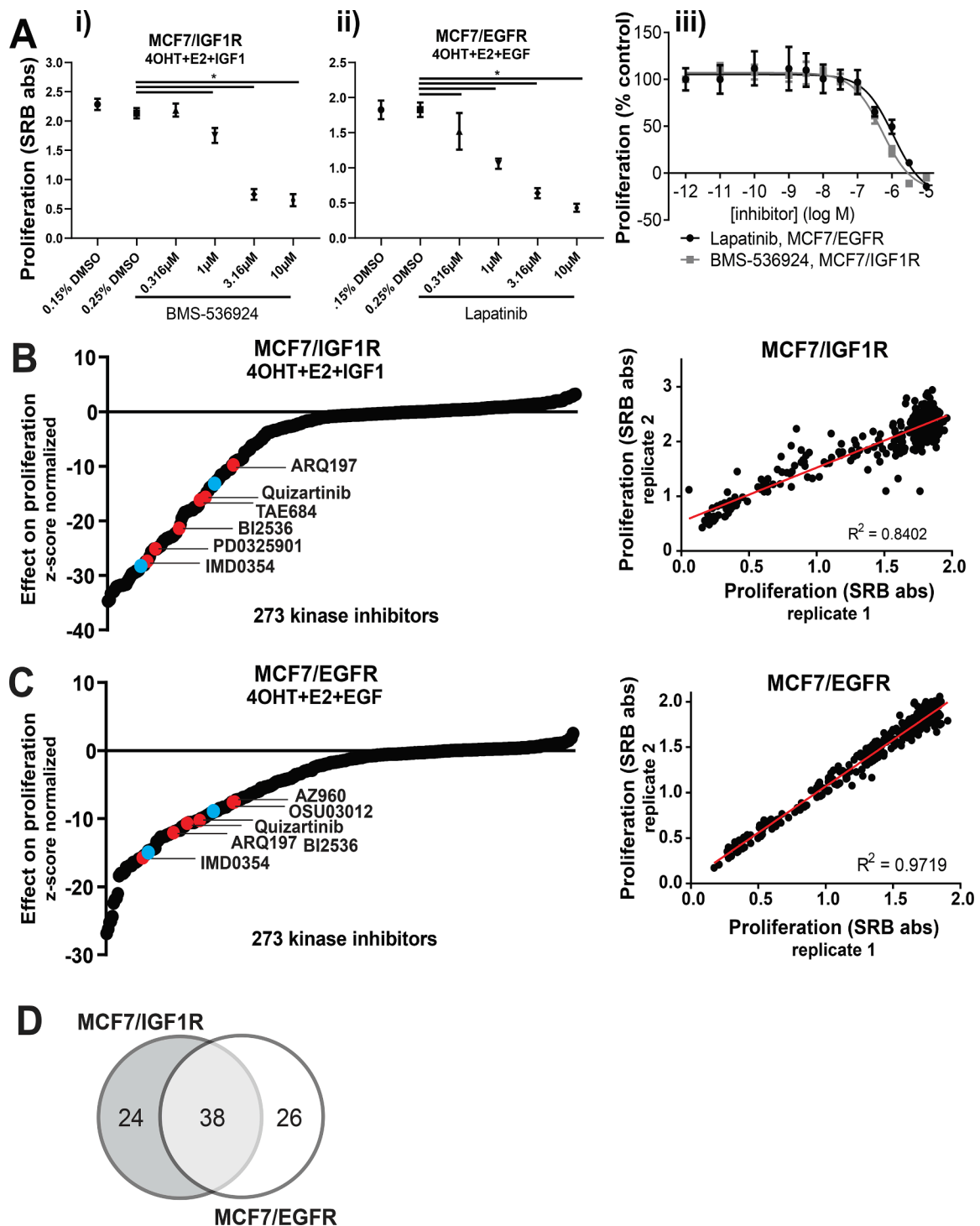
Fig. 2. Kinase inhibitor screen for the identification of kinases that antagonize IGF1R and EGFR mediated antiestrogen resistance. A) IGF1R inhibitor BMS-536924 response of MCF7/IGF1R cells (i) and EGFR inhibitor lapatinib response of MCF7/EGFR cells (ii) under tamoxifen resistant (TamRes; 11,12]) conditions (RPMI phenol-red free medium with 5 % v/v CDFBS, 1 μM 4OHT, 1 nM E2 and 100 ng/ml IGF1 or EGF) on the 4 different screening plates; and complete dose response curves of both inhibitors on cell proliferation under TamRes conditions (iii). Data are expressed as mean ± SD, n = 8, and are from two independent experiments. Significance was determined by one-way ANOVA followed by Dunnett's test. Significant differences between conditions are indicated by horizontal lines; * at p < 0.0001. B) Kinase inhibitor screen of MCF7/IGF1R cells under TamRes condition. Effects on cell proliferation are presented as Z-scores (see Materials section). Compounds for further study are indicated with red dots; blue dots indicate 1 μM (upper dot) and 3.16 μM (lower dot) BMS-536924. The right graph represents the reproducibility between two independent screens performed on different days (correlation coefficient R² = 0.84. C) Kinase inhibitor screen of MCF7/EGFR cells under TamRes conditions. Compounds of further study are indicated with red dots; blue dots indicate 1 μM (upper dot) and 3.16 μM (lower dot) lapatinib. The right graph represents the reproducibility between two independent screens performed on different days (correlation coefficient R² = 0.97). D) Venn diagram showing overlapping and differentially effective kinase inhibitors in MCF7/IGF1R and MCF7/EGFR cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Kinase inhibitors that effectively antagonize AE resistance in MCF7/EGFR and/or MCF7/IGF1R cells.

24 kinase inhibitors specific for MCF7/IGF1R cells		38 common inhibitors for MCF7/IGF1R and MCF7/EGFR cells		25 kinase inhibitors specific for MCF7/EGFR cells	
Compounds	Targets	Compounds	Targets	Compounds	Targets
GDC-0068	Akt1/2/3	MK-2206 dihydrochloride	Akt1/2/3	MK-5108 (VX-689)	Aurora kinase A
TAE684 (NVP-TAE684)[1]	**ALK**	PD 0,332,991 (Palbociclib)	CDK 4/6	MLN8054	Aurora kinase A/B
Hesperadin	Aurora Kinase B	**ARQ 197 (Tivantinib)**	**c-Met**	MLN8237 (Alisertib)	Aurora kinase A/B
NVP-ADW742	IGF-1R	**Quizartinib (AC220)**	**FLT3**	SNS-314	Aurora kinase A/B/C
TAK-733	MEK1	Tyrphostin AG 879 (AG 879)	HER2	Gefitinib (Iressa)	EGFR
AZD6244 (Selumetinib)	MEK1	Mubritinib (TAK-165)	HER2	Erlotinib HCl	EGFR
PD0325901	**MEK1/2**	**IMD 0354**	**IKKβ**	Desmethyl Erlotinib	EGFR
GSK1120212 (Trametinib)	MEK1/2	Temsirolimus (Torisel)	mTOR	Afatinib (BIBW2992)	EGFR, HER2
AZD8330	MEK1/2	WYE-354	mTOR	CI-1033 (Canertinib)	EGFR, HER2
AS703026	MEK1/2	WAY-600	mTOR	AZD8931	EGFR, HER2, ErbB3
PD318088	MEK1/2	Deforolimus (Ridaforolimus)	mTOR	BMS-599626 (AC480)	EGFR, HER2, ErbB4
WYE-125132 [2]	mTOR	Rapamycin (Sirolimus)	mTOR	AST-1306	EGFR, HER2, ErbB4
AZD8055 [2]	mTOR	Everolimus (RAD001)	mTOR	Lapatinib Ditosylate	EGFR, HER2, ErbB4
R406	Syk	Ku-0063794	mTORC 1/2	Dacomitinib (PF299804)	EGFR, HER2, ErbB4
GSK1838705A	ALK, IR, IGF-1R	BKM120 (NVP-BKM120)	PI3K p110αβγδ	**AZ 960**	**JAK2**
PF-03814735	Aurora Kinase A/B, FLT1, FAK, TrkA	BYL719	PI3Kα	**OSU-03012**	**PDK-1**
PF-02341066 (Crizotinib)	c-Met, ALK	ZSTK474	PI3Kαβγδ	GSK461364	PLK1
GSK1904529A	IR, IGF-1R	PIK-90	PI3Kαβγδ	Vandetanib (Zactima)	VEGFR2
TAK-901	JAK3, c-SRC, CLK2, FGR, YES1	BI6727 (Volasertib)	PLK1	AEE788 (NVP-AEE788)	EGFR, HER2, c-Abl, FLT1, c-Fms
Torin 2 [2]	mTOR, ATM, ATR, DNA-PK	ON-01910	PLK1	Neratinib (HKI-272)	EGFR, HER2, KDR
NVP-BGT226	mTOR, PI3Kαβγ	HMN-214	PLK1	Pelitinib (EKB-569)	EGFR, Src, MEK/MAPK
INK 128	mTOR, PI3Kαγδ	**BI 2536**	**PLK1/2/3**	Masitinib (AB1010)	Kit, Lyn B, PDGFRa/b
Torin 1 [2]	mTORC1/2, DNA-PK, PI3Kγ	KX2-391	Src	PP-121	PDGFR, Hck, VEGFR, mTOR, Src
NVP-TAE226	PYK2, FAK, IR, IGF-1R, c-Met	A-674563	Akt1, CDK2, PKA, GSK-3β	PKI-402	PI3Kαβγδ, mTOR
		GSK690693	Akt1/2/3, PKCη, PKCθ, PrkX	Saracatinib (AZD0530)	Src, LCK, YES, EGFR, Lyn
		PCI-32765 (Ibrutinib)	BTK, BLK, Bmx, CSK, FGR		
		PHA-793887	CDK 1/2/4/5/7/9, GSK-3β		
		PHA-848125	CDK 2, TrkA, CDK7, CDK4, CDK5		
		Amuvatinib (MP-470)	c-Kit, PDGFRα, FLT3		
		Triciribine	Akt, HIV-1		
		Foretinib (GSK1363089, XL880)	MET, KDR, Tie-2, VEGFR, RON		
		OSI-027	mTOR, mTORC1/2, PI3Kγ, DNA-PK		
		AZD2014	mTOR, p-Akt, pS6		
		WYE-687	mTOR, PI3Kα		
		BEZ235 (NVP-BEZ235)	mTOR, PI3Kαβγδ, ATR		
		GDC-0941	PI3Kαβγ, mTOR, C2β		
		GSK1059615	PI3Kαβγδ, mTOR		
		BIBF1120 (Vargatef)	VEGFR1/2/3, LCK, FLT3, PDGFR, FGFR		

[1] Inhibitors in boldface were selected for further evaluation; [2]these inhibitors are toxic to MCF7/EGFR cells.

concentrations (0.316 – 10 μM) of BMS-536924 (for MCF7/IGF1R cells) or lapatinib (for MCF7/EGFR cells), five DMSO (VWR International; Amsterdam, the Netherlands) controls, and 4OHT only. The entire screen was repeated independently on a different day. Data from the inhibitor screen was analyzed by unbiased sample-based analysis [39] in which SRB absorbance values of all individual wells samples were converted to z-scores:

$$z - score = \frac{SRB\ value - mean\ (SRB\ values\ of\ DMSO\ controls)}{standard\ deviation\ (SRB\ values\ of\ DMSO\ controls)}$$

Kinase inhibitors with a z-score lower than 1 μM BMS-536924 or 1 μM lapatinib were selected for further study.

Immunoblotting. Cells were plated and starved in 12- or 6-well plates and treated with indicated stimuli. After stimulation, cells were placed on ice, washed thrice with ice-cold phosphate-buffered saline (PBS), before being lysed either directly in sample buffer (125 mM Tris/HCl pH 6.8, 20% glycerol, 4% SDS and 0.2% bromophenol blue) or in lysis buffer (1% w/v sodium deoxycholate, 5 mM Tris/HCl (pH 7.5), 15 mM NaCl, 0.1% SDS, 1% v/v NP-40, 0.4 mM EDTA), hereafter protein concentration was determined and sample buffer was added. Proteins were separated on 7.5–15% acrylamide gels (Bio-Rad; Hercules, CA, USA) by electrophoresis, subsequently transferred to PVDF membranes (Merck & Co., Inc; Kenilworth, NJ, USA). Membranes were blocked with 5 % w/v bovine serum albumin (BSA) or ELK non-fat dry milk (FrieslandCampina; Amersfoort, the Netherlands) in Tris-buffered saline tween-20 (TBST) buffer (100 mM Tris, pH 7.4, 500 mM NaCl, 0.05% w/v Tween
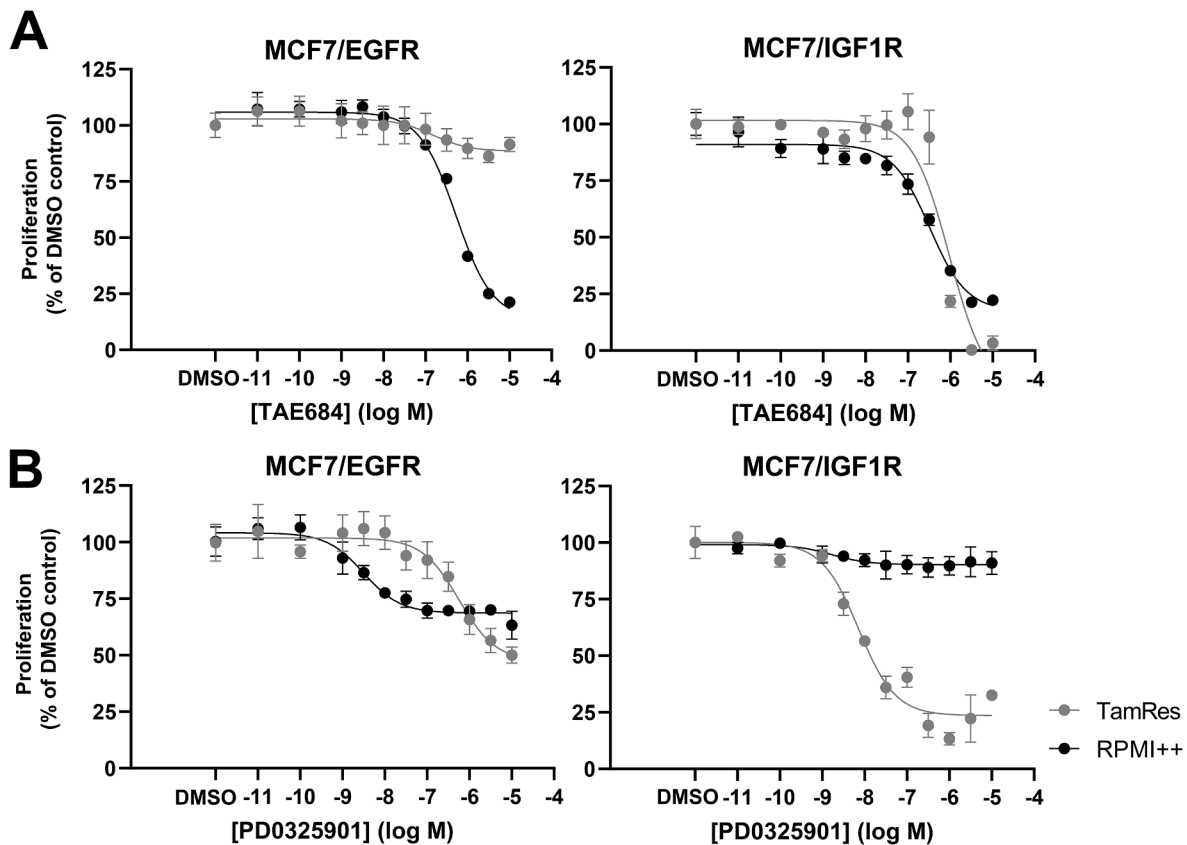
Fig. 3. ALK and MEK1/2 inhibitors effects in MCF7/IGF1R, MCF7/EGFR and parental MCF7 cells in standard RPMI complete medium and under IGF1R/ EGFR-induced tamoxifen resistance conditions. A) Effect of ALK inhibitor TAE684 on MCF7/IGF1R and MCF7/EGFR cell proliferation in standard RPMI complete medium (RPMI++) and under TamRes conditions (phenol red free RPMI × medium with 5% v/v CDFBS, 1 μM 4OHT, 1 nM E2 and 100 ng/ml IGF1 or 100 ng/ml EGF respectively). B) Effects of MEK1/2 inhibitor PD0325901 on MCF7/IGF1R and MCF7/EGFR cell proliferation in standard RPMI complete medium (RPMI++) and under TamRes conditions. Data presented are mean ± SD (n = 4) of one of two independent experiments.

20), then stained with appropriate primary and secondary antibodies in 1 % w/v BSA or ELK. Protein bands were visualized using horseradish peroxidase (HRP, Thermo Fisher Scientific; Waltham, MA, USA) or fluorescent imaging on an ImageQuant LAS4000 machine, (GE Healthcare Europe GmbH; Eindhoven, the Netherlands). Detecting reagents ECL and ECL Prime were from Fisher Scientific (Thermo Fisher Scientific; Waltham, MA, USA) and VWR International (Amsterdam, the Netherlands), respectively. Tubulin was used as a loading control and phosphorylated (p) AKT and pMAP1/3 signals were normalized to the mean of all pAKT or pMAPK1/3 signals on a blot.

siRNA knockdown. For siRNA transfection, 10,000 cells were seeded per well in a 96-well plate (day 0), transfected with 50 nM siRNA (all from Dharmacon; Pittsburg, PA, USA) using INTERFERin® transfection reagent (Westburg/PolyPlus; Leusden, the Netherlands) in starvation medium. Transfection was performed for 72 h and cells were treated as indicated and allowed to proliferate for another 96 h.

Cell cycle analysis. 200.000 cells per well (in TamRes medium) or 300.000 cells per well (in standard RPMI/DMEM complete medium) were plated in a 12-well plate, starved in 5 % w/v CDFBS or kept in normal medium and treated with the indicated conditions. After 24 h cells were harvested for FACS analysis. Briefly, cells were washed once in PBS/EDTA (all cells from supernatant were kept), harvested from the plate using trypsin/EDTA and combined with the cells from supernatant. Cells were spun down (1.000 rpm, 5 min, 4 °C), suspended in ice-cold PBS/EDTA and then fixed with the addition of 96% ethanol. Fixed cell samples were able to be kept for up to 7 days at −20 °C before preparation for FACS analysis. Cells were rehydrated in PBS, stained for 15 min with 3 μM DAPI (Invitrogen; Grand Island, NY, USA in staining buffer (100 μM Tris, 150 mM NaCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$), filtered

for single cell suspension and subsequently FACS analyzed on a FACS CantoII flow cytometer. Analysis of cell cycle status was performed using FlowJo® software (BD Bioscience; Franklin Lakes, NJ, USA).

High content imaging. Cells were plated in a μ-clear 96 wells imaging plate, then starved in 5 % v/v CDFBS medium for 48 h. Before treatment, cells were incubated with Hoechst 33,342 (0.1 ng/μL; Invitrogen; Grand Island, NY, USA) for 1 h, thereafter treated as indicated with the addition of AnnexinV-Alexa633 (0.5 μg/ml) and propidium iodide (PI, 0.05 μM) (Invitrogen; Grand Island, NY, USA). After treatment the plate was imaged at 24, 48, 72 and 96 hr on a Nikon C1 confocal microscope, for all three fluorophores. Afterwards, the plates were fixed, stained once more with Hoechst for nuclei counting and stained with SRB for proliferation readout. Image analysis was performed using CellProfiler [40].

RNA sequencing and bioinformatics. Cells were seeded overnight in 6-well plates and treated in triplicate for 6 h in TamRes culturing condition medium or standard RPMI complete medium with kinase inhibitors at indicated concentrations, or vehicle. RNA was isolated with RNeasy Plus Mini Kit as described by the manufacturer (Qiagen; Redwood City CA, United States). cDNA libraries were prepared from the samples with the Illumina (San Diego, CA, USA) TruSeq Stranded mRNA Library Prep Kit. The libraries were sequenced according to the Illumina TruSeq v3 protocol on an Illumina HiSeq2500 sequencer using paired-end 100 bp reads. Alignment was performed against the human GRCh38 reference genome using the STAR aligner (version 2.4.2a) [41]. Marking duplicates, sorting and indexing were performed using sambamba (version 0.6.6) [42]. Gene expression was quantified using the FeatureCounts software (version 1.4.6) [43] based on the ENSEMBL gene annotation for GRCH38 (release 84). RNA-Seq data were normalized using the TMM
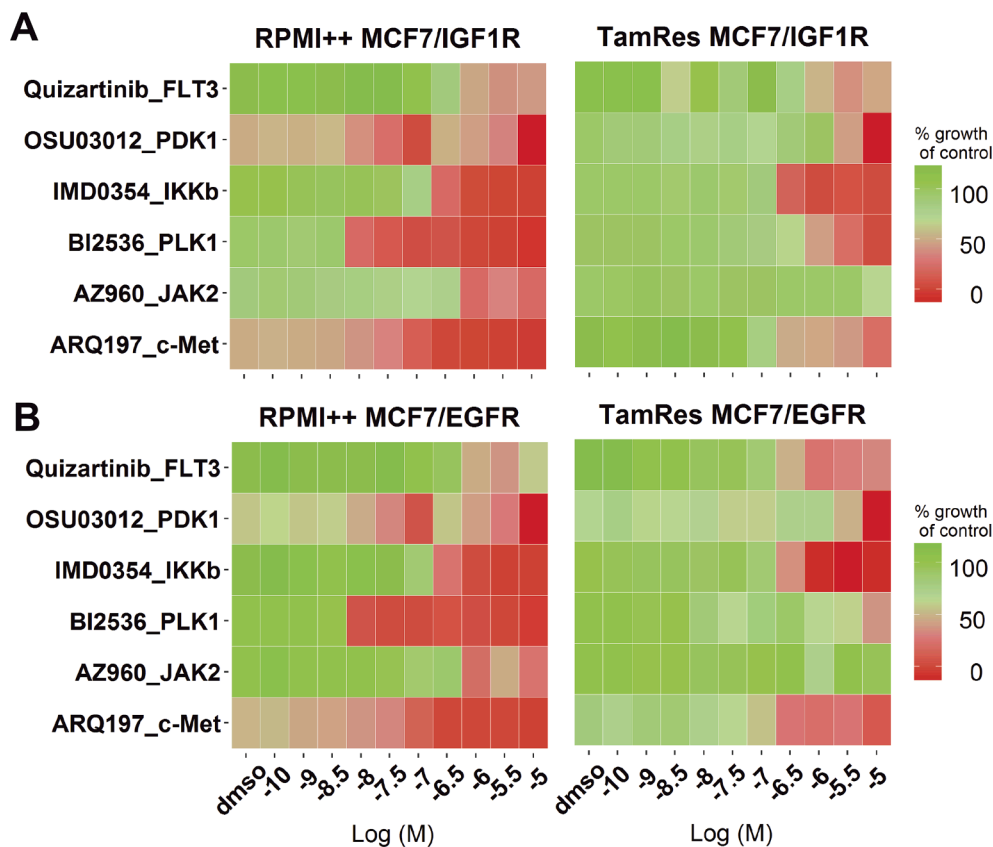
Fig. 4. Proliferation inhibition profiles of 6 selected kinase inhibitors. A) Proliferation inhibition profiles in MCF7/IGF1R cells, in full (RPMI++) and AE resistance medium (TamRes). B) Proliferation inhibition profiles in MCF7/EGFR cells, in standard RPMI complete medium (RPMI++) and tamoxifen resistance medium (TamRes). Data presented are the mean of three independent experiments. Colors indicate inhibitory effect.

(trimmed-mean of M−values) method from the Bioconductor package EdgeR [44], followed by quantile normalization and were then log2 transformed. Statistical significance for differentially expressed genes (DEGs) was calculated by the adjusted p-value procedure of Benjamini & Hochberg [45].

Analysis of altered cellular signaling pathways, biofunctions and upstream regulators. Differentially expressed genes between two different experimental conditions were analyzed with the software package Ingenuity Pathway Analysis (IPA, Qiagen; Redwood City CA, United States) for altered signaling pathways, biofunctions and upstream regulators. Significance and z-scores of altered upstream regulators, biofunctions and diseases were calculated as described [46].

Tissue microarrays staining and evaluation. Tissue microarrays of formalin-fixed, paraffin-embedded primary breast tumor specimens were prepared and immunohistochemically stained according to the procedures described previously [47]. The staining was performed after 20 min antigen retrieval at pH9.0, with primary mouse antibody against MEK1/2 (Cell Signaling, Danvers, MA, USA; clone L38C12), and primary rabbit antibody against Ser 221 phospho-MEK1/2 (pMEK) (Cell Signaling; Danvers, MA, USA; clone 166F8), and incubation overnight (1:25 and 1:100 dilution respectively) at 4 °C. Subsequently, slides were incubated with EnVision plus anti-mouse and anti-rabbit antibodies (K4001 and K4003, DAKO; Santa Clara, CA, USA) and staining was visualized using diaminobenzidine. MEK and pMEK staining were scored for quantity and intensity by two independent observers as described by us before [27]. The study has been approved by the medical ethics committee of the Erasmus MC Rotterdam, the Netherlands (MEC 02.953).

Statistical analysis. Statistical analyses were performed with Graph-Pad Prism software (San Diego, CA, USA), unless stated otherwise. The *t*-test or one-way ANOVA with Dunnett's test was used to determine difference between conditions; p-values were two sided. The relation between human tumor tissue staining and clinical benefit was analyzed with Pearson's Chi-square test.

3. Results

Identification of kinase inhibitors that antagonize antiestrogen resistance in MCF7/IGF1R and MCF7/EGFR cells.

Increased expression and activity of EGFR and IGF1R is one of the mechanisms that underlies AE resistance in ER + breast cancer. To identify novel kinase inhibitors that could antagonize RTK-mediated AE resistance, we performed kinase inhibitor screens including 273 well-characterized inhibitors, targeting 42 established cancer-related protein kinases (Fig. 1A). The screens (Fig. 1B) were performed in two established AE resistant ER + breast cancer cell lines that overexpress IGF1R (MCF7/IGF1R) or EGFR (MCF7/EGFR), under IGF1/EGF-induced tamoxifen resistance conditions. 4OHT and fulvestrant remain fully active against E2-induced proliferation in these MCF7/IGF1R and MCF7/EGFR cells, but these cells are highly resistant against the antiestrogens when stimulated with IGF1 or EGF, respectively [11,12].

In both screens we used cell proliferation as an endpoint and BMS-536924 (IGF1R and insulin receptor inhibitor) and lapatinib (EGFR and HER2 inhibitor) as positive controls, because these compounds effectively antagonized AE resistance mediated by IGF1 and EGF receptor activation, respectively (Fig. 2A, i-iii). All kinase inhibitors were tested at 1 μM in the screens. A majority of the kinase inhibitors did effect cell proliferation and demonstrated similar effects in repeated screens (Fig. 2B and C; see Table T1 [dataset] [48] for all data of individual kinase inhibitors). As a selection threshold for further study of individual kinase inhibitors, as reference we used the degree of effect that 1 μM BMS-536924 or 1 μM lapatinib had. This revealed 24 kinase
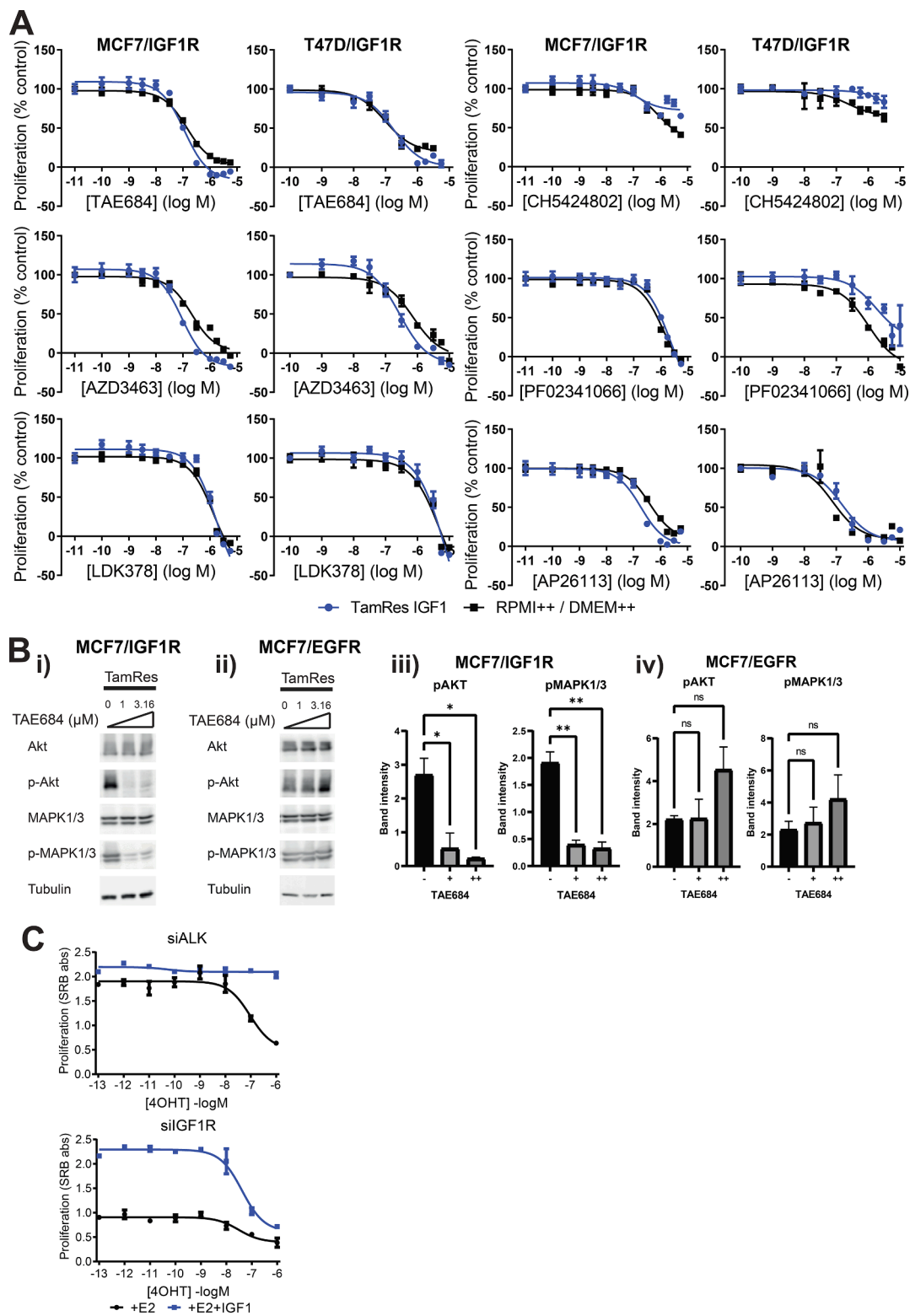
Fig. 5. Effect of various ALK inhibitors on IGF1R signaling-mediated antiestrogen resistance. A) Inhibition profiles of 6 different ALK inhibitors in both MCF7/IGF1R and T47D/IGF1R cells in standard RPMI/DMEM complete medium (RPMI++/DMEM++) and under IGF1-induced tamoxifen resistance (TamRes IGF1) conditions. Data shown is mean ± SD (n = 4) and representative of two independent experiments. B) Effect of ALK inhibition on downstream IGF1R and EGFR signaling as determined by Western blotting for pAKT and pMAP1/3 (i, ii), including quantification of two independent experiments (iii, iv). Cells were cultured in 5% CDFBS + E2 + 4OHT medium and subsequently treated for 60 min either IGF1 (i) or EGF (ii) in combination with an increasing dose of TAE684. Tubulin was used as a loading control. Significance was determined by one-way ANOVA followed by Dunnett's test; * indicates significantly different from DMSO control at p < 0.025; ** at p < 0.005; ns = not significant. C) Effect of siRNA knockdown of ALK (siALK) or IGF1R (siIGF1R) on antiestrogen resistance in MCF7/IGF1R cells. Data is mean ± SD of a representative of two independent experiments.

Table 2
Reported IC50 of different ALK inhibitors for several receptor tyrosine kinases.

	ALK	IGF1R	EGFR
TAE684 [76]	3 nM	10–20 nM	- -
CH5424802 [77]	1.9 nM	>5 μM	>5 μM
AP26113 [78]	0.62 nM	46 nM	129 nM
PF02341066 [79]	3.3 nM	0.78 μM	2.7 μM*

* IC50 for EGFR mutants G719C and L861Q.

inhibitors that antagonized AE resistance specifically in MCF7/IGF1R cells, 26 which specifically affected MCF7/EGFR cells, and 38 suppressing proliferation in both cell lines (Fig. 2D and Table 1).

Interestingly, despite the fact that IGF1 and EGF both activate the MEK/MAPK and PI3K/AKT/mTOR pathways in our cells [11,12], only in MCF7/IGF1R cells we observed antagonism of AE resistance by a large panel of MEK inhibitors (as well as several ALK inhibitors) while PI3K and mTOR inhibitors antagonized AE resistance in both MCF7/IGF1R and MCF7/EGFR cells, except for four inhibitors that antagonized AE resistance in MCF7/IGF1R cells but were toxic in MCF7/EGFR cells (Table 1). As expected, in MCF7/EGFR cells most EGFR inhibitors reverted EGF-mediated AE resistance, but also several inhibitors of Aurora kinases (Table 1). For our further studies we prioritized kinase inhibitors not directly implicated in IGF1R or EGFR signaling that antagonized AE resistance either in MCF7/IGF1R, or in MCF7/EGFR cells, or in both type of cells, including inhibitors of ALK, MEK1/2, c-Met, FLT3, IKKβ, PLK1/2/3, JAK2 or PDK-1 (Table 1); kinase inhibitors directly involved in cell cycle progression were excluded.

Validation of AE antagonizing effect of candidate kinase inhibitors in MCF7/IGF1R and MCF7/EGFR cells. Eight kinase inhibitors were further studied for concentration response effects: the ALK inhibitor TAE684, the MEK 1/2 inhibitor PD0325901, the FLT3 inhibitor quizartinib, the PDPK1 inhibitor OSU03012, the IKK-β inhibitor IMD0354, the PLK1 inhibitor BI2536, the JAK2 inhibitor AZ960, and the c-Met inhibitor ARQ197. We performed our validation in MCF7/IGF1R and MCF7/EGFR cells. Moreover, besides the tamoxifen resistant conditions used in our screens, we also included standard RPMI complete medium to investigate possible direct overt toxicity. Consistent with the primary screen, the ALK inhibitor TAE684 inhibited proliferation of MCF7/IGF1R cells under IGF1-induced tamoxifen resistance conditions. However, TAE684 had a similar effect on MCF7/IGF1R cells in standard RPMI complete medium, indicating that this is not specifically an AE resistance antagonizing effect (Fig. 3A). TAE684 also inhibited cell proliferation in MCF7/EGFR cells in standard RPMI complete medium, but not under EGF-induced tamoxifen resistance conditions (Fig. 3A).

The MEK inhibitor PD0325901 did not inhibit MCF7/IGF1R cell proliferation in standard RPMI complete medium, however, it rendered MCF7/IGF1R cells sensitive to AE treatment again under IGF1-induced tamoxifen resistance conditions. PD0325901 only partially inhibited cell proliferation of MCF7/EGFR cells, both under EGF-induced tamoxifen resistance and standard RPMI complete medium conditions (Fig. 3B). Thus, EGFR-driven AE resistance was not significantly affected by PD0325901. None of the other six kinase inhibitors showed specific AE antagonizing effects, because all of them also inhibited cell proliferation in MCF7/IGF1R and MCF7/EGFR in standard RPMI complete medium. (Fig. 4A, B).

Together, our results indicate that the ALK inhibitor TAE684 inhibits cell proliferation mediated by IGF1 signaling but not by EGF signaling and that the MEK inhibitor PD0325901 antagonizes AE resistance in MCF7/IGF1R cells but not in MCF7/EGFR cells.

Effect of other ALK inhibitors on IGF1R signaling-mediated antiestrogen resistance. Because the ALK inhibitor TAE684 showed promising effects on inhibition of cell proliferation in MCF7/IGF1R cells under IGF1-induced tamoxifen resistance conditions, we evaluated the effects of five additional different ALK inhibitors. Four showed similar inhibitory effects on MCF7/IGF1R cell proliferation as TAE684 under IGF1-

induced tamoxifen resistance conditions and in standard RPMI complete medium. CH5424802 was the only ALK inhibitor that did not completely inhibit MCF7/IGF1R cell proliferation (Fig. 5A). Similar inhibitory effects for these ALK inhibitors were observed in T47D/IGF1R cells (Fig. 5A). Some of the ALK inhibitors target multiple receptor tyrosine kinases including IGF1R and EGFR, with IC50 values effective in the low to high nM range for IGF1R inhibition (Table 2). Only CH5424802 seems a highly selective ALK inhibitor. Given the limited effect of CH5424802 on IGF1R signaling mediated AE, it is likely that the effect of other ALK inhibitors could be related to IGF1R inhibition. In support of this, the non-selective ALK inhibitor TAE684 prevented phosphorylation of the IGF1R downstream targets AKT and MAPK1/3 in MCF7/IGF1R cells (Fig. 5Bi); no inhibitory effect was observed on downstream EGFR signaling in MCF7/EGFR cells (Fig. 5Bii). Next, we tested whether siRNA knockdown of *ALK* or *IGF1R* would affect AE resistance in MCF7/IGF1R cells. While knockdown of *IGF1R* allowed a 4OHT concentration-dependent inhibition of proliferation in IGF1-induced tamoxifen resistance conditions (as expected), this was not observed upon knockdown of *ALK* (Fig. 5C). Together, these data strongly suggest that the less selective ALK inhibitors we tested effectively antagonize IGF1R signaling-mediated AE resistance by direct inhibition of IGF1R rather than selective ALK inhibition.

Effect of different MEK inhibitors on IGF1R signaling-mediated AE resistance. In our kinase library screen, we observed that various MEK inhibitors antagonized AE resistance of MCF7/IGF1R cells. Therefore, we also assessed the effect of six other selective MEK inhibitors, including PD0325901, on antagonizing AE resistance. Besides PD0325901, three other MEK inhibitors, trametinib, TAK-733 and selumetinib, showed concentration-dependent antagonism of AE resistance in MCF7/IGF1R, without greatly affecting the proliferation in standard growth medium (Fig. 6A). Almost identical effects were observed in T47D/IGF1R cells (Fig. 6A). PD184352 and U0126 were only effective at high concentrations and inhibited cell proliferation both under IGF1-induced tamoxifen resistance conditions and in standard RPMI complete medium (Fig. 6A). Next, we evaluated the effect of PD0325901 on IGF1-mediated signaling. PD0325901 effectively prevented phosphorylation of MAPK1/3 (Fig. 6Bi) as expected from a MEK inhibitor because MEK is upstream from MAPK1/3. PD0325901 also effectively blocked EGF-mediated MAPK1/3 phosphorylation in MCF7/EGFR cells (Fig. 6Bii), despite the fact that MEK inhibitors are ineffective in antagonizing AE resistance in these cells (Table T1)[dataset][48]. Together these data strongly support the role of MEK inhibition as an effective approach to antagonize AE resistance mediated by IGF1R signaling.

Antagonisms of IGF1R signaling-mediated AE resistance by MEK inhibition is caused by cell cycle arrest. Next, we evaluated how the four effective MEK inhibitors antagonize IGF1R signaling-mediated AE resistance in MCF7/IGF1R and T47D/IGF1R cells. All four MEK inhibitors caused a drastic inhibition of cell cycle progression under IGF1-induced tamoxifen resistance conditions both in MCF7/IGF1R and T47D/IGF1R cells, with the majority of cells remaining in the G_1/G_0 phase (Fig. 7); no effect of MEK inhibitors on cell cycle progression was observed in standard RPMI or DMEM complete medium (Fig. 7). Importantly, these effects were similar in MCF7/IGF1R and T47D/IGF1R cells but were not observed in standard RPMI complete medium. Trametinib, PD0325901 and TAK-733 were most potent in modulating these signaling events. The effects on cell cycle progression by all four MEK inhibitors were not associated with major onset of cell death, as determined by high content imaging (Fig. 8A-C). Only some onset of apoptosis was observed by MEK inhibitors in MCF7/IGF1R cells in TamRes medium, and in T47D/IGF1R cells in standard DMEM complete medium (Fig. 8C).

MEK inhibitors affect the IGF1R signaling-mediated cell cycle programs under antiestrogen resistance conditions. To gain further insight in the effect of MEK inhibition on MCF7/IGF1R cells, we performed a transcriptome analysis using RNA sequencing. MCF7/IGF1R cells were
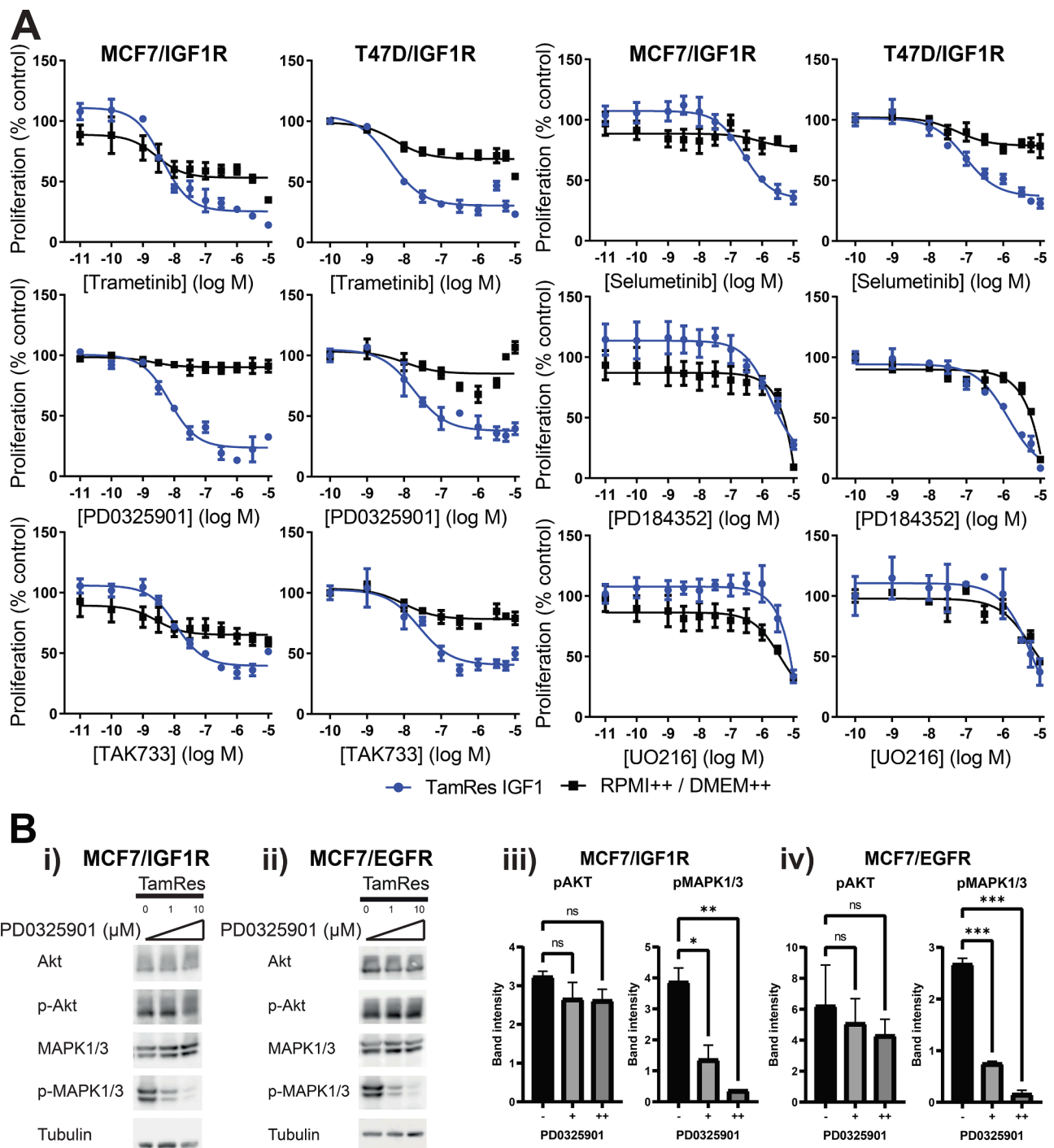
Fig. 6. Effect of different MEK inhibitors on antiestrogen resistance in MCF7/IGF1R and T47D/IGF1R cells. A) Inhibition profiles of 6 different MEK inhibitors in both MCF7/IGF1R and T47D/IGF1R cells after 96 h treatment in standard RPMI/DMEM complete medium (RPMI++/ DMEM++) and under IGF1-induced tamoxifen resistance (TamRes IGF1) conditions. Data shown is mean ± SD (n = 4) and representative of two independent experiments. B) Effect of PD0325901 on signaling in MCF7/IGF1R (i) and MCF7/EGFR (ii) cells, respectively as determined by Western blotting for pAKT and pMAPK1/2, including quantification of two independent experiments (iii, iv). Cells were cultured in 5% CDFBS + E2 + 4OHT medium and subsequently treated for 60 min with either IGF1 or EGF in combination with an increasing dose of PD0325901. Tubulin was used as a loading control. Significance was determined by one-way ANOVA followed by Dunnett's test; * indicates significantly different from DMSO control at $p < 0.025$; ** at $p < 0.005$; *** at $p < 0.001$; ns = not significant.

cultured in IGF1-induced tamoxifen resistance medium or standard RPMI complete medium in the absence or presence of PD0325901 or selumetinib. In the presence of PD0325901, 2677 genes were differentially expressed (DEG) under IGF1-induced tamoxifen resistance conditions compared to DMSO control with a Log2 fold change (FC) $\geq |0.5|$; selumetinib caused the differential expression of 2639 genes under IGF1-induced tamoxifen resistance conditions compared to DMSO

control. Because we were particularly interested in genes that are exclusively regulated by the MEK inhibitors under IGF1-induced tamoxifen resistance conditions, we identified the DEGs by PD0325901 and selumetinib that were significantly differentially expressed solely under IGF1-induced tamoxifen resistance conditions compared to DMSO controls. There were 1220 and 1253 such DEGs for PD0325901 and selumetinib, respectively (Fig. 9A, Table T2 and T3
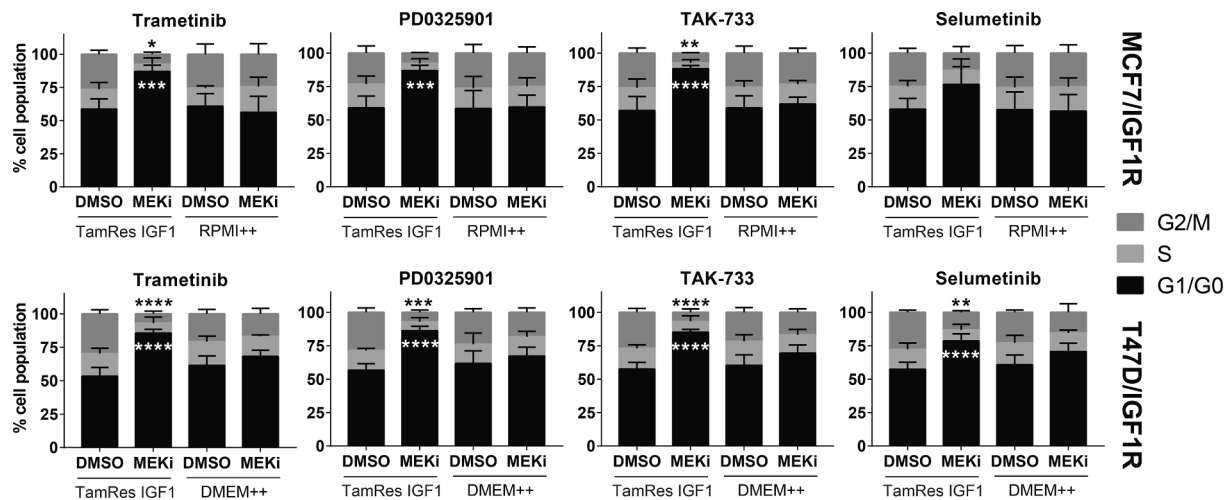
Fig. 7. MEK inhibition causes cell cycle arrest under IGF1-mediated antiestrogen resistant conditions. Cell cycle analysis of MCF7/IGF1R and T47D/IGF1R cells treated with different MEK inhibitors (MEKi) in IGF1-induced tamoxifen resistance medium (TamRes IGF1) or standard RPMI/DMEM complete medium (RPMI++/DMEM++). Trametinib and selumetinib were used at 10^{-6}M and PD0325901 and TAK-733 were used at $10^{-5.5}$M. Cells were treated for 96 h followed by flow cytometric analysis. Data shown are mean $\pm$ SD of three independent experiments. Significance was determined by Student's t-test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

[dataset][48]). The number of DEGs similarly regulated by PD0325901 and selumetinib treatment was 774 (335 up and 439 down regulated) (Fig. 9B, Table T4 [dataset][48]). The top five downregulated genes included *MMP10, SERPINE1, F2RL1, AKAP12* and *CLDN1* and the top five upregulated genes included *SEMA3F, RPL23AP87, TENT5B, TMEM229B* and *INAVA* (Table T4 [dataset][48]).

Next, we used Ingenuity Pathway Analysis (IPA) to discover the biological meaning of the regulation of the DEGs by the MEK inhibitors. This revealed common affected signaling networks by PD0325901 and selumetinib under IGF1-induced tamoxifen resistance conditions, with "cellular development, cellular growth and proliferation, hereditary disorder" being most significant (Fig. 9C). 22 DEGs in this program are commonly regulated by both MEK inhibitors (Fig. 9D). Of these 22 DEGs, 16 genes are lower expressed compared to control condition, and 6 are higher expressed (Fig. 9D, Table T5 [dataset][48]). Among the 16 lower expressed genes are 5 that may promote cell survival or cell cycle progression (*FABP5, GNL3L, NUMBL, TNFAIP8, ZFAND6*) (Table T6 [dataset][48]); interestingly, also *RRAS2* and *RAB31* were decreased and associated with breast cancer [49-51]. Among the 6 higher expressed genes are two (*ING4, ZNF385A*) that can interact with TP53 and may promote TP53 dependent expression cell cycle arrest genes (Table T6 [dataset][48]). In conjunction with the network analysis, we also determined strong modulation of the IPA biofunction description "Cell Cycle Progression": 56 DEGs were similarly affected by both MEK inhibitors (Table T7 [dataset][48]). This involved upregulation of the *CDKN2D* and *CCNG2* that both inhibit CDK4/6 kinase activation; and downregulation of *RUNX1, RUNX2, KLF10* and *CD44*. Two networks were altered after PD0325901 and selumetinib under IGF1-induced tamoxifen resistance conditions that share the (partial) label "Cellular Movement". There were 12 overlapping DEGs in this network (out of 35) (Fig. 9E; Table T10 [dataset][48]) that included e.g. *ITGA2, ADAM9, CMTM8* and *RAP1B* and may regulate integrin-mediated cell signaling under IGF1-induced tamoxifen resistance conditions. We also observed networks that were rather MEK inhibitor specific. Notably, two "cell cycle" networks in the top ten were altered after selumetinib treatment but not after PD0325901 treatment (Tables T8 and T9 [dataset][48]). The first network was centered around the downregulation of *MYC* (Fig. 10), a well-known regulator of cell cycle progression that is activated by ERα activation. The second network involved various known cell cycle regulators that were downregulated including *TCF7, FOXP3, PIM2* and *FBXW7* (Fig. 11). Finally, we also performed an upstream

regulator analysis in IPA (Table T11 [dataset][48]). Confirming the validity of this approach, MEK and ERK were predicted upstream regulators. Eight other potential upstream regulators of gene expression changes were identified for both MEK inhibitors, including two regulators that are predicted to be activated (CST5 and *let-7*), and six that are inhibited (NUPR1, PGR, JUN, TREM1, PDGF BB and ETV1).

Clinical relevance of MEK protein expression. Given the role of MEK modulation in IGF1R-mediated antiestrogen resistance, we investigated the clinical relevance of our findings by evaluating the expression of MEK, pMEK and IGF1R by tissue microarray staining in primary breast cancer tissue (Fig. 12). We selected 219 patients with primary operable ER + breast cancer, who developed metastatic breast cancer and were treated with first-line tamoxifen, and for which detailed clinical follow-up are available [47]. Strong stainings for MEK and pMEK were observed in 15 (7%) and 37 (17%) patients, respectively (Table 3, Fig. 12A and B), indicating a substantial proportion of patients with increased levels of activated MEK. In total 83 (38%) patients stained positive for IGF1R (Fig. 12C) of which 5% had strong MEK and 13% strong pMEK staining. The staining patterns were also related to tamoxifen treatment outcome, with clinical benefit observed in 142 patients and no clinical benefit (i.e., progressive disease or stable disease < 6 months) in 77 patients.

Strong pMEK staining showed a significant relation with outcome (p = 0.024, Pearson's Chi-square test, Table 3), MEK and IGF1R did not. The strong pMEK staining was more often seen in ER-positive patients with no clinical benefit than in patients with clinical benefit, compared to no/weak pMEK staining (Table 3). No significant association with progression-free survival was observed, with a hazard ratio of 1.16 (95% CI: 0.97–1.38, P = 0.113).

4. Discussion

The aim of this study was to identify candidate therapeutic regimes that could overcome tamoxifen resistance in ER + breast cancer that is mediated by increased activity of either IGF1R or EGFR. We performed screens with 273 kinase inhibitors targeting 42 different kinases to identify novel targets to antagonize IGF1R- or EGFR-driven AE resistance important. We revealed that both MEK and ALK inhibitors can reverse IGF1R signaling-mediated AE resistance, with the effect of MEK inhibitors being more potent and selective. MEK inhibitors blocked cell cycle progression under IGF1R signaling dependent AE resistance
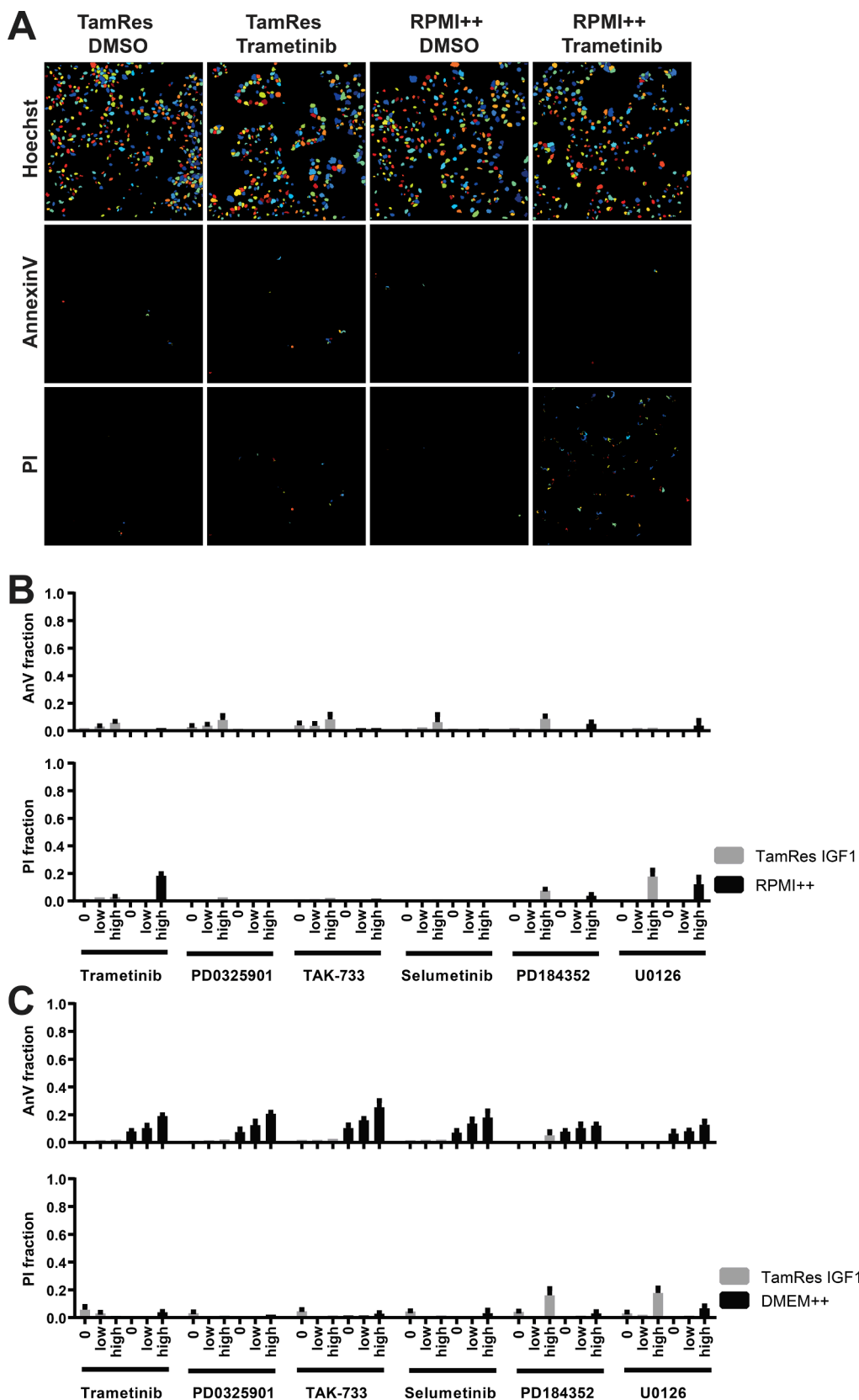
Fig. 8. Effect of MEK inhibitors on cell viability in MCF7/IGF1R and T47D/IGF1R cells. A) MCF7/IGF1R cells were treated with trametinib or DMSO for 96 h in IGF1-induced tamoxifen resistance medium (TamRes IGF1) or standard RPMI complete medium (RPMI++) followed by confocal microscopy-based high content imaging as described in Material and Methods. Shown are representative images for nuclear staining (Hoechst33342); apoptotic cell death (AnnexinV-Alexa633) (AnV) and necrotic cell death (propidium iodide) (PI) in MCF7/IGF1R cells after trametinib treatment. Shown are the digital masks of the cells/nuclei. Different colors were added to these masks to aid in the identification and counting of cells/nuclei. B) MCF7/IGF1R cells were treated with different MEK inhibitors at different concentrations in IGF1-induced tamoxifen resistance medium (TamRes IGF1) or standard RPMI complete medium (RPMI++) for 96 h followed by high content imaging and analysis after Hoechst33342/AnV/PI staining. Data represents the AnV positive cells (AnV fraction) and PI positive cells (PI fraction) shown as mean ± SD of quadruplicate wells in MCF7/IGF1R cells and C) T47D/IGF1R cells were treated and analyzed as indicated under B). Concentrations of inhibitors used: trametinib: 10^{-8} M and 10^{-6} M, PD0352901 and TAK-733: $10^{-7.5}$ M and $10^{-5.5}$ M, selumetinib and PD184352: $10^{-6.5}$ M and 10^{-5} M, U0126: 10^{-6} M and 10^{-5} M.

conditions with suppression of Aurora kinases and RB activation. Transcriptomics revealed that MEK inhibition suppressed transcriptional programs that involve cell survival, cell cycle progression and cell migration program. Strong pMEK staining showed a significant

correlation with no clinical benefit of first-line tamoxifen treatment for patients with metastasized ER + breast cancer, suggesting possible clinical benefit of MEK inhibitors.

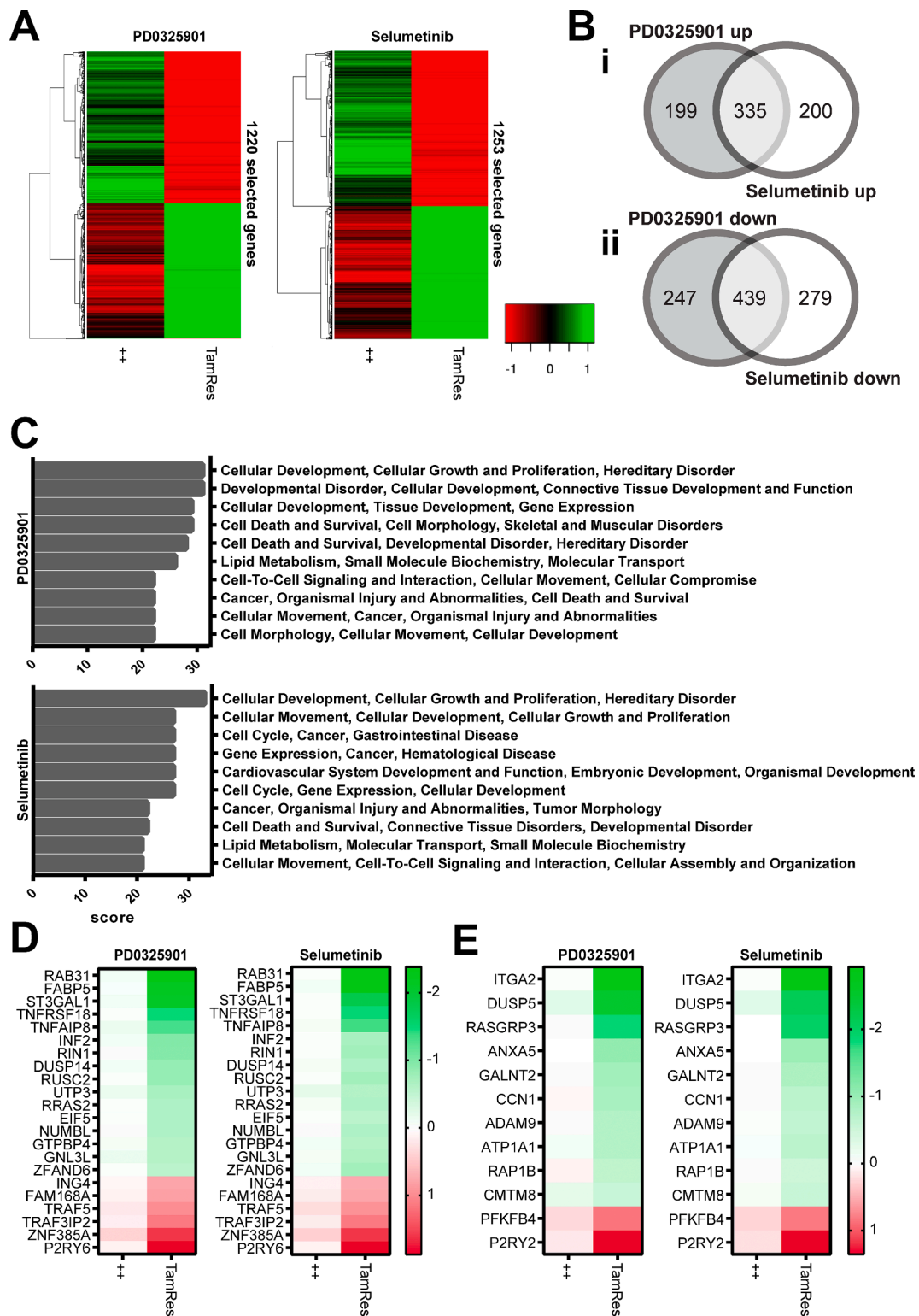In our kinase inhibitor screens, only two RTK inhibitors antagonized

Fig. 9. Transcriptome analysis reveals effects of MEK inhibitors on IGF1R-mediated signaling pathways in MCF7/IGF1R cells under IGF1-induced tamoxifen resistance conditions. A) Heatmap of up- and downregulated genes by PD0325901 (PD0) (1 μM) and selumetinib (Sel) (1 μM) compared to DMSO control under standard RPMI complete medium (++) and IGF1-induced tamoxifen resistance (TamRes) conditions after 6 h stimulation with the MEK inhibitors B) Venn diagrams illustrating up (i) and down (ii) regulated DEGs after PD0325901 and selumetinib treatment and the overlap between these genes. C) Top ten of altered signaling networks for both PD0325901 (upper panel) and selumetinib (lower panel). D) Heatmap of 22 DEGs (out of 35) in the first "cellular development, cellular growth and proliferation" network that are similarly regulated by PD0325901 and selumetinib. E) Heatmap of 12 DEGs in the "cellular movement" network that are similarly regulated by PD0325901 and selumetinib. Colors represent log2 values of fold changes compared to control.
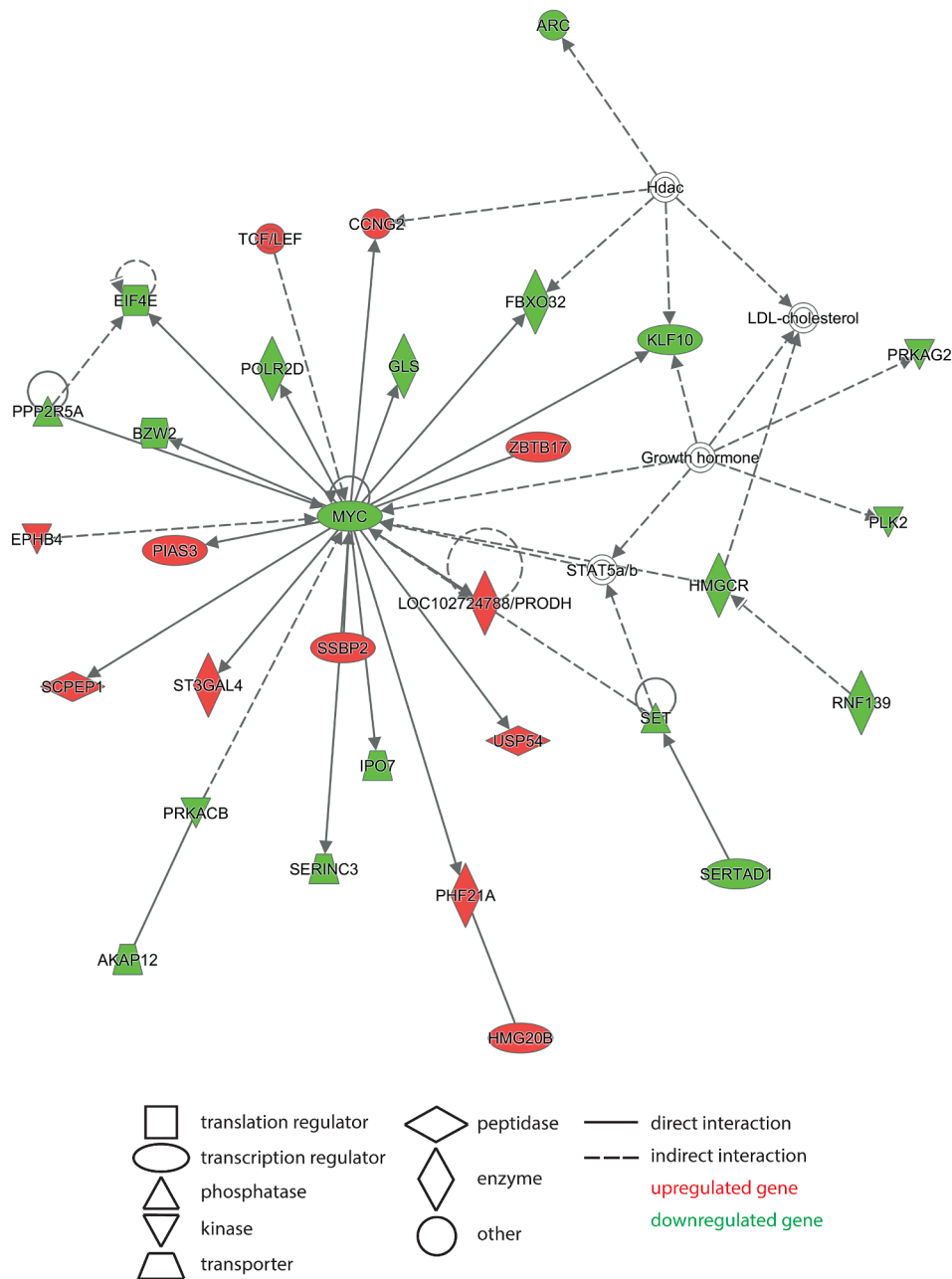
Fig. 10. Cell cycle network altered in MCF7/IGF1R cells after selumetinib treatment in IGF1-induced tamoxifen resistance medium. Graphical representation of the first "cell cycle" signaling network regulated by selumetinib under IGF1-induced tamoxifen resistance growth conditions.

IGF1R-dependent AE resistance: the MEK inhibitor PD0325901 and ALK inhibitor TAE684. The MEK inhibitor PD0325901 antagonized AE resistant cell proliferation under IGF1-induced tamoxifen resistance conditions in MCF7/IGF1R cells, while it did not affect cell proliferation in standard RPMI complete medium. The ALK inhibitor TAE684 inhibited cell proliferation in standard RPMI complete medium in MCF7/EGFR and MCF7/IGF1R cells, whereas it selectively decreased IGF1-induced and not EGF-induced AE resistant cell proliferation.

In validation experiments with five additional ALK inhibitors or siRNA knockdown of *IGF1R* or *ALK*, only non-selective ALK inhibitors that also target IGF1R or *IGF1R* knockdown antagonized IGF1-induced tamoxifen resistance. This was not observed for the selective ALK inhibitor CH5424802 or *ALK* knockdown. Thus, ALK inhibitor effects on IGF1R-mediated AE resistance are likely due to off-target inhibition of IGF1R activity. However, ALK inhibition may still have clinical benefits. ALK is an RTK that belongs to the insulin receptor (IR) superfamily, like

IGF1R [52]. The *ALK* gene has been shown to be deregulated via mutations [53,54], amplifications [55,56] and translocations [57,58] mostly in neuroblastoma and lung cancer and also in inflammatory tumors [59]. ALK downstream signaling converges with IGF1R signaling where it also depends on the activation of IR substrates IRS1 and IRS2, thereby initiating cell survival, cell cycle progression, proliferation and angiogenesis signaling components [60,61]. Signaling downstream of these RTKs involves three main signaling pathways: the JAK-STAT3 pathway [62], the PI3K-AKT pathway [63] and the RAS-MAPK pathway [64]. Inhibition of ALK, whether or not in combination with targeting IGF1R, has already been proposed for non-small cell lung cancer (NSCLC) treatment [65], and new ALK inhibitors are currently under clinical development. Our data suggest that mono-targeting of ALK may not be sufficient to confer the desired effect, because *ALK* knockdown or treatment with the selective ALK inhibitor CH5424802, which does not target IGF1R, did not antagonize IGF1-induced AE
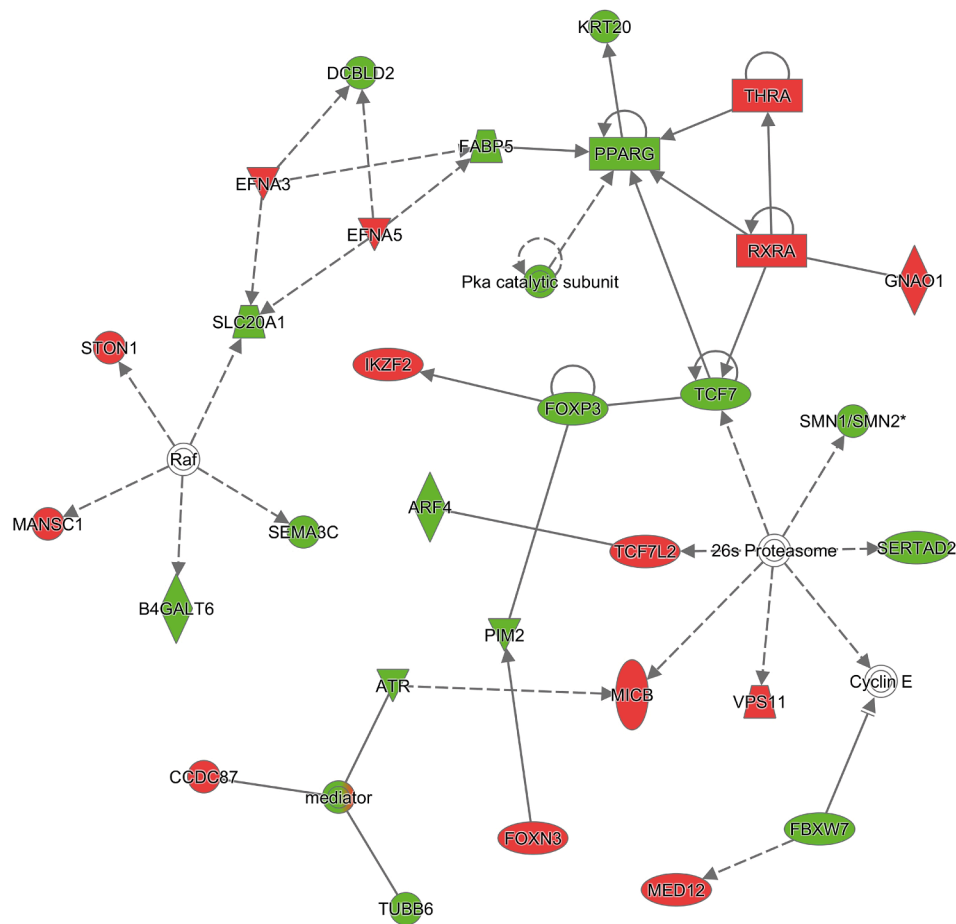
Fig. 11. Second cell cycle network altered in MCF7/IGF1R cells after selumetinib treatment in IGF1-induced tamoxifen resistance medium. Graphical representation of the second "cell cycle" signaling network regulated by selumetinib under IGF1-induced tamoxifen resistance growth conditions. Symbols and colors are as in Fig. 10.
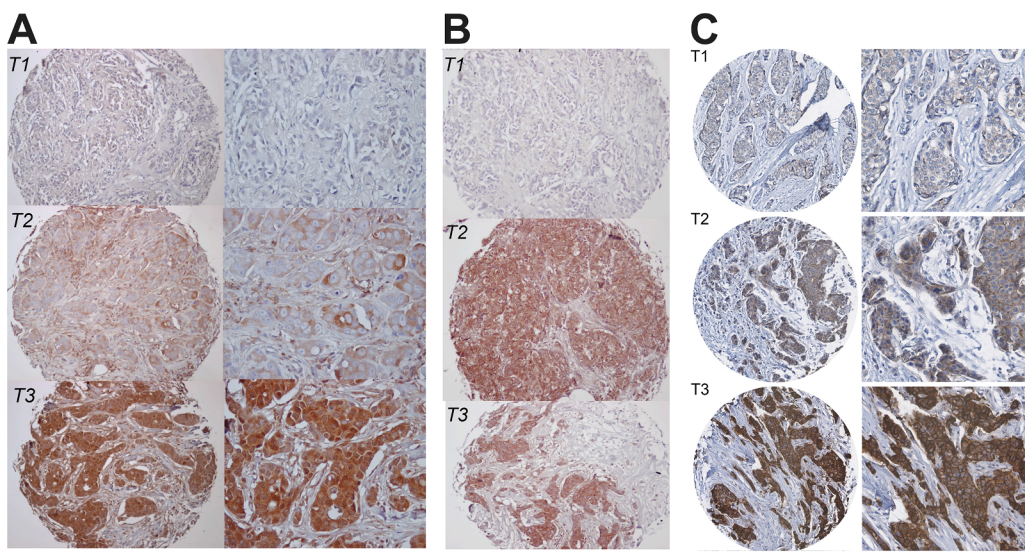
Fig. 12. Immunohistochemical staining for MEK, pMEK and IGF1R. Tumor tissue microarrays were stained immunohistochemically with antibodies against MEK (A), pMEK (B), and IGF1R (C). Tumor sections are shown with weak (T1), moderate (T2), and strong staining (T3) for MEK and IGF1R. The pMEK tumor sections show weak (T1) and strong (T2, T3) staining.

resistance. The ALK inhibitors TAE684 and AZD3463, which also target IGF1R, were equally effective in antagonizing AE resistant cell proliferation of both MCF7/IGF1R and T47D/IGF1R cell lines as the IGF1R inhibitor BMS-536924. Given the crosstalk between ALK and IGF1R, targeting both IGF1R and ALK with dual specific RTKs might be a viable treatment option to tackle AE resistance.

Table 3

Primary breast cancer tissue array staining for (p)MEK and IGF1R and clinical benefit of 219 ER-positive breast cancer patients who received first-line tamoxifen.

	Total	Patients with clinical benefit *	Patients with no clinical benefit **	Pearson's Chi-Square p-value
	219	142 (65%)	77 (35%)	
MEK				
strong staining	15	11 (73%)	4 (27%)	0.475
no/weak staining	204	131 (64%)	73 (36%)	
pMEK				
strong staining	37	18 (49%)	19 (51%)	0.024
no/weak staining	182	124 (68%)	58 (32%)	
IGF1R				
positive	83	51 (61%)	32 (39%)	0.411
negative	136	91 (67%)	45 (33%)	

Tumor tissue microarrays were stained immunohistochemically with primary antibodies against MEK pMEK, and IGF1R, followed by incubation with secondary EnVision® plus anti-mouse and anti-rabbit antibodies, and visualization using diaminobenzidine.

* Clinical benefit of tamoxifen treatment: complete and partial response, and stable disease > 6 months.

** No clinical benefit of tamoxifen treatment: progressive disease and stable disease < 6 months.

In our study we identified the MEK inhibitors PD0325901, trametinib, TAK-733 and selumetinib as potent antagonist of AE resistant cell proliferation of MCF7/IGF1R cells under IGF1-induced tamoxifen resistance conditions. Interestingly, no effect of these MEK inhibitors was observed in standard RPMI complete medium. This indicates that the IGF1R activation by IGF1 initiates specific MEK-dependent signaling that drives a transcriptional program that can evoke antiproliferative signaling mediated by tamoxifen under high estrogen levels. Although, MEK1 and MEK2 are major signaling components downstream of various RTKs, the MEK inhibitors did not at all antagonize EGFR-mediated AE resistance, indicating differential modulation of AE resistance by IGF1R and EGFR signaling.

Under the IGF1-induced tamoxifen resistance conditions, combination treatment of 4OHT and MEK inhibitors caused a cell cycle arrest in the G_0/G_1 phase of MCF7/IGF1R cells. Modulation of cell cycle progression was also reflected in gene expression profiles: the combination of 4OHT treatment and MEK inhibition affected a network of genes involved in cellular development, growth and proliferation. Interestingly, among the lower expressed genes are two genes implicated in breast cancer development and metastasis: *RRAS2* and *RAB31* [49-51].

Our transcriptomics analysis revealed that the MEK-inhibitors impacted on the expression of genes that have previously been linked to ER + breast cancer progression and cancer stem cell-ness, including *RUNX1,* and *RUNX2* [66,67], as well as epithelial-mesenchymal transition, including *TCF7, KLF10* and *TGFB1* [68-70]. The transcriptomics data suggest that MEK inhibitors reverse transcriptional changes induced by IGF1R-mediated signaling under tamoxifen resistant conditions. This was not only related to modulation of cell cycle regulated genes, but also to the expression levels of various migration and invasion-related genes including integrins (*ITGA2*, *ITGAV*, *ITGB1*, *ITGB5*, *ITGB6*), cell adhesion-related genes including, *CLDN1*, *RDX*, *PXN*, *TSPAN5* and *FN1*. Thus, this treatment may also decrease integrin-mediated signaling, thereby indirectly impacting on cell cycle progression.

The MEK inhibitor sensitivity of IGF1R-overexpressing breast cancer cells under tamoxifen resistant conditions may well connect to our previous findings on a role for PAK2 in AE resistance. We reported that PAK2 mediates the IGF1R-induced resistance to tamoxifen and

fulvestrant in MCF7/IGF1R and T47D/IGF1R cells, and that high expression of *PAK2* in ER + metastatic breast cancer patients is correlated with unfavorable outcome after first-line tamoxifen monotherapy [27]. Group A p21-activated kinases, such as PAK2, are important (co) activators of the Ras/Raf/MEK/MAPK cascade [71,72], and it has been shown that PAK2 may be responsible for MEK1/2 Ser 217/221 phosphorylation (in mouse keratinocytes [73]). We hypothesize that PAK2 is involved in the phosphorylation of MEK and inhibition of MEK phosphorylation antagonizes IGF1R mediated AE resistance.

In a recent clinical trial, a combination of selumetinib and fulvestrant, did not improve outcome for patients with breast cancer progressing after aromatase inhibitor therapy [74]. To our knowledge, selumetinib in combination with AE therapy has not clinically been applied in tamoxifen and/or raloxifen resistant ER + breast cancer, whether IGF1R positive or not. It should also be noted that selumetinib was not the most potent AE resistance reverting compound for our MCF7/IGF1R and T47D/IGF1R cells; other MEK inhibitors such as PD0325901 and trametinib might be more effective in clinical studies. Interestingly, while our study was ongoing, Hew et al. [75] reported that selumetinib in combination with tamoxifen treatment, reverses AE resistance in an ER+, AE resistant ovarian cancer cell line *in vitro* and in mouse xenografts. This might indicate that MEK inhibition can be applied in a broader context to reverse AE in ER + cancer cells.

In conclusion, we have identified MEK inhibition as an important strategy to overcome IGF1R signaling-mediated AE resistance in breast cancer. Our work suggests that the responsiveness to combined MEK inhibitor and antiestrogen therapy depends on specific RTK expression such as IGF1R. Therefore, future patient selection in clinical trials could be based on RTK expression status and, thereby, optimize treatment outcome of combined MEK inhibitor/AE therapy. Our screens have been limited to the targeting of 42 kinases, leaving additional opportunities for novel kinase inhibitors targeting other kinases as alternatives to alleviate RTK-mediated AE resistance.

CRediT authorship contribution statement

L. Wester: Investigation, Conceptualization, Formal analysis, Data curation, Writing – original draft. **S. Venneker:** Investigation. **M. Hazenoot:** Resources, Investigation. **C. Pont:** Investigation. **E. Koedoot:** Formal analysis. **A.M. Timmermans:** Investigation. **J.W.M. Martens:** Supervision, Writing – review & editing. **M.P.H.M. Jansen:** Conceptualization, Formal analysis, Writing – original draft, Funding acquisition. **C.E.M. Kockx:** Investigation, Formal analysis, Writing – review & editing. **W.F.J. van IJcken:** Investigation, Formal analysis, Writing – review & editing. **J.H.N. Meerman:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Funding acquisition. **Y. Zhang:** Supervision, Writing – review & editing. **B. van de Water:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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