Combination of mTORC1/2 inhibitor vistusertib plus fulvestrant *in-vitro* and *in-vivo* targets estrogen receptor positive endocrine resistance breast cancer.

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Abstract

Background: Endocrine therapies are still the main strategy for the treatment of estrogen-receptor positive (ER+) breast cancers (BC) but resistance remains problematic. Cross-talk between ER and PI3K/AKT/mTORC has been associated with ligand-independent transcription of ER. We have previously reported the antiproliferative effects of the combination of everolimus (an mTORC1 inhibitor) with endocrine therapy in resistance models, but potential routes of escape via AKT signalling can lead to resistance, therefore the use of dual mTORC1/2 inhibitors have met with significant interest.

Methods: To address this, we tested the effect of vistusertib, a dual mTORC1 and mTORC2 inhibitor, in a panel of endocrine resistant and sensitive ER+ BC cell lines, with varying *PTEN*, *PIK3CA* and *ESR1* mutation status. End-points included proliferation, cell signalling, cell cycle and effect on ER-mediated transcription. Two patient-derived xenografts (PDX) modelling endocrine resistance were used to assess the efficacy of vistusertib, fulvestrant or the combination on tumour progression and biomarker studies were conducted using immunohistochemistry and RNA-seq technologies.

Results: Vistusertib caused a dose-dependent decrease in proliferation of all the cell lines tested and reduced abundance of mTORC1, mTORC2 and cell cycle markers, but caused an increase in abundance of EGFR, IGF1R and ERBB3 in a context dependent manner. ER-mediated transcription showed minimal effect of vistusertib. Combined therapy of vistusertib with fulvestrant showed synergy in two ER+ PDX models of resistance to endocrine therapy and delayed tumour progression after cessation of therapy.

Conclusions: These data support the notion that models of acquired endocrine resistance may have a different sensitivity to mTOR inhibitor/endocrine therapy combinations.

Keywords: breast cancer, estrogen receptor, mTORC1/2 signaling, vistusertib, endocrine resistance.

Background

The largest proportion of patients diagnosed with primary breast cancer (BC) have tumours which develop in response to the female hormone estrogen. Classically, patients with estrogen receptor (ER) positive BC are treated with endocrine therapy such as aromatase inhibitors (AI), which block estrogen synthesis, or with estrogen antagonists such as tamoxifen or fulvestrant. Despite the efficacy of these agents, resistance to endocrine therapy remains a major clinical problem (reviewed by [1]). *In vitro* and *in-vivo* studies suggest that cross-talk between the ER and growth factor signalling pathways can circumvent the need for steroid hormone. However, direct targeting of growth factors implicated in resistance has been met with limited success, largely as a result of tumour heterogeneity (reviewed [2]).

More recently, clinical studies have focused on targeting downstream of growth factor signalling, either by direct perturbation of PI3K/mTOR or CDK4/6 within the G1/S checkpoint. De-regulation of the PI3K/AKT/mTOR pathway has been strongly implicated in resistance to endocrine therapy. Loss of the tumour suppressor *PTEN* can lead to up-regulation of PI3K activity and has been associated with resistance to tamoxifen. Furthermore, up-regulation of growth factor signalling via IGFR can similarly increase activity, whilst loss of *LKB1* can activate mTOR in a growth factor independent manner. The PI3K/AKT/mTOR can directly activate ER in a ligand-independent manner via phosphorylation of AF-1 at serine 167 of the ER. Furthermore, AKT has been shown to alter the ER-cistrome (genome-binding pattern) effectively changing the ER-transcriptional program [3]. These bi-directional interactions between hormonal and

kinase signalling pathways potentiate pro-survival signals allowing BC cells to escape endocrine therapy blockade.

Based upon these observations, targeting this pathway clinically in combination with endocrine therapy has proven attractive. The BOLERO-2 study, in which patients who had progressed on a non-steroidal AI were randomised to receive the steroidal AI exemestane alone or in combination with the mTORC1 inhibitor everolimus, showed a doubling in progression free survival in response to the combination [4], an observation supported by the phase II TAMRAD trial which showed everomilus in combination with tamoxifen was superior to single agent [5].

Despite the efficacy of these agents, negative feedback-loops exist downstream of mTORC1 and lead to rapid tumour re-wiring resulting in increased activation of IGFR1-dependent AKT activity, which in the long term may limit their effectiveness. In recent years, new generation dual mTORC1/2 inhibitors have been developed which have the potential to negate the mTORC1 associated feedback-loops [6], a concept recently tested in the MANTA trial [7].

In this study, we explored the relevance of the dual mTORC1/2 inhibitor, vistusertib, in endocrine resistant and sensitive BC cell lines, as well as in patient derived xenograft (PDX) models and showed combination with fulvestrant had superior antiproliferative effects compared with fulvestrant alone. Furthermore, in a fulvestrant resistant PDX model, vistusertib resensitised the tumour to the antiproliferative effect of fulvestrant.

Methods

Antibodies and Reagents

The following primary antibodies were used in this study for immunoblotting: pRB^{ser780} (CST-3590), pRB^{ser807} (CST-8516), total-RB (CST-9309), cyclin D1 (CST-2922), cyclin D3 (CST-2936), pAKT^{ser473} (CST-9271), pAKT^{Thr308} (CST-9275), total-AKT (CST-9272), pEGFR^{Tyr1068} (CST-3777), total-EGFR (CST-2232), pERBB2^{Tyr1248} (CST-2243), total-ERBB2 (CST-4290), pERBB3^{Tyr1222} (CST-4784), pIGF1R^{Tyr1135} (CST-3918), pS6K$^{Ser235/236}$ (CST-2211), total-S6K (CST-2217), Raptor (CST-2280), RheB (CST-13879), p4EBP1$^{Thr37/46}$ (CST-2855), 4EBP1 (CST-9452), pSIN1^{Thr86} (CST-14716), SIN1 (CST-12860), pER^{ser167} (CST-5587), Rictor (CST-2114) and Deptor (SCT-11816) were purchased from Cell Signalling Technology. p107 (sc-318), p130 (sc-317), total-ER (sc-8002, F-10), ERBB3 (sc-415), IGF1R (sc-713) were purchased from Santa Cruz Biotechnology; β-tubulin (T-9026) were from Sigma-Aldrich; Ki67 from Clinisciences. The following antibodies were used for immunohistochemistry: pERK1/2$^{Thr202/4}$ (CST-4370), pAKT^{ser473} (CST-4060), pS6K$^{Ser235/6}$ (CST-4858), pmTOR^{Ser2448} (CST-2976) and p4EBP1$^{Thr37/46}$ (CST-2855) were purchased from Cell Signalling Technology. Ki67 was purchased from clinisciences. Reagents were obtained from the following sources: 17-β-estradiol (E2) and 4-hydroxytamoxifen (4-OHT) from Sigma-Aldrich; fulvestrant from Tocris; neratinib and vistusertib from SelleckChem.

Cell Culture

Human BC cell lines MCF7, SUM44, HCC1428, and T47D were obtained from the American Type Culture Collection, USA and Asterand. All cell lines were banked in

multiple aliquots to reduce the risk of phenotypic drift and identity confirmed using short tandem repeat (STR) analysis. Cells were routinely screened for mycoplasma contamination. Cells were maintained in phenol red-free RPMI1640 containing 10% foetal bovine serum (FBS) and 1nM estradiol (E2). Long-term estrogen derived (LTED) equivalents, modelling relapse on an AI were generated, as reported previously [8] and were maintained in phenol red-free RPMI1640 containing 10% charcoal-dextran stripped FBS (DCC). Tamoxifen-resistant (TAMR) MCF7 cells were generated by growing wild-type MCF7 long-term in the presence of RPMI1640 containing 10% DCC + 0.01nM E2 + 100nM 4-OHT. Fulvestrant resistant (ICIR) MCF7 and MCF7 LTED cell lines were generated by growing parental cells long-term in the presence of RPMI1640 containing 10% DCC + 1nM E2 + 100nM fulvestrant or RPMI1640 containing 10% DCC + 100nM fulvestrant, respectively. Palbociclib resistant (PalboR) cell lines were generated and maintained, as previously described [9, 10]. All cell lines were stripped of steroids for 48-72-hours prior to the start of experiments.

Proliferation Assays

Cells were seeded into 96-well tissue culture plates and allowed to attach overnight. Monolayers were then treated with increasing concentrations of the drugs and after 72-hours cell viability was determined using the CellTitre-Glo® Luminescent Cell Viability Assay (Promega), according to the manufacture's protocol. Values were expressed as relative luminescence compared to the vehicle treated control. Non-linear regression analysis was used to fit the curves and IC_{50} values were calculated using PRISM 7 software (Graphpad). To determine the nature of the interaction between vistusertib and

fulvestrant, combination studies were performed by using Chou and Talalay' s constant ratio combination design and quantified using Calcusyn software (BIOSOFT, Cambridge, UK) [11]. The combination indices (CI) were obtained by using mutually nonexclusive Monte Carlo simulations. In this analysis, CI scores significantly lower than 1 were defined as synergistic; CI > 1, as antagonistic; and a CI = 1, as additive.

Immunoblotting

All cells were grown in the presence of RPMI1640 containing 10% DCC for 3 days prior to seeding. Cells were seeded into dishes, allowed to attach overnight and treated with the appropriate drugs the following day. After 24 hours treatment, total protein was extracted and immunoblotting carried out, as previously described [8].

Real-time Quantitative PCR

mRNA from treated cells and from HBCx34 OvaR PDX models (n=30; [12]) was extracted using RNeasy Mini Kit (Qiagen), quantified and reverse-transcribed with SuperScriptIII First Strand Synthesis System (Invitrogen). Taqman gene expression assays (Applied Biosystems) were used to quantify *TFF1* (Hs00907239_m1), *PGR* (Hs01556702_m1), *GREB1* (Hs00536409_m1), *PDZK1* (Hs00275727-m1) and *ESR1* (Hs01046818_m1), *EGFR* (Hs01076090_m1), *ERBB2* (Hs01001580_m1), *ERBB3* (Hs00176538_m1), *IFG1R* (Hs00609566_m1) and/or *IRS1* (Hs00178563_m1) together with *FKBP15* (Hs00391480_m1) as housekeeping gene to normalise the data. The relative quantity was determined using ΔΔCt, according to the manufacturer's instructions (Applied Biosystems).

In vivo Patient-derived Xenografts

HBCx22 OvaR and HBCx34 OvaR PDX models resistant to endocrine therapy were established as stated previously [12], in accordance with the French Ethical Committee. Efficacy studies were carried out to determine the anti-tumour activity of vistusertib alone and combined to fulvestrant administered over 90 days. The treatment groups (10-12 mice per arm) received either vistusertib (15 mg/kg daily by oral gavage) or fulvestrant (5mg/mouse suspended in corn oil by weekly subcutaneous injection into the flank). These concentrations are in keeping with previous studies [6] and clinical achievable doses [13] for vistusertib. For the combination group, fulvestrant was dosed 2 hours before administration of vistusertib. The control groups received both vehicles. To assess whether treatment with vistusertib alone or in combination with fulvestrant could further delay tumour progression, five mice from each group were followed for an additional 40 days after drug withdrawal.

Tumour diameters were measured using calipers and volumes were calculated as $V = a \times b^2/2$, where "*a*" is the largest diameter and "*b*" is the smallest. Percent change in tumour volume was calculated for each tumour as (Vf - V0/V0) x 100, where V0 is the initial volume (at the beginning of treatment) and Vf is the final volume (at the end of treatment). Tumour regression (R) was defined as a decrease in tumour volume of at least 50% taking as reference the baseline tumour volume [14].

Tumour volumes were expressed relative to the initial starting volume (relative tumour volume (RTV)). Tumour growth inhibition (TGI) from the start of treatment was calculated as the ratio of the mean RTV between control and treated groups measured at

the same time. Because the variance in mean tumour volume data increases proportionally with volume (and is therefore disproportionate between groups), data were log-transformed to limit any size dependency before statistical evaluation. Statistical significance of TGI was calculated by the paired Student t test by comparing the individual RTVs in the treated and control groups.

Immunohistochemistry

In order to assess biomarker changes, a pharmacodynamic study was performed for 4 days of treatment with vistusertib, fulvestrant or a combination of the two drugs in the HBCx22 OvaR PDX model. Mice were sacrificed at 4 hours after the final treatment and tumours resected. Excised tumours were fixed in 10% neutral buffered formalin, paraffin embedded and tissue microarrays (TMA) were built from the blocks. Three xenografts from each treatment group and two tissue cores per tumour were included in the TMA. Sections from the TMA were cut and stained for the expression of biomarkers, as previously described [12]. The immunohistochemically stained TMA sections were digitally scanned at ×20 with a Hamamatsu NanoZoomer- XR whole-slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan). The quality of the images was checked manually and the images were analysed with Visiopharm integrator system (VIS) version 2018.9.3.5303 (Visiopharm A/S) using VIS ready to use automated image analysis algorithms (APPs).

RNA-seq

Excised tumours from HBCx34 OvaR PDX sacrificed mice were used for a gene expression study (n=12; 3 mice by group). Libraries were created after using Truseq Stranded mRNA Library Prep Kit (Illumina) and sequenced using the NextSeq500 (Illumina). RNA-seq data was aligned to human GRCh38 reference genome using STAR Aligner (star v2.6.1a) [15], read count for each gene were calculated with htseq (v0.6.1) [16]. Genes were compared for differential expression between the different treatments using edgeR [17], and were considered to be statistically expressed when absolute fold-change >= 2 and FDR < 5%. These significantly expressed gene lists were subject to further functional annotation using Ingenuity Pathway Analysis (IPA) to identify altered pathways due to the corresponding treatments. For individual pathways, the Benjamini–Hochberg procedure was used to the calculate false discovery rate (FDR) in order to adjust for multiple testing. RNA-seq data supporting the findings was deposited in the NCBI (http://ncbi.nlm.nih.gov/geo/) with reference PRJNA564917.

Results

Inhibitory effects of vistusertib on BC cell proliferation

We tested the antiproliferative effect of vistusertib in a panel of isogenic cell lines modelling sensitivity or resistance to endocrine therapy (MCF7, SUM44, HCC1428 and T47D) for which the *PIK3CA*, *PTEN* and *ESR1* mutation status was previously established [18, 19]. Assays were conducted in the presence of E2, to model the effects of vistusertib as a monotherapy, or in the absence of E2, to model the combination with an AI in the primary setting. MCF7 cells showed a concentration dependent decrease in

proliferation in the presence of E2 with an IC_{50} of 20nM. In the absence of E2, minimal further antiproliferative effect was evident from the addition of vistusertib and the IC_{50} was increased (Figure 1a, **Additional File 1: Table 1a)**. In an extended panel of ER+ cell lines, in the presence of E2, vistusertib sensitivity varied with IC_{50} values between 30-500nM (**Additional File 2: Figure S1a and Additional File 1: Table 1a**). Removal of E2 caused a drop in proliferation in all cell lines, as expected. Addition of vistusertib further reduced cell viability in a dose dependent manner (IC_{50} values between 40-700nM; **Additional File 2: Figure S1a and Additional File 1: Table 1a**). In order to assess the effect of vistusertib in cell lines modelling resistance to an AI, escalating concentrations were tested in two MCF7 LTED models in the presence or absence of E2. Of note, the MCF7 LTED^{Y537C}, which harbour a hotspot *ESR1* mutation in the ligand-binding domain, showed sensitivity with an IC_{50} of 50nM in the presence or absence of E2, in keeping with their ligand independent phenotype (Figure 1b). Contrastingly, MCF7 LTED^{wt} showed an IC_{50} slightly higher (75nM) (Figure 1c). Three further LTED cell lines were assessed. HCC1428 LTED expressing wild-type (wt) *ESR1*, SUM44 LTED harbouring *ESR1*^{Y537S} and T47D LTED which lose ER expression showed varying IC_{50} values between 65-350nM (**Additional File 2: Figure S1b and Additional File 1: Table 1a**).

We further assessed sensitivity to vistusertib in cell lines modelling resistance to tamoxifen (TAMR) or fulvestrant (ICIR). In keeping with the previous data, both models showed a concentration-dependent decrease in proliferation with IC_{50} values of 85nM and 50nM, respectively (Figure 1d-e and **Additional File 1: Table 1b**). Finally, we assessed the effect of escalating doses of fulvestrant both in the presence or absence of a fixed

concentration of vistusertib in MCF7 LTED^{wt} and MCF7 LTED^{Y537C} cell lines (Figure 1f-g and **Additional File 1: Table 1c**). In both cell line models, the combination with vistusertib appeared synergistic with combination index below 1.

These data suggest that vistusertib may provide benefit in combination with an AI in patients with *de novo* endocrine resistance and showed efficacy in models of acquired endocrine resistance irrespective of *ESR1* mutation status or ESR1 protein abundance.

Effect of vistusertib on receptor tyrosine kinase and downstream signalling pathways.

Previous studies have shown that blockade of mTORC1 can lead to feedback loops via IGFR and ERBB signalling networks [20, 21] (Figure 2a). In order to test the effect of targeting both mTORC1 and mTORC2, we examined the effect of vistusertib upon key protein targets within the mTOR pathway. Immunoblot analysis of the MCF7 and LTED derivatives was assessed (Figure 2b). Vistusertib caused a decrease in expression of pS6RP$^{Ser235/6}$, p4EBP1$^{Thr37/46}$ and pAKT^{Ser473} and an increase in Deptor and pSin1 together with a decrease in abundance of Cyclin D1, D3 and pRB indicative of cell cycle arrest. Treatment with fulvestrant alone or in combination with vistusertib reduced abundance of both phosphorylated and total ER. Despite the dual blockade of mTORC1/2, feedback loops via IGF1R and ERBB family members were evident but appeared cell line specific. For instance, MCF7 LTED^{wt} showed marked increases in pIGF1R and pAKT^{Thr308} in response to vistusertib. To test if the effect of vistusertib was persistent beyond a 24 hours period, we performed a time course experiment and showed

a gradual increase in abundance of pEGRF, pIGF1R and pSin1 markers up to 96 hours of treatment (**Additional File 3: Figure S2**)

Effect of vistusertib alone or in combination with fulvestrant on ER-mediated transcription

Evidence suggests that cross-talk between PI3K/AKT/mTOR impacts on ER function as a transcription factor. Indeed, mTORC1 via S6RP has been shown to phosphorylate ER at serine 167 [22]. We therefore assessed the effects of vistusertib on ER-mediated transcription. The relative expression of a panel of estrogen-regulated genes (ERGs: *TFF1*, *PGR*, *GREB1* and *PDZK1*) was evaluated in the presence or absence of E2. In MCF7 and in both MCF7 LTED derivatives, treatment with vistusertib under DCC conditions, caused subtle or no changes in expression of ERGs that was gene- and cell-specific (**Additional File 4: Figure S3**). Similarly, in the presence of 0.01nM of E2, vistusertib caused small changes in the expression of the ERGs for all the three cell lines tested, but fulvestrant alone or in combination with vistusertib consistently reduced expression of all the ERGs when compared with the vehicle control (Figure 3). These data suggest that vistusertib does not impact in ER-mediated transcription.

Vistusertib in combination with fulvestrant impedes tumour progression in human BC PDX models of acquired endocrine resistance

In order to assess the effect of vistusertib alone or in combination with fulvestrant *in vivo*, we adopted two PDX models of acquired endocrine resistant BC. HBCx34 OvaR is an ER+ PDX which is resistant to E-deprivation and tamoxifen but sensitive to the anti-

proliferative effects of fulvestrant [12] (Figure 4). After a period of 64 days, all treatments showed over a 95% reduction in tumour volume (fulvestrant: 97.6%, p=0.004; vistusertib: 96.2%, p<0.0001; combination: 99.7%, p<0.0001) compared to vehicle control (Figure 4a and **Additional File 5: Figure S4**). Vistusertib showed greater efficacy than fulvestrant as a monotherapy over the first 50 days (adjusted p-value=0.005) and appeared similar to the combination over this time period. At the end of treatments, all xenografts were in regression or complete response in the combination arm (% of tumour volume change $\leq$ 50%), against 4 xenografts in the fulvestrant-treated group (Figure 4a).

Analysis of the combination of vistusertib and fulvestrant appeared the most effective showing a significant increase in efficacy compared to fulvestrant alone (p=0.0001, Mann-Whitney test, Figure 4a).

In order to further explore the impact of vistusertib alone or in combination with fulvestrant, tumours were resected at the end of the study and subjected to RNA-seq. Fulvestrant showed the greatest impact on gene expression (1456 upregulated and 1077 downregulated genes) versus vistusertib (291 upregulated and 174 downregulated genes) when compared with vehicle control (Figure 4b). Noteworthy, the number of gene changes as a result of the combination largely reflected that seen for fulvestrant (1717 upregulated and 1412 downregulated genes) indicating the mitogenic driver within this PDX remains ER. In order to identify canonical pathways affected by these treatments, we conducted ingenuity pathway analysis (IPA; FDR< 5%) using differentially expressed genes (FDR < 5% and fold-change >=2; **Additional File 6: File S1**). Fulvestrant showed a dominant effect on cell cycle and estrogen-mediated S-phase entry both as a

monotherapy or in combination with vistusertib. Contrastingly, single agent vistusertib showed no impact on ER-mediated S-phase entry. Treatment with vistusertib showed minimal although significant enrichment of EGF, ERBB, and ERK/MAPK signalling compared with vehicle control (**Additional File 6-File S1**). In order to explore this further, we carried out targeted qRT-PCR (Figure 4c). Treatment with fulvestrant significantly reduced expression of *TFF1*, *PGR*, *GREB1*, *IRS1* but increased expression of *EGFR*, *ERBB2* and *ERBB3*. Contrastingly, vistusertib had minimal effect on expression of *ESR1*, *GREB1* and *PGR*; however, it significantly reduced *TFF1* but not to the degree seen with fulvestrant or the combination. Noteworthy, vistusertib significantly increased expression of *EGFR* but not *ERBB2*, *ERBB3* or *IGF1R*.

In order to further explore the efficacy of the combination of vistusertib with fulvestrant, a second PDX model, HBCx22 OvaR, was assessed. HBCx22 OvaR is an ER+ model showing partial resistance to fulvestrant and harbours a 24 base-pair in-frame deletion in exome 13 in *PIK3R1* [12] (Figure 5). As expected, single agent fulvestrant had no significant impact on tumour progression compared to vehicle control, confirming the resistant phenotype. Vistusertib as a monotherapy delayed tumour progression by 54.5% ($p=0.04$) compared to vehicle control. The combination of vistusertib plus fulvestrant was the most effective treatment with tumour volumes 84.7% lower than vehicle control ($p=0.0002$) (Figure 5a). After 93 days of treatment, the therapies were withdrawn and the tumour volumes assessed for a further 40-days in order to establish the efficacy of the drugs in delaying tumour progression (Figure 5b). Removal of therapies showed sustained anti-tumour effect in the combination group, whilst, tumours treated with vistusertib alone showed significant progression.

In order to assess dynamic changes, three mice per arm were sacrificed after 4 days of therapy and tissue sections were subjected to immunohistochemical analysis. Treatment with vistusertib or vistusertib in combination with fulvestrant revealed suppression of $pAKT^{Ser473}$, $p4EBP1^{Thr37/46}$ and $pS6RP^{Ser235/6}$, as well as a slight but noticeable decrease in $pmTOR^{Ser2448}$ (Figure 5c and **Additional File 7: Figure S5a**). Furthermore, fulvestrant reduced expression of $pERK1/2^{Thr202/4}$ both alone and in combination with vistusertib. In contrast to our *in vitro* analysis, no alteration in abundance of pEGFR and pIGFR were evident in response to vistusertib alone, whilst pEGFR was significantly suppressed by the combination with fulvestrant (**Additional File 7: Figure S5b**). Noteworthy, assessment of Ki67 showed the greatest reduction when the combination of vistusertib and fulvestrant was used (**Additional File 7: Figure S5a**).

Taken together, these data suggest the combination may provide greater efficacy than fulvestrant alone in ER+ acquired endocrine resistant disease.

Effectiveness of vistusertib in combination with pan-ERBB inhibitors and in models of resistance to palbociclib

As increased feedback loops via ERBB and IGF1R family members were evident *in vitro* and from our gene expression analysis, we assessed sensitivity of MCF7-$LTED^{wt}$ cell lines to the antiproliferative effect of vistusertib, or fulvestrant combined with the pan-ERBB inhibitor neratinib, or the combination of all three agents (Figure 6a). Fulvestrant and neratinib enhanced the antiproliferative effect of vistusertib, however, the triple combination was most effective. These data further support previous observations in

which the triple combination targeting three cellular nodes: ERBB, ER and mTORC1 showed greatest antiproliferative effect [20].

More recently, CDK4/6 inhibitors have become the standard of care in the treatment of endocrine resistant ER+ BC. Despite their efficacy, not all patients benefit and many will eventually relapse with acquired resistance. Studies suggest that cross-talk exists between CDK4 and the mTOR pathway via pTSC2 [23] and that blockade of mTORC1/2 may delay onset of resistance to CDK4/6 inhibition [24]. To assess this, we treated three palbociclib resistant cell line models (MCF7-^{PalboR}, MCF7 LTED-^{PalboR} and T47D-^{PalboR}) (Figure 6b) with escalating concentrations of vistusertib with or without fulvestrant. All three cell lines showed sensitivity to mTORC1/2 blockade. The addition of fulvestrant further enhanced the antiproliferative effect. Taken together these data suggest mTORC1/2 blockade remains effective after acquisition of resistance to palbociclib.

Discussion

Cross-talk between the PI3K/AKT/mTOR pathway and ER is well documented and targeting this pathway with mTORC1 inhibitor, everolimus, has shown marked efficacy [25]. However, negative feedback loops have been identified leading to activation of growth factor signalling pathways and reduced drug sensitivity [21] [20]. In this study, we assessed the efficacy of the mTORC1/2 inhibitor, vistusertib, *in vitro* and in PDX models of endocrine resistance. In summary, we show that vistusertib as a monotherapy had little impact on global gene expression compared to fulvestrant and did not significantly impact on ER-mediated transactivation. These findings are in contrast to

previous studies which have shown that inhibition of PI3K leads to an open chromatin state at estrogen target loci resulting in enhanced ER-mediated transactivation, supporting the concept of combined PI3K and endocrine therapies [26]. However, our observations are in keeping with a recent study which explored the impact of mTORC suppression on the genome wide recruitment of ER which showed no alteration in binding patterns compared to vehicle control [24]. This would suggest that direct cross-talk may be restricted to PI3K and AKT [26] [3].

Vistusertib as a single agent significantly suppressed the abundance of pS6 and p4EBP1 both *in vitro* and *in vivo*. In contrast to our previous studies with everolimus [20] [21], vistusertib decreased abundance of $pAKT^{ser473}$, whilst increasing $pAKT^{thr308}$ indicative of efficient suppression of both mTORC1 and mTORC2 activity. In addition, AZD2014 may display different target engagement properties from everolimus, which may in turn lead to different clinical efficacy. Nonetheless, we found evidence of increased expression of pEGFR and pIGF1R in a context specific manner suggesting that tumour re-wiring and feedback loops previously associated with poor response to mTORC1 suppression, were evident. However, despite this, cell proliferation was significantly reduced both *in vitro* and *in vivo*. Moreover, the enhanced expression of growth factor receptors, in particular members of the ERBB family, were far more pronounced with fulvestrant.

There are two underlying mechanisms by which EGFR can be increased in this context. Firstly, suppression of mTOR leads to loss of phosphorylated TCS2 and suppression of S6, leading to the removal of the negative feedback loop resulting in increased expression of EGFR [23]. Conversely, ER is known to cross-talk with EGFR/ERBB2 and studies

suggest that ER sequesters the coactivators AIB1 and SRC1 leading to the suppression of ERBB2 signalling, whilst in the presence of fulvestrant, downregulation of ER-function would lead to the converse [27] [28]. Despite this early re-wiring, the combination of vistusertib and fulvestrant showed enhanced anti-tumour activity which was maintained even after cessation of drug in PDX model resistant to fulvestrant.

It is noteworthy, in our HBCx34 model which is PTEN competent and ER+, that ER expression remains the dominant mitogenic driver. In this context, mTORC1/2 suppression is sufficient to impede tumour progression, most likely as the PI3K pathways is not hyperactivated. In addition, this PDX is sensitive to fulvestrant and thus combining blockade of ER and mTORC1/2 significantly impedes tumour progression. Contrastingly, HBCx22 shows hyperactivation of the PI3K/AKT/mTOR pathway as a result of a *PIK3R1* frameshift and despite continuing to express high levels of ER, is resistant to fulvestrant. In this setting, monotherapy targeting ER or mTORC1/2 is insufficient to have prolonged anti-tumour effect whilst the combination targeting both pathways suppresses tumour progression even after cessation of therapy.

The recent MANTA trial explored the concept of targeting both ER and mTORC1/2 in patients with primary and secondary AI therapy resistant disease. Patients were randomised to single agent fulvestrant versus fulvestrant in combination with vistusertib or everolimus. Although not significant, the combination of vistusertib plus fulvestrant showed a trend towards improved progression free survival in the first year compared to fulvestrant as a single agent (median 7.6-8.0 versus 5.4 months). However, the combination of fulvestrant plus everolimus appeared superior increasing progression free survival from 5.4 to 12.3 months [7]. The lack of a significant effect of the combination

of vistusertib plus fulvestrant compared to everolimus may reflect the differences in target engagement properties for the two compounds, or alternatively different dependency of patients who have relapse on AI therapy on mTORC1 signalling. These data are in contrast to those seen in our PDX models and one explanation could be that prior treatment influences responses to secondary combinations. For instance, the most powerful antiproliferative effects seen in our study was associated with resistance to fulvestrant. This suggests that in patients with acquired resistance, previous lines of endocrine therapy should be considered to guide treatment choices

Lastly, as noted, CDK4/6 inhibitors are changing the face of therapy for ER+ BC ([29, 30], however, not all patients will respond, and many will acquire resistance. Previous studies have shown that the combination of mTORC1/2 inhibition with a CDK4/6 inhibitor enhances E2F suppression and delays onset of resistance as well as circumventing it [24]. In order to corroborate these observations we assessed vistusertib sensitivity in a panel of cell lines with acquired resistance to palbociclib [9, 10]. Unlike the previous study, our cell lines utilised different resistance mechanisms including loss of RB copy number (T47D-^{PalboR}) and tumour re-wiring via increased growth factor signalling (MCF7-^{PalboR} and MCF7-LTED^{PalboR}). Vistusertib effectively suppressed the proliferation of all models tested and this effect was enhanced by the addition of fulvestrant. These data provide further support for the concept that mTORC1/2 inhibitors may provide utility after acquisition of resistance to CDK4/6 inhibitors.

Conclusion

In summary, our data suggests that suppression of mTORC1 and mTORC2 has no significant impact on ER-mediated transcription but combination therapy with fulvestrant shows synergistic benefit. Patients with secondary acquired resistant ER+ BC may have

different sensitivities to mTOR inhibition in combination with endocrine therapy. Finally, mTORC1/2 inhibitors may provide utility after relapse on CDK4/6 inhibitors.

List of abbreviations

ER: estrogen receptor; ER+: estrogen receptor positive; BC: breast cancer; AI: aromatase inhibitors; E2: estradiol; LTED: long-term estrogen deprived; FBS: fetal bovine serum; DCC: dextran charcoal; 4-OHT: 4-hydroxytamoxifen; RTK: receptor tyrosine kinase; PDX: patient derived xenografts; FBS: foetal bovine serum; RVT: relative tumour volume; TGI: tumour growth inhibition; TMA: tissue microarray; TAMR: tamoxifen-resistance; ICIR: fulvestrant-resistance; ERGs: estrogen regulated genes.

Declarations

Ethics Approval: *In-vivo* studies were carried out in accordance with French Ethical Committee.

Consent of publication: All authors approved the final version of this manuscript.

Availability of Data and materials: RNA-seq data supporting the finding from this manuscript was deposited in the NCBI (http://ncbi.nlm.nih.gov/geo/) with reference PRJNA564917.

Competing of interests: SRJ, MD and LAM receive academic funding from Pfizer, Puma Biotechnology Inc. and AstraZeneca. MD receives honoraria from Myriad Genetics and speaker's bureau of Roche, is a consultant and advisory board member of Radius, GTx and Orion Pharma and has received remuneration from the ICR rewards to Inventors Schemes. SRJ is a consultant/ independent contractor for AstraZeneca,

Novartis, Pfizer, OBI, Eli Lilly and Company and is on the speaker's bureau for OBI and Puma. No potential conflict of interests was disclosed by the other authors. SCC is an employee of AstraZeneca.

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Authors' contribution: SP, MFL, RR, NS, SC-J, MH, AD and EM performed experimental work; SP, MFL, RR, ES, LZ, QG and L-AM analysed and interpreted the data; SP, MFL, RR and L-AM wrote the manuscript; SRJ, MD, SCC and L-AM conceived and design the study; L-AM supervised the study.

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Figure Legends

Figure 1. Effect of vistusertib alone or in combination with endocrine agents in several cell line models of endocrine sensitivity and resistance BC. (a-c) Effect of escalating doses of vistusertib on proliferation of (**a**) MCF7, (**b**) MCF7 LTED^{Y537C} and (**c**) MCF7 LTED^{wt} cell lines in the absence and in the presence of 0.01nM E2. (**d-e**) Effect of escalating doses of vistusertib on proliferation of (**d**) tamoxifen (MCF7 TAMR) and (**e**) fulvestrant resistant (MCF7 ICIR and MCF7 LTED ICIR) cell lines. (**f-g**) Effect of escalating doses of fulvestrant in the presence or absence of 75nM of vistusertib on both (**f**) MCF7 LTED^{wt} and (**g**) MCF7 LTED^{Y537C} (left panels) and respective combination index heatmaps (right panels). Data are expressed as luminescence relative to vehicle control. Cell viability was analysed using a CellTiter-Glo assay. Error bars represent mean ± SEM.

Figure 2. Effect of vistusertib on RTKs and downstream signalling pathways. (a) Schematic representation of the PI3K/AKT/mTOR signalling pathway and cross-talk with RTKs. (**b**) Effect of vistusertib alone on in combination with fulvestrant on mTORC1, mTORC2, cell cycle, ER and RTKs targets, both in the presence or absence of 0.01nM E2.

Figure 3. Effect of vistusertib alone or in combination with fulvestrant in ER-mediated transcription. MCF7, MCF7 LTED^{wt} and MCF7 LTED^{Y537C} were treated in the presence of 0.01nM E2 with vistusertib, fulvestrant or the combination for 24-hours and effects on *TFF1, PGR, GREB1* and *PDZK1* were assessed by RT-qPCR. Error bars represent means ± SEM. Vist= vistusertib; Fulv= fulvestrant, Vist + Fulv= combination treatment.

Figure 4. Effect of vistusertib alone or in combination with fulvestrant on tumour progression in HBCx34 OvaR PDX models. (a) Long-term study assessing changes in tumour volume over 64 days of treatment in HBCx34 OvaR. HBCx34 OvaR is an ER+ PDX model which is resistant to E-deprivation and tamoxifen but sensitive to the anti-proliferative effects of fulvestrant. Mice were treated with vehicle control, fulvestrant, vistusertib or the combination and data shows median tumour volume (mm^3). Bars represent % of volume change at the end of treatment compared with baseline, for each individual animal. (**b**) Venn diagram showing the intersect of genes up and downregulated for the different treatments by RNA-seq analysis; tumours of three

699 animals by group were evaluated. (**c**) Effect of vistusertib (n=10), fulvestrant (n=8) or the
700 combination (n=3) in relation to vehicle (n=9) upon relative RNA expression of ERGs
701 and RTKs by RT-qPCR. Error bars represent means ± SEM. Statistical analysis was
702 performed using Anova with Dunnett's multiple comparisons test. [#]Tendency to
703 difference between groups by t-test. Vist= vistusertib; Fulv= fulvestrant, Vist + Fulv=
704 combination treatment.
705
706 **Figure 5. Effect of vistusertib alone or in combination with fulvestrant on tumour**
707 **progression in HBCx22 OvaR PDX models.** (**a**) Long-term study assessing changes in
708 tumour volume over 93 days of treatment in HBCx22OvaR. HBCx22 OvaR is an ER+
709 model that shows partial resistance to fulvestrant. Mice were treated with vehicle control,
710 fulvestrant, vistusertib or the combination. Data represents mean relative tumour volume
711 ± SEM. (**b**) Effect of vistusertib alone or in combination with fulvestrant on tumour
712 growth of individual mice over a period of 93 days. Treatments were withdrawn and
713 tumour growth reassessed for a further 40 days to establish the efficacy of the drugs in
714 delaying tumour progression. (**c**) Immunohistochemical analysis of several markers
715 following treatment for a period of 4 days with either vehicle, vistusertib (Vist),
716 fulvestrant (Fulv) or the combination of both (Vist + Fulv). Tumours were harvest 4
717 hours after last treatment. Statistical analysis was performed using ANOVA with
718 Dunnett's multiple comparisons test. [#]Tendency to difference between groups by t-test.
719
720 **Figure 6. Effect of vistusertib in combination with neratinib/ fulvestrant in cell line**
721 **models of endocrine and palbociclib resistance BC.** (**a**) Effect of escalating doses of

vistusertib in combination with fulvestrant (1nM) (Fulv) and neratinib (500nM) on proliferation of MCF7 LTED^{wt} cell lines in the presence of 0.01nM E2. Data are expressed as percentage of viable cells relative vehicle control. (**b**) Effect of escalating doses of vistusertib with or without fulvestrant (1nM) on proliferation of palbociclib resistant cell lines MCF7^{PalboR}, MCF7 LTED^{PalboR} and T47D^{PalboR} cell lines. Data expressed as luminescence. Error bars represent mean ± SEM.

Additional Files

Additional File 1: Table S1a-c. IC_{50} values for antiproliferative effect of (**a**) vistusertib for several endocrine sensitive and resistant cell line models both in the presence or absence of 0.01nM E2, (**b**) vistusertib in cell line models of resistance to tamoxifen (TAMR) and fulvestrant (ICIR); (**c**) fulvestrant alone or in combination with 75nM of vistusertib in the presence of 0.01nM E2.

Additional File 2: Figure S1. Effect of vistusertib in models of endocrine sensitive and resistant BC. (a) Effect of escalating doses of vistusertib on proliferation of endocrine sensitive (HCC1428, T47D and SUM44) and (**b**) endocrine resistant (HCC1428 LTED, T47D LTED and SUM44 LTED^{Y537S}) cell line models both in the absence and in the presence of 0.01nM E2. Data are expressed as relative luminescence and represented as fold-change relative to vehicle DCC control for each cell line condition.

Additional File 3: Figure S2. Effect of vistusertib on RTKs and downstream signalling pathways over a time course of 96 hours. MCF7 LTED^{wt} were treated for a time-course period of 24, 48, 72 and 96 hours with or without vistusertib (100nM) in the presence or absence of E2 (0.01nM).

Additional File 4: Figure S3. Effect of vistusertib in ER-mediated transcription. MCF7, MCF7 LTED^{wt} and MCF7 LTED^{Y537C} were treated in the absence of E2 with vehicle or vistusertib for 24 hours and effects on *TFF1, PGR, GREB1* and *PDZK1* were assessed by RT-qPCR (n=2 biological and n=3 technical replicates). Error bars represent means ± SEM. Note, as MCF7 LTED^{wt} do not express *PGR*, this was excluded from the analysis.

Additional File 5: Figure S4. Effect of vistusertib alone or in combination with fulvestrant on tumour progression in HBCx34 OvaR PDX models. (a) Assessment of tumour volume in individual animals treated with vehicle, fulvestrant, vistusertib or the combination.

Additional File 6: File S1. Ingenuity pathway analysis of the HBCx34 OvaR PDX models at the end of the study

Additional File 7: Figure S5. Representative immunohistochemistry images of (a) expression of Ki67, mTOR, pAKT^{ser473}, p4EBP1, pS6 and pERK1/2 and (b) pEGFR and pIGF1R in HBCx22 OvaR PDX models following treatment for a period of 4

days with either vehicle, vistusertib (Vist), fulvestrant (Fulv) or the combination of both (Vist + Fulv).