Targeting tumour re-wiring by triple blockade of mTORC1, epidermal growth factor and estrogen receptor signalling pathways in endocrine resistant breast cancer

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Abstract

Background: Endocrine therapies are the mainstay of treatment for estrogen-(E)-receptor positive (ER+) breast cancer (BC). However, resistance remains problematic largely due to enhanced cross-talk between ER and growth-factor pathways, circumventing the need for steroid-hormones. Previously, we reported the antiproliferative effect of everolimus (RAD001-mTORC1 inhibitor) with endocrine therapy in resistance models however, potential routes of escape from treatment via ERBB2/3 signalling were observed. We hypothesised combined targeting of three cellular nodes: ER, ERBB and mTORC1 may provide enhanced long-term clinical utility.

Methods: A panel of ER+ BC cell lines adapted to E-deprivation (LTED) and expressing $ESR1^{wt}$ or $ESR1^{Y537S}$, modelling acquired resistance to an aromatase-inhibitor (AI), were treated *in-vitro* with a combination of RAD001 and neratinib (pan-ERBB inhibitor) in the presence or absence of estradiol (E2), tamoxifen (4-OHT) or fulvestrant (ICI182780). End points included proliferation, cell signalling, cell cycle and effect on ER-mediated transactivation. An i*n-vivo* model of AI-resistance was treated with monotherapies and combinations to assess the efficacy in delaying tumour progression. RNA-seq analysis was performed to identify changes in global gene expression as a result of the indicated therapies.

Results: Here, we show RAD001 and neratinib (pan-ERBB inhibitor) caused a concentration-dependent decrease in proliferation, irrespective of the *ESR1* mutation status. Combination of either agent with endocrine therapy further reduced proliferation but the maximum effect was observed with a triple combination of RAD001, neratinib and endocrine therapy. In the absence of E, RAD001 caused a reduction in ER-mediated transcription in the majority of the cell lines, which

associated with a decrease in recruitment of ER to an E-response element on the *TFF1* promoter. Contrastingly, neratinib increased both ER-mediated transactivation and ER recruitment, an effect reduced by addition of RAD001. *In-vivo* analysis of an LTED model showed the triple combination of RAD001, neratinib and fulvestrant was most effective at reducing tumour volume. Gene-set-enrichment-analysis revealed addition of neratinib negated the EGF/EGFR feedback loops associated with RAD001.

Conclusions: Our data supports the combination of therapies targeting ERBB2/3 and mTORC1 signalling together with fulvestrant in patients who relapse on endocrine therapy and retain a functional ER.

Keywords: breast cancer, estrogen receptor, neratinib, everomilus, endocrine resistance.

Background

Breast cancer (BC) is the most common malignancy in women, responsible for over 522,000 deaths in 2012 [1]. The majority of the BCs at primary diagnosis are estrogen receptor-alpha positive (ER+) and depend on estrogen (E) for their growth and progression. Endocrine therapies targeting estrogenic stimulation of tumour growth have been developed clinically and have shown success, reducing mortality of ER+ BC. These include tamoxifen, which competes with E for the ER; fulvestrant (ICI182780), which binds to ER and targets it for degradation; and aromatase inhibitors (AIs), which block the conversion of androgens to E [2]. Despite the initial effectiveness of these approaches, many patients eventually relapse with either intrinsic or acquired resistance and in most cases continue to express ER [3, 4]. Studies suggest that *ESR1* mutations within the ligand binding domain of the receptor

and/or cross-talk between ER and various cellular kinases allow the receptor to circumvent the need for steroid hormone [5]. In recent years, emphasis has been placed on co-targeting both the ER and the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTORC) pathway, known to phosphorylate and activate ER in a ligand-independent manner [6], to avoid or reverse these resistance mechanisms.

The combination of the rapalogue everolimus (RAD001) with exemestane, as third line therapy in ER+/ERBB2- negative patients who relapsed on prior endocrine therapy, was reported from the BOLERO-2 trial to increase median progression-free survival (PFS) from 4.1 to 10.6 months compared to exemestane alone [7]. Nonetheless, it is clear that blockade of a single protein in a complex signalling cascade, even if a critical downstream effecter, is unlikely to provide a total or prolonged growth inhibition partly as a result of early rewiring. For instance, a negative feedback-loop exists downstream in the PI3K/AKT/mTORC pathway such that mTORC1 inhibition leads to a reduction in S6K1 activity, which in turn allows IRS1/2 expression to be increased with associated enhanced activation of IGFR1-dependent AKT activity [8]. Furthermore, mTORC1 blockade has also been shown to induce enhanced ERBB2/3 signalling [9], as well as ERK1/2 [8, 10], creating potential routes of escape negating the anti-tumour effectiveness of mTORC1 blockade and limiting long-term effectiveness (Fig. 1). This may account for the short-term clinical remissions and lack of stable disease, often with re-bound growth at the time of further disease progression. As such, it rational to explore targeting of the mTORC1 with vertical blockade of growth factor receptors, such as those governing ERBB signalling (Fig. 1).

In this study, we assessed the effect of the combined therapy of mTORC1 inhibitor, RAD001, with additional co-blockade of ERBB signalling with neratinib, an irreversible pan-ERBB receptor tyrosine kinase (RTK) inhibitor [11] [12] [13] in human BC cell models of endocrine sensitive and resistant disease with varying *ESR1*, *PIK3CA* and *ERBB2* mutation status. Analysis showed that triple blockade of the three signalling nodes had greater efficacy than monotherapies both *in-vitro* and *in-vivo* and that the triple combination was well tolerated in xenograft model.

Methods

Reagents

Primary antibodies against phospho-EGFR^{tyr1068} (CST-3777), total-EGFR (CST-2232), phospho-ERBB2^{tyr1248} (CST-2247), phospho-ERBB3^{tyr1222} (CST-4784), total-ERK1/2 (CST-9102), phospho-AKT^{ser473} (CST-9271), total-AKT (CST-9272), phospho-S6$^{ser240/244}$ (CST-5364), total-S6 (CST-2217), phospho-ER^{ser167} (CST-5587), phospho-Rb$^{ser807/811}$ (CST-8516), CDK4 (CST-2901) and cyclinD1 (CST-2922) were purchased from Cell Signaling, Inc.; total-ERBB3 (sc-415), ER-alpha (sc-8002, F-10) and PARP (sc-8007) were purchased from Santa Cruz; phospho-ERK1/2 and α-tubulin (T-9026) were obtained from Sigma and total-ERBB2 from Millipore. Secondary antibodies (anti-mouse and anti-rabbit horseradish peroxidase) were obtained from Dako. 17 β-estradiol (E2) and 4-hydroxytamoxifen (4-OHT) were purchased from sigma and fulvestrant (referred to as ICI) was obtained from Trocis, UK. Neratinib (PB272) was provided by Puma Biotechnology and Pfizer. Everolimus (RAD001) was purchased from Selleck. All chemicals, unless otherwise stated, were purchased from Sigma, UK. All tissue culture grade plastics were obtained from Nunc, UK.

155

156 **Tissue Culture**

157 The human BC cell lines were obtained from the ATCC Rockville, USA or Asterand
158 and authenticity was confirmed by STR. Cells were aliquoted to prevent phenotypic
159 drift and routinely tested for *Mycoplasma* contamination. Wild-type (wt)-MCF7, wt-
160 HCC1428 and wt-SUM44 cell lines were cultured in phenol-red free RPMI medium
161 supplemented with 10% fetal bovine serum (FBS) and 1nM E2. MCF7, HCC1428 and
162 SUM44 cells adapted to long-term E deprivation (LTED) and modeling resistance to
163 an AI were maintained in phenol-red free RPMI medium containing 10% dextran
164 charcoal stripped serum (DCC) in the absence of E2 [14]. Cells were passaged twice
165 weekly and fed every 48 to 72-hours. MCF7-LTED and HCC1428-LTED are
166 homozygote for *ESR1*^{wt}, whilst SUM44-LTED are heterozygote for *ESR1*^{Y537S}.

167

168 **Cell proliferation assays**

169 Wt-MCF7, wt-SUM44, wt-HCC1428 and their LTED derivatives were seeded in 10%
170 DCC medium into 96-well plates. Cell monolayers were left to acclimatize for 24-
171 hours before treatment with the drug combinations for 6-days with a treatment change
172 on day-3. Cell viability was determined using the CellTiter-Glo® Luminescent Cell
173 Viability Assay (Promega), according to the manufacture's protocol.

174

175 **Transcription assays**

176 Cell lines were seeded in 24-well plates in DCC medium and left to acclimatize for
177 24-hours. The following day transfection was performed using Fugene (Promega) with
178 0.1μg of E-response-element-linked-luciferase (EREIItkluc) and 0.1μg of β-
179 galactosidase (pCH110) reporter constructs [15]. Luciferase (Promega) and β-

galactosidase (GalactoStar, Applied Biosystems) activity were measured using a luminometer.

Western Blotting

Whole cell extracts were generated, as described previously [16]. Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Whatman). Antigen-antibody interactions were detected with Amersham ECL detection reagents (GE Healthcare).

Chromatin Immunoprecipitation

Wt-HCC1428 and HCC1428-LTED cells were cross-linked in 1% formaldehyde at room temperature for 10min and then quenched with 125mM glycine. Samples were then lysed, sonicated and chromatin was immunoprecipitated by overnight incubation at 4°C with ER (HC-20, sc-546) or IgG antibodies pre-bound with Protein G magnetic dynabeads (Invitrogen). Chromatin was washed vigorously with RIPA buffer and reversed cross-linked by an overnight incubation in elution buffer at 65°C. DNA was digested with RNase and Proteinase K, purified, precipitated with phenol chloroform and eluted in Tris-HCl pH8.0. Real-time qPCR was performed using *TFF1* oligos forward: 5′ GGC CAT CTC TCA CTA TGA ATC ACT TCT GCA 3′ and reverse: 5′ GGC AGG CTC TGT TTG CTT AAA GAG CGT TAG 3′.

Ion Torrent

DNA was amplified using Ion AmpliSeq™ Library Kit 2.0 (Life Technologies), digested, Ion Xpress™ Barcode adapters ligated and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). Libraries were quantified by qPCR using an

Ion Library Quantification Kit (Life Technologies), templated on the Ion OneTouch2 System (Life Technologies) and sequenced on the Ion PGM System (Life Technologies). Reads were aligned by the PGM server with standard settings to the reference genome hg19, samtools v1.2 was used to calculate the on-target coverage. IonReporter™ (v4.4) was used for mutation calling (parameters: Data Quality Stringency= 12, Downsample To Coverage= 4000, SNP/InDel/MNP Min Cov Each Strand= 50, SNP/InDel/MNP Min Variant Score= 15, SNP/InDel/MNP Min Coverage= 250, Hotspot Min Variant Score= 6, Hotspot Min Coverage= 150). All mutations called were manually reviewed in IGV and included in the analysis if they had a VAF ≥1%.

Human tumour xenografts modelling relapse on AI therapy

In-vivo studies were carried out in ovariectomized 8- to 12-week old female BALB/c FOX nude mice, in accordance with Home Office guidelines and approved by the Institute of Cancer Research Ethics Committee. MCF72a-LTED tumour xenografts were initiated by implantation of cells (10^7) combined with matrigel (1:1) into the left flank. Tumours were established in the absence of E. Once tumours reached a diameter of 7-8mm, animals were assigned to treatment groups with no statistically significant differences in mean volume before treatment. Animals were treated with either vehicle, fulvestrant administrated subcutaneously weekly (5mg/Kg in olive oil), neratinib (40mg/Kg in 0.5% hydroxypropyl methylcellulose (HPMC)/0.4%Tween 80) or RAD001 (2mg/Kg in 0.5%HPMC/0.4%Tween80) administrated daily by oral gavage for a total of 41-days. Drugs were supplied alone or in the combinations indicated. Tumour growth was assessed weekly in all arms by caliper measurements of the two large diameters. Volumes were calculated according to the formula: $a \times b^2 \times \pi/6$, where

a and b are orthogonal tumour diameters. Tumour volumes were then expressed as mean fold-change in volume at the start of treatment. The study operator was blinded to the treatments.

A second short-term study to address changes in gene expression was performed. Tumours from three mice per treatment were harvested 6-hours post-final drug administration following five full days of therapy. Tumours were snap frozen in liquid nitrogen for gene expression analysis.

Immunohistochemical Analysis

Tumour fragments were formalin fixed and parafin embedded. Sections were stained for ER using anti-ER antibody (6F11, Novocastra, UK) [17]

RNA-seq

Libraries were created after Ribo-zero rRNA removal kit (Illumina) using NEBNext Ultra Directional RNA (NEB) and sequenced using the HiSeq2500 (paired end 100bp v4 chemistry). Tophat (v2.1) and Cuffdiff (v2.2.1) [18] with default parameters were used for alignment and differential expression analysis. Genes which have a fold change greater than 50% compared to vehicle in any condition were mapped to KEGG pathway graphs using Pathview [19]. Gene set enrichment analysis (GSEA) [20] was used to identify gene sets that were significantly up/down regulated in each treatment. [19]. The data supporting this study has been deposited in the NCBI gene expression omnibus (GSE112401).

Statistical Analysis

Statistical analysis was performed using Student's t-test or one-way ANOVA with Tukey's to adjust for multiple comparisons. For xenograft studies, overall statistical differences were calculated using the Wilcoxon signed-rank test if the variance was not equal and failed the normality test, otherwise paired t-tests were used.

Results

Effect of RAD001 or neratinib alone or in combination with endocrine therapy on cell growth

Endocrine sensitive and LTED BC cell lines retaining ER expression and with varying levels of *EGFR, ERBB2, ERBB3* and *FRAP1* expression [21] and differing *PIK3CA*, *ERBB2* and *ESR1* mutation status (**Additional File S1: Figure S1a,b**) were assessed for their sensitivity to escalating doses of RAD001 (Fig. 2a) or neratinib (Fig. 2b) in the presence or absence of E2. The addition of RAD001 to wt cell lines, in the absence of E2 showed minimal additional antiproliferative activity compared with E-deprivation alone. Contrastingly, in the presence of E2, RAD001 caused a concentration-dependent decrease in proliferation of all wt cell lines tested. On the whole, even at the highest concentration of RAD001 (50nM), the antiproliferative effect was inferior to that seen with E-deprivation alone. Contrastingly, all LTED models showed a concentration dependent decrease in proliferation in the absence of E2 with varying degrees of sensitivity. Noteworthy, MCF7-LTED and SUM44-LTED, which harbour an $ESR1^{Y537S}$ mutation, appeared most sensitive with IC_{50} values of 1.5 and 0.5nM, respectively (Fig. 2a & **Additional File S1: Figure S1c**).

279 Escalating concentrations of neratinib caused a hormetic (bell shaped) proliferation
280 curve in all wt cell lines tested in the absence of E2, with mid-range doses causing c.
281 2-3 fold increase in proliferation. IC_{50} values for neratinib were not achieved in this
282 setting (Fig. 2b and **Additional File S1: Figure S1d**). LTED derivatives in the
283 absence of E2 showed IC_{50} values of 900nM for MCF7-LTED and SUM44-LTED and
284 400nM for HCC1428-LTED. Addition of E2, increased the sensitivity of all wt cell
285 lines with wt-MCF7 having the lowest recorded IC_{50} (300nM) (Fig. 2b and **Additional**
286 **File S1: Figure S1d**).
287
288 We subsequently assessed the interaction between RAD001 or neratinib with
289 escalating doses of 4-OHT and ICI (**Additional File S2: Figure S2 and Additional**
290 **File S3: Figure S3**). In the presence of exogenous E2, ICI and 4-OHT caused a
291 concentration dependent decrease in proliferation in all wt and LTED cells. For all cell
292 lines tested, RAD001 enhanced sensitivity to 4-OHT and ICI, with the exception of
293 the HCC1428-LTED, in which no further antiproliferative effect was detected when
294 RAD001 was combined with 4-OHT (**Additional File S2: Figure S2,b**). Similar
295 responses were observed when neratinib was combined with the 4-OHT or ICI, with
296 the exception of the wt-HCC1428 with ICI and HCC1428-LTED with 4-OHT, in
297 which neratinib showed minimal impact, particularly at higher concentrations (>1nM)
298 (**Additional File S3: Figure S3a,b**).
299
300 **Dual blockade of mTORC1 and ERBB signalling in combination with endocrine**
301 **therapy enhances anti-proliferative effectiveness**
302 As altered growth factor signalling has been associated with mTORC1 blockade
303 providing a route of resistance to long-term inhibition of this kinase [9], we examined

304 the strategy of combining RAD001 with neratinib in the presence of continued
305 endocrine therapy. In order to assess this, suboptimal concentrations of each agent
306 were combined in the presence or absence of E2. For all the cells lines tested, both in
307 the presence and absence of E2, the combination of RAD001 and neratinib showed
308 superior anti-proliferative effect compared to either agent alone (Fig. 3).
309
310 In order to assess the effect of combining mTORC1 and ERBB suppression with
311 endocrine therapy, cell lines were treated with sub-optimal concentrations of RAD001
312 or neratinib alone or in combination, with escalating doses of 4-OHT or ICI. The
313 combination of RAD001 and neratinib enhanced the efficacy of both endocrine agents,
314 particularly at the lower concentration range (**Additional File S4: Figure S4a,b**).
315
316 **Effect of the combination of RAD001 and neratinib on cell signalling**
317 In order to investigate the effect of RAD001 and neratinib alone or in combination
318 with endocrine agents on cellular signal transduction pathways, parental (endocrine
319 sensitive) and LTED cell lines were treated with drug combinations indicated for 24-
320 hours ± E2, 4-OHT or ICI (Fig. 4). As expected, phosphorylation of S6 was
321 dramatically suppressed by RAD001 alone or in combination with neratinib in all cell
322 lines tested. Contrastingly, neratinib caused cell line specific effects on members of
323 the ERBB family. For instance, neratinib caused a significant downregulation in total
324 ERBB2 in all cell lines and reduced phosphorylated EGFR in MCF7-LTED, wt-
325 SUM44 and wt-HCC1428, as well as phosphorylated ERBB3 in wt-MCF7, wt-
326 SUM44 and HCC1428-LTED. Furthermore, RAD001, caused an upregulation of
327 phosphorylated AKT in all cell lines tested and increased phosphorylation of ERK1/2
328 in wt-SUM44, and to a lesser degree in SUM44-LTED, wt-HCC1428 and HCC1428-

LTED, indicative of rapid re-wiring previously associated with resistance to mTORC1 inhibition [8] [9] [10]. Noteworthy, in the majority of cell lines, the combination of RAD001 with neratinib suppressed the upregulation of phosphorylated AKT and ERK1/2 (Fig. 4).

In order to investigate the impact of RAD001 or neratinib combined with 4-OHT or ICI versus the triple combination, on cell cycle progression, we assessed the abundance of pertinent cell cycle proteins. The combination of endocrine therapy with either RAD001 or neratinib decreased levels of phosphorylated RB, CyclinD1 and CDK4 to a greater extent than the endocrine therapies alone. However, as expected, the greatest degree of inhibition was evident with triple combination concomitantly blocking mTORC1, ERBB and ER-signalling, an effect most evident with ICI (Fig. 4). No substantial increase in cleaved-PARP was evident suggesting minimal impact on apoptosis.

Effect of RAD001 alone or in combination with neratinib on ER-transactivation

The majority of the patients who relapse on endocrine therapy retain expression of ER. *In-vitro* data has shown that ER can be phosphorylated in a ligand-independent manner, circumventing the need for steroid hormone. Major pathways associated with this include ERBB/ERK1/2 and PI3K/AKT/mTOR [6]. To assess whether interactions between the drugs impacted on E-independent transactivation, endocrine sensitive and LTED cell lines were transiently transfected with an ERE-luciferase reporter construct and treated with either RAD001, neratinib or the combination with or without E2, 4-OHT or ICI (Fig. 5a and **Additional File S5: Figure S5**). Under E-deprived (DCC) conditions, mimicking the effects of an AI, neratinib caused a significant enhancement

in ER/ERE mediated transcription compared to the vehicle control in all cell lines tested ($p \leq 0.03$) with the exception of SUM44-LTED, which showed a trend to significance ($p=0.1$). RAD001 alone suppressed ER-mediated transcription to varying extents across the cell lines. Most notably, wt-SUM44 and SUM44-LTED together with the HCC1428-LTED appeared most sensitive with a drop in ER-mediated transcription of approximately 50% compared to vehicle control. In contrast, wt-MCF7 and their LTED derivatives, as well as wt-HCC1428 were unaffected. Of note, the combination of RAD001 and neratinib appeared to negate the neratinib driven increase in ER-mediated transcription in several of the models ($p \leq 0.03$). However, transactivation remained higher than that seen with RAD001 alone and indeed the combination did not reduce the effect of neratinib in wt-HCC1428 or MCF7-LTED.

In order to address the enhanced ER/ERE-mediated transactivation in response to neratinib, chromatin immunoprecipitation was performed in wt-HCC1428 and HCC1428-LTED cells, which showed differential responses to neratinib when combined with RAD001 (Fig. 5a). ChIP analysis of ER recruitment in wt-HCC1428 showed enrichment at the *TFF1* promoter in response to neratinib compared to vehicle control. The combination of RAD001 and neratinib had no significant impact on recruitment, in keeping with the ER/ERE-mediated transcription analysis (Fig. 5a,b). Contrastingly, HCC1428-LTED showed enhanced recruitment of ER in response to neratinib, which was significantly reduced by the addition of RAD001 ($p<0.001$) (Fig. 5b), suggesting context specific impacts on ER-mediated transcription were responsible for these events.

Suboptimal concentrations of 4-OHT or ICI caused between a 40-60% reduction in ER-transactivation in all cell lines, with the exception of HCC1428-LTED in response to 4-OHT where the reduction did not meet statistical significance. Similarly, SUM44-LTED, which harbours a Y537S mutation in *ESR1*, showed no response to either 4-OHT or ICI. The combination of RAD001 or neratinib with endocrine therapy showed no further reduction in ER-mediated transcription compared to endocrine therapy alone in all cell lines tested with the exception of the SUM44 models. In this setting, RAD001 in combination with 4-OHT or ICI caused a significant reduction in ER-mediated transcription. However, the addition of neratinib showed no impact and indeed the triple combination impeded ER-mediated transactivation to a similar degree as RAD001 when combined with either endocrine agent. This suggests the wt-SUM44 and SUM44-LTED are particularly sensitive to cross talk between ER and mTORC1 signalling. Indeed, the combination of 4-OHT with RAD001 significantly reduced pER^{ser167} and total ER (**Additional File S5: Figure S5)**.

Effect of RAD001 alone or in combination with neratinib and/or fulvestrant *in-vivo*

To assess the effect of the drugs as monotherapies or combinations on tumour volume *in-vivo*, mice were implanted with MCF72a-LTED ER+ tumour cells, which grow independently of exogenous E and model relapse on an AI (details regarding generation of this model are shown in **Additional File S6: Figure S6a).** Animals were treated with vehicle, monotherapy (RAD001, neratinib or fulvestrant), dual or triple therapy combinations. The mean fold-change in tumour volume for each treatment was expressed relative to the start of treatment (Fig. 6a). Tumour volumes for the control vehicle group increased 1.8 times over the treatment period (p=0.1). All

monotherapies caused a reduction in tumour volume by day 41 compared to the start of treatment (RAD001: 36%, p=0.03; neratinib: 23%, p=0.6; fulvestrant: 37%, p=0.03). Dual combination therapies showed a further reduction in tumour volume (RAD001+neratinib: 73%, p=0.03; RAD001+fulvestrant: 72%, p=0.004; neratinib+fulvestrant: 65%, p=0.004). Triple combination of RAD001, neratinib plus fulvestrant was the most effective resulting in an 80% inhibition in tumour growth (p=0.008). Assessment of mouse weights showed the drug combinations had no significant effect during course of the study (Fig. 6b).

In order to assess dynamic changes in gene expression in response to RAD001, neratinib or the combinations with fulvestrant, a second short-term xenograft study was carried out followed by RNA-seq. Differentially expressed genes were subjected to pathway analysis (**Additional File S6: Figure S6b).** As expected, RAD001 increased *AKT* and *ERK* expression, which was reduced by the addition of neratinib. *ER* expression was elevated with neratinib treatment compared to RAD001 and fulvestrant. Noteworthy, *CoR* expression was elevated in the dual (RAD001 plus neratinib) and triple combination. Furthermore, global effects on proliferation showed a greater reduction with the dual and triple combinations compared to single agents. This was further supported by the assessment of *E2F* target genes (**Additional File S6: Figure S6c)**. As expected, the triple combination of RAD001, neratinib and fulvestrant suppressed the expression of cell cycle associated genes (*CCNE1*, *CCNL1*, *CDK3*, *CDK7* and *CDK9*) when compared with RAD001 alone or in combination with neratinib, in keeping with the longer-term xenograft study (**Additional File S7: Figure S7a**). Based on the pathway analysis, we used gene set enrichment analysis (GSEA) to assess the dynamic changes in EGFR/ERBB2 signalling after blockade

with neratinib, RAD001 or the combinations. Neratinib reduced expression of genes associated with EGF/EGFR activation of ERK signalling [22] ($p=0.004$) (Fig. 6c) contrastingly, RAD001 significantly induced expression of this gene set ($p=0.03$) (Fig. 6d), an observation in support of rapid re-wiring associated with resistance to mTORC1 inhibition. As expected, the combination of neratinib and RAD001 significantly reduced expression of the EGFR/ERK gene set ($p=0.003$) (Fig. 6e). Addition of fulvestrant to the double combination showed a further trend in the reduction of this ($p=0.09$) (Fig. 6f). Finally, assessment of the fulvestrant alone or in combination with RAD001 showed no impact on EGFR/EGF regulated genes, however the addition of neratinib significantly reduced the EGFR/ERK gene set ($p<0.0001$) (**Additional File S7: Figure S7b**). Taken together, this suggests that the addition of neratinib negates the EGF/EGFR feedback loop, providing further support for the antiproliferative effect seen with the triple combination, highlighting the potential utility of concomitantly targeting three cellular signalling nodes.

Discussion

In-vitro and *in-vivo* analysis of tumours that are resistant to endocrine therapy suggests complex interplay between cell signalling molecules, which cooperate to govern escape mechanisms. Treatment with small molecule inhibitors of pertinent pathways may provide clinical benefit. For instance, recent studies have shown that blockade of mTORC1 signalling in combination with AI therapy, causes a marked increase in PFS in patients with metastatic ER+ BC (BOLERO-2) [7], however, relapse remains a significant clinical issue.

In order to identify potential pathways attributed to lack of response to RAD001, we previously carried out a molecular study in cell lines adapted to LTED, modelling the patient cohort on the BOLERO-2 study. We showed that RAD001 induced a feedback-loop via ERBB2/3, which could potentiate resistance [9]. In further support, Carracedo and colleagues [10] showed a similar up-regulation of ERK1/2 in response to mTORC1 inhibition. Furthermore, studies using ERBB inhibitors have highlighted resistance pathways via up-regulation of PI3K/mTORC/AKT signalling [23] [24], suggesting a high degree of cross talk between these two pivotal cellular signal transduction pathways. In addition, both PI3K/mTORC/AKT and ERK1/2 have been implicated in the ligand-independent activation of ER, leading to resistance to endocrine therapy [6]. Based upon these observation, we hypothesised simultaneous blockade of all three cellular nodes may provide potential benefit circumventing the resistance seen with individual therapies. In order to test this, we assessed the combination of neratinib, a pan-ERBB inhibitor, with the mTORC1 inhibitor, RAD001, in the presence of various endocrine agents in models mimicking endocrine sensitive and AI resistant disease.

Surprisingly, treatment of endocrine sensitive BC cells with neratinib in the absence of exogenous E2 generated a hormetic response curve, with lower concentration of the drug causing a marked increase in proliferation and associated ER-mediated transactivation. Previous clinical studies have reported mixed benefit of the combination of AI with EGFR or ERBB2 blockade in primary or naïve advanced BC and in some cases have shown a trend towards poorer outcome [25] [26]. Furthermore, this observation is not only evident with targeted EGFR and ERBB2 RTKs but also with pan-ERBB inhibitors, such as AZD8931, in which, a recent phase II randomised study in combination with an AI in women with endocrine naïve advanced BC

provided no benefit compared to anastrozole alone and did not delay endocrine resistance in this patient population [27]. Noteworthy, treatment of LTED cell lines also showed enhanced ER/ERE-mediated transcription and recruitment of ER to target promoters in response to neratinib. However, in contrast to the parental cell lines, proliferation decreased. The decrease in proliferation is in keeping with clinical studies, which suggest that patients, who have acquired resistance to endocrine therapy via up-regulation of EGFR/ERBB2, may benefit from pan-ERBB inhibition, as they become more reliant on growth factor signaling as the mitogenic driver [26].

In contrast to E-deprivation, the combination of neratinib with 4-OHT or ICI showed enhanced antiproliferative effect in the majority of parental cell lines. However, although the combination outperformed either treatment alone at the concentrations tested, the magnitude of benefit was less than would be expected from additive benefit from either treatment alone. These data are in keeping with previous *in-vitro,* as well as clinical studies, assessing the combination of EGFR blockade with gefitinib to delay the onset of endocrine resistance [28] [29]. The mechanism underlying this remains unclear, but *in vitro* studies suggest tamoxifen-bound-ER binds co-repressor molecules allowing the ERBB2 promoter to sequester SRC1 and AIB1 leading to transcription of ERBB2 potentially providing the target for RTK inhibition [30].

Treatment with RAD001 showed differential effects on cell proliferation. Most notably, the LTED derivatives showed lower IC_{50} values compared to their parental cell lines with the exception of HCC1428. *PIK3CA* mutation status was not a governing factor of sensitivity, as both SUM44 and HCC1428 harbour the wt-gene. Wt-SUM44 in the absence of exogenous E2 showed no response to RAD001 however,

this was attributed to the fact that in E-deprived conditions, the majority of cells are in cell cycle arrest as such further perturbation provides little effect. This was confirmed by the observation in the presence of E2 where the IC_{50} was c. 3nM, similar to that seen in MCF7-LTED. These data again show that mutation status is not the governing feature of sensitivity and cellular context remains more informative and are in keeping with the translational study of BOLERO2, which showed that *PIK3CA* mutations were not in themselves predictive of clinical benefit to mTORC1 inhibitors [31]. Treatment with RAD001 decreased ER-mediated transcription, as a result of reduced S6 kinase activity, and subsequent phosphorylation of ER^{ser167}, which was particularly notable in wt-SUM44 and their LTED derivative. Allosteric inhibition of mTORC1 led to an increase in phosphorylated AKT, indicative of the previously observed S6 feedback loop [8]. Furthermore, in certain cell lines ERK1/2 was also elevated. This may indicate that phospho-ERK activation following mTORC1 inhibition occurs via cross-talk with the PI3K-RAS signalling pathway [10]. In selected cell lines, evidence suggested that enhanced ERBB signalling maybe responsible for the observed ERK activation. Indeed, GSEA analysis showed RAD001 increased expression of EGF/EGFR associated genes which was significantly suppressed by the addition of neratinib. In keeping, our *in-vivo* study showed concordant data in which the triple combination significantly reduced tumour volume.

Taken together, these data support the combination of mTORC1 blockade with inhibition of ERBB signalling and ER-function in ER+ BC, highlighting the potential clinical utility. Further support for the dual blockade of both mTORC1 and ERBB signalling comes from a recent phase I clinical trial piloting the combination of

neratinib with temsirolimus, in which, antitumoral activity in patients with advanced BC was evident [32].

Conclusions

In conclusion, our results provide support for the combination of RAD001 together with neratinib and endocrine therapy to re-sensitise endocrine resistant tumours to the antiproliferative effects of endocrine therapy. Most notably, the combination with ICI, disabling both the ER and AKT axis appeared superior. Furthermore, even within this restricted panel of cell lines, the heterogeneity of response highlights the need to identify common adaptive nodes.

Abbreviations

ER: estrogen receptor; ER+: estrogen receptor positive; BC: breast cancer; AI: aromatase inhibitors; E2: estradiol; E: estrogen; LTED: long-term estrogen deprived; FBS: fetal bovine serum; DCC: dextran charcoal; 4-OHT: 4-hydroxytamoxifen; ICI: fulvestrant; RTK: receptor tyrosine kinase; GSEA: gene set enrichment analysis; PFS: progression free survival.

Declarations

Ethics Approval: *In-vivo* studies were carried out in accordance with Home Office guidelines and approved by the Institute of Cancer Research Ethics 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 will be deposited with the NCBI gene expression omnibus (GEO) (http://ncbi.nlm.nih.gov/geo/).

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Authors' contribution: L-AM, MD, SRJ, RECJ, FA-C and ASL conceived and designed study; RR, SP, AR, SKG, JN-B and NS performed experiments; AT performed the xenografts experiments; ES analysed RNA-seq data; LAM and RR interpreted data and wrote the manuscript.

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719 **Figure Legends**

720 **Figure 1. Simplified schematic diagram of the pathways described in this study.**
721 **(a)** Growth factor signalling (IGFR and ERBB) leads to activation of PI3K and
722 phosphorylation of AKT. AKT inhibits TCS1/2 resulting in upregulation of mTORC1.
723 In parallel, mTORC1 can also be upregulated by the RAS-RAF-MEK-ERK signalling
724 pathway. ERK phosphorylates and inactivates TCS2 also leading to mTORC1
725 activation. S6K1 activity increases as a result of mTORC1 activation. S6K1
726 suppresses mTORC2 and IRS1. ER is also a target of S6K1 leading to phosphorylation
727 of serine 167. (**b**) Inhibition of mTORC1 with everolimus suppresses S6K1 removing
728 the negative feedback loop causing a rise in IRS1 and AKT activity via loss of
729 suppression on mTORC2. Increased AKT activity suppresses TCS1/2 and increases
730 expression of growth factor receptors (ERBB2/3) enhancing RAS-RAF-ERK
731 signalling. (**c**) The dual blockade of ERBBs (neratinib) and mTORC1 signalling
732 (everolimus) may suppress the two feedback loops described in panel B. Yellow
733 shows normal mTORC signalling cascade; blue represent activated proteins; red
734 represents inhibited proteins; dotted lines show loss of normal feedback loops.

735

736 **Figure 2. Antiproliferative effect of (a) RAD001 and (b) neratinib in endocrine**
737 **resistant and sensitive BC cell lines.** Cells were treated in absence or presence of
738 exogenous E2 (0.01nM) and doubling concentrations of RAD001 or neratinib.
739 Treatments were performed at day 1 and day 3 after seeding. After 6 days of treatment,
740 cell viability was analyzed by using a cell titer-glo assay. Data are expressed as fold-
741 change relative to DCC control. Error bars represent mean ± SEM.

742

Figure 3. Antiproliferative effect of RAD001, neratinib or their combination in endocrine resistant and sensitive BC cell lines. Cell lines were treated with vehicle, suboptimal concentrations for each drug alone or in combination, both in the absence and presence of 0.01nM exogenous E2. After 6 days of treatment, cell viability was analysed using cell titer-glo and data expressed as fold-change relative to vehicle control. Error bars represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.
Concentrations used in DCC: wt-MCF7 (0.75nM RAD001; 2000nM neratinib); MCF7-LTED (0.75nM RAD001; 500nM neratinib); wt-SUM44 (0.75nM RAD001; 2000nM neratinib); SUM44-LTED (0.4nM RAD001; 500nM neratinib); wt-HCC1428 (12.5nM RAD001; 1200nM neratinib); HCC1428-LTED (3nM RAD001; 250nM neratinib). Concentrations used in E2: wt-MCF7 (1.5nM RAD001; 200nM neratinib); MCF7-LTED (1.5nM RAD001; 300nM neratinib); wt-SUM44 (0.37nM RAD001; 450nM neratinib); SUM44-LTED (0.37nM RAD001; 250nM neratinib); wt-HCC1428 (1.5nM RAD001; 500nM neratinib); HCC1428-LTED (3nM RAD001; 250nM neratinib)

Figure 4. Effect of RAD001, neratinib or their combination with endocrine agents on cell signalling pathways governing cell cycle. Endocrine resistant and sensitive BC cell lines were treated for 24-hours with the drug combinations indicated. Whole-cell extracts were assessed for expression on S6 kinase, ERK1/2, AKT and ERBB signalling together with markers of cell cycle and apoptosis by immunoblotting. IC_{50} values were used for RAD001 and neratinib together with standard concentrations of E2 (0.01nM), 4-OHT (10nM) and ICI (1nM) (with exception of HCC1428-LTED, in which 10nM was used). ERBB pathways are highlighted in pink, ERK1/2 in blue, mTORC1/AKT in green and cell cycle in orange.

768 wt-MCF7 (2nM RAD001; 500nM neratinib); MCF7-LTED (4nM RAD001; 750nM
769 neratinib); wt-SUM44 (3nM RAD001; 700nM neratinib); SUM44-LTED (3nM
770 RAD001; 700nM neratinib); wt-HCC1428 (3nM RAD001; 1000nM neratinib);
771 HCC1428-LTED (10nM RAD001; 500nM neratinib).
772
773 **Figure 5. Effect of RAD001, neratinib or their combination on ER-mediated**
774 **transactivation and recruitment of the ER-basal transcription machinery.** (**a**) Cell
775 lines were co-transfected with EREIItkLuc and pCH110 and treated for 24-hours with
776 RAD001 and neratinib in the absence of E2 (DCC). Luciferase activity was
777 normalized by β-galactosidase from triplicate wells and fold-changes expressed
778 relative to the DCC control. (**b**) ChIP analysis to determine the effect of neratinib,
779 RAD001 or the combination on recruitment of ER to the *TFF1* promoter in wt-
780 HCC1428 and HCC1428-LTED. Error bars represent mean ± SEM. *p<0.05;
781 **p<0.01; ***p<0.001.
782 Concentration used for transactivation assay and ChIP: wt-MCF7 (2nM RAD001;
783 500nM neratinib); MCF7-LTED (4nM RAD001; 650nM neratinib); wt-SUM44 (3nM
784 RAD001; 700nM neratinib); SUM44-LTED (3nM RAD001; 700nM neratinib); wt-
785 HCC1428 (3nM RAD001; 700nM neratinib); HCC1428-LTED (10nM RAD001;
786 300nM neratinib).
787
788 **Figure 6. Effect of the RAD001 and neratinib alone or in combination with**
789 **endocrine therapy *in-vivo*.** (**a**) Long-term study assessing the relative mean changes
790 in tumour volume over 41 days of treatment and (**b**) Effect of drug regimes on animal
791 weight. Error bars represent mean ± SEM (n=7-9 animals per group). RAD, RAD001
792 (2mg/Kg); Ner, neratinib (40mg/Kg); ICI, fulvestrant (5mg/Kg). (**c-f**) GSEA

enrichment plots for 198 genes known to be induced by sustained activation of ERK in response to EGF activity. Plots show the profile of the running Enrichment Score and positions of GeneSet Members on the Rank Ordered List for rank gene lists generated from the comparison of (**c**) neratinib vs. vehicle; (**d**) RAD001 vs. vehicle; (**e**) RAD001 + neratinib vs. RAD001; (**f**) RAD001 + neratinib + ICI vs. RAD001 + neratinib.

Additional Files

Additional File S1: Figure S1. IC_{50} values for antiproliferative effect of RAD001 and neratinib in relation to the *ESR1*, *ERBB2* and *PIK3CA* mutational status in endocrine resistant and sensitive BC cell lines. (**a**) Mutational or wt status is depicted in grey and white, respectively, for *ESR1*, *ERBB2* and *PIK3CA*. (**b**) Varying degrees of expression of genes encoding proteins targeted by fulvestrant, neratinib and RAD001 showing heterogeneity in the cell lines tested. (**c-d**) Cells were treated in absence or presence of exogenous estradiol (E2) (0.01nM) and doubling concentrations of (**c**) RAD001 or (**d**) neratinib. Treatments were performed at day 1 and day 3 after seeding. After 6 days of treatment, cell viability was analyzed by using a cell titer-glo assay and IC_{50} values were plotted.

Additional File S2: Figure S2. Antiproliferative effect of RAD001 in combination with endocrine agents (a) 4-OHT and (b) ICI. Endocrine resistant and sensitive BC cell lines were treated with a combination of RAD001 (3nM) and increasing concentrations of (**a**) 4-OHT or (**b**) ICI for 6 days with media change at day3. Cell viability was analyzed by using a cell titer-glo assay. Data are expressed as fold-change relative to vehicle control. Error bars represent mean ± SEM.

Additional File S3: Figure S3. Antiproliferative effect of neratinib in combination with endocrine agents (a) 4-OHT and (b) ICI. Endocrine resistant and sensitive BC cell lines were treated with a combination of neratinib (500nM in wt-MCF7 and MCF7-LTED; 700nM in wt-SUM44, SUM44-LTED and wt-HCC1428; 300nM in HCC1428-LTED) and increasing concentrations of (**a**) 4-OHT or (**b**) ICI for 6 days with media change at day 3. Cell viability was analyzed by using a cell titer-glo assay. Data are expressed as fold-change relative to vehicle control. Error bars represent mean ± SEM.

Additional File S4: Figure S4. Antiproliferative effect combination of RAD001 and neratinib together with endocrine agents (a) 4-OHT and (b) ICI. Endocrine resistant and sensitive BC cell lines were treated with a combination of RAD001 and neratinib and increasing concentrations of (**a**) 4-OHT or (**b**) ICI for 6 days with media change at day 3. Cell viability was analyzed by using a cell titer-glo assay. Data are expressed as fold-change relative to vehicle control. Error bars represent mean ± SEM. wt-MCF7 (1.5nM RAD001; 200nM neratinib); MCF7-LTED (1.5nM RAD001; 300nM neratinib); wt-SUM44 (0.37nM RAD001; 450nM neratinib); SUM44-LTED (0.37nM RAD001; 250nM neratinib); wt-HCC1428 (1.5nM RAD001; 500nM neratinib); HCC1428-LTED (3nM RAD001; 250nM neratinib).

Additional File S5: Figure S5. Effect of RAD001, neratinib or their combination with endocrine agents on ER-mediated transactivation and ER signalling. Cell lines were co-transfected with EREIItkLuc and pCH110, and treated for 24-hours with the drug combinations indicated. IC_{50} values were used for RAD001 and neratinib together with standard concentrations of E2 (0.01nM), 4-OHT (0.1nM) and ICI

(0.1nM). Luciferase activity was normalized by β-galactosidase from triplicate wells and fold-changes expressed relative to the E2 control. Error bars represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001. wt-MCF7 (2nM RAD001; 500nM neratinib); MCF7-LTED (4nM RAD001; 650nM neratinib); wt-SUM44 (3nM RAD001; 700nM neratinib); SUM44-LTED (3nM RAD001; 700nM neratinib); wt-HCC1428 (3nM RAD001; 700nM neratinib); HCC1428-LTED (10nM RAD001; 300nM neratinib).
Western blot was used to assess changes in phosphorylation of the ER in response to RAD001, neratinib or their combination together with endocrine agents. IC_{50} values were used for RAD001 and neratinib together with standard concentrations of E2 (0.01nM), 4-OHT (10nM) and ICI (1nM) (with exception of HCC1428-LTED, in which 10nM was used). wt-MCF7 (2nM RAD001; 500nM neratinib); MCF7-LTED (4nM RAD001; 750nM neratinib); wt-SUM44 (3nM RAD001; 700nM neratinib); SUM44-LTED (3nM RAD001; 700nM neratinib); wt-HCC1428 (3nM RAD001; 1000nM neratinib); HCC1428-LTED (10nM RAD001; 500nM neratinib).

Additional File S6: Figure S6. Assessment of dynamic changes in gene expression in response to RAD001, neratinib or the combinations with fulvestrant. (**a**) MCF72a cells, which were previously engineered to express aromatase (*CYP19*) [33] were implanted into ovariectomised mice under androstenedione support. In this setting, MCF72a cells convert androstenedione in to estrogen to drive proliferation. Once tumours developed, androstenedione was withdrawn. After a lag phase tumour growth occurred synonymous with ligand-independence. Assessment of the MCF72a-LTED showed continued expression of ER and proliferation in the absence of exogenous estrogen providing a model of AI-relapse. (**b**) Changes to gene expression

(log2 difference Drug – Vehicle) as detected by RNA-seq for five drug combinations (neratinib, RAD001, ICI, neratinib+RAD001 and neratinib+RAD001+ICI) were mapped to KEGG pathway graphs using Pathview (https://bioconductor.org/packages/release/bioc/html/pathview.html). Genes with a fold-change greater than 50% compared to vehicle in any condition were selected in order to expand the list of differentially expressed genes, allowing the identification of subtle changes in gene expression for example kinases or transcription factors that might have significant impact on downstream gene expression. A heatmap for each gene in shown. (**c**) Assessment of expression of E2F target genes in response to neratinib and RAD001.

Additional File S7: Figure S7. Assessment of dynamic changes in expression of cell cycle regulatory genes. (a) Log_2 differences in *CCNE1, CCNL1, CDK3, CDK7* and *CDK9* gene expression following treatment with RAD001, RAD001 + neratinib and RAD001 + neratinib + fulvestrant (ICI), compared to vehicle. (**b**) GSEA enrichment plots for 198 genes known to be induced by sustained activation of ERK in response to EGF activity. Plots show the profile of the running Enrichment Score and positions of GeneSet Members on the Rank Ordered List for rank gene lists generated from the comparison of ICI vs. vehicle; neratinib + ICI vs. ICI; RAD001 + ICI vs. ICI; RAD001 + neratinib + ICI vs. RAD001 + ICI.