Enhanced sensitivity to rapamycin following long-term oestrogen deprivation in MCF-7, T-47-D and ZR-75-1 human breast cancer cells

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Abstract

Human breast cancer cells (MCF-7, T-47-D and ZR-75-1) can adapt to circumvent any reduced growth rate during long-term oestrogen deprivation, and this provides three model systems to investigate mechanisms of endocrine resistance in breast cancer. In this paper we report consistent differences in the effects of three growth inhibitors following long-term oestrogen deprivation in all three cell models. Long-term oestrogen deprivation of MCF-7, T-47-D and ZR-75-1 cells resulted in reduced growth inhibition by PD98059 (2–10 µg/ml), implying a loss of dependence on mitogen-activated protein kinase pathways for growth. The growth inhibitor LY294002 (2–10 µM) inhibited growth of both oestrogen-maintained and oestrogen-deprived cells with similar dose-responses, implying continued similar

dependence on phosphoinositide 3-kinase (PI3K) pathways with no alteration after adaptation to oestrogen independent growth. However, by contrast, long-term oestrogen deprivation resulted in an increased sensitivity to growth inhibition by rapamycin, which was not reduced by readdition of oestradiol. The enhanced inhibition of long-term oestrogen-deprived MCF-7-ED, T-47-D-ED and ZR-75-1-ED cell growth by combining rapamycin with LY294002 at concentrations where each alone had little effect, offers preclinical support to the development of therapeutic combinations of rapamycin analogues with other PI3K inhibitors in endocrine-resistant breast cancer.

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Introduction

The long-term efficacy of endocrine therapy of breast cancer is compromised by the remarkable ability of human breast cancer cells to circumvent any form of imposed growth inhibition, be it through inhibition of oestrogen synthesis using aromatase inhibitors or through antagonism of oestrogen action using antioestrogens (Miller 1996, Lonning 2004). Over the years, cell culture models have been developed where the effects of long-term oestrogen deprivation can be reproduced *in vitro* (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Murphy *et al.* 1989, Daly & Darbre 1990a, Masamura *et al.* 1995, Stephen *et al.* 2001, Chan *et al.* 2002) in order to investigate molecular mechanisms, which might provide therapeutic targets. The models based on long-term growth of MCF-7, T-47-D or ZR-75-1 human breast cancer cell lines under conditions of oestrogen deprivation have demonstrated that cells that are initially dependent on oestrogen for their proliferation can adapt over time and learn to grow at the same rate in the absence of oestrogen as they grew originally only in the presence of oestrogen (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Murphy *et al.* 1989, Daly & Darbre 1990a, Jeng *et al.* 1998, Stephen *et al.* 2001, Chan *et al.* 2002, Staka *et al.* 2005). In all models except for one (Murphy *et al.* 1989), there is no loss of oestrogen receptor (ER)

number or function and in fact ER levels increase in line with the known downregulation of ER by oestrogen (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Daly & Darbre 1990a, Jeng *et al.* 1998, Chan *et al.* 2002, Staka *et al.* 2005, Shaw *et al.* 2006). Furthermore, oestrogen-regulated genes, such as *pS2* and progesterone receptor, do not become constitutively high like the growth rate but rather remain at a low level of expression that can still be upregulated by readdition of oestrogen (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Daly & Darbre 1990a, Jeng *et al.* 1998, Shaw *et al.* 2006).

The molecular basis for this growth adaptation in MCF-7 cells has been suggested to result from the increased ER levels causing the cells to become hypersensitive to low levels of oestradiol (E₂) remaining in the oestrogen-deprived medium (Jeng *et al.* 1998, Santen *et al.* 2005). However, although the long-term oestrogen-deprived MCF-7 cells develop the ability to grow under certain conditions at lower concentrations of E₂, which can be inhibited by correspondingly lower levels of antioestrogen (Masamura *et al.* 1995, Jeng *et al.* 1998, Chan *et al.* 2002), it remains unclear as to why the upregulated growth in the selection medium can no longer be fully inhibited with the antioestrogens tamoxifen or fulvestrant in MCF-7 cells (Staka *et al.* 2005, Martin *et al.* 2005, Shaw *et al.* 2006) or in T-47-D and ZR-75-1 cells (Daly & Darbre 1990a).

An alternative explanation for this upregulated basal growth has been suggested to result from alterations to growth factor signalling pathways and early studies found alterations to levels of several growth factors, their receptors and binding proteins in the long-term oestrogen-deprived MCF-7 cells (Lippman & Dickson 1990, Herman & Katzenellenbogen 1994, Chan *et al.* 2002) and T-47-D and ZR-75-1 cells (Daly & Darbre 1990a,b). Increased levels of the insulin-like growth factor receptor type 1 (IGF1R) and enhanced sensitivity to IGFs in oestrogen-deprived MCF-7 cells (Stephen *et al.* 2001), and acquired sensitivity to stimulation rather than inhibition by transforming growth factor β in T-47-D cells (Daly & Darbre 1990b) have demonstrated that alterations may vary at the growth factor/growth factor receptor level, and more recent work has focused on the common signal transduction pathways rather than the initial receptor changes. Studies of the signalling pathways used by growth factor receptor tyrosine kinases such as the IGF1R have reported raised levels of components of both the mitogen-activated protein kinase (MAPK; Shim *et al.* 2000, Santen *et al.* 2001, Yue *et al.* 2002, Martin *et al.* 2003) and the phosphoinositide 3-kinase (PI3K; Yue *et al.* 2003, Sabnis *et al.* 2005) pathways in oestrogen-deprived MCF-7 cells which might enable either growth in low levels of growth factors not stripped from the serum or growth to become independent of external growth factors. Alternatively, alterations to phosphorylation of ER by signalling kinases might enable ligand-independent ER activation (Yamnik *et al.* 2009).

Inhibitors of the MAPK or PI3K pathways have been shown to inhibit growth of MCF-7 cells (Yue *et al.* 2003, 2005, Santen *et al.* 2008). The inhibitor PD98059 (Alessi *et al.* 1995) has been shown to exert its effects on cellular proliferation by inhibiting the phosphorylation of components of the MAPK pathway. Also, increased levels of the activated phosphorylated p44/42MAPK (ERK1/2) have been suggested to play a functional role in the upregulated basal growth in long-term oestrogen-deprived MCF-7 cells (Shim *et al.* 2000, Santen *et al.* 2001, Yue *et al.* 2002, Martin *et al.* 2003). The cell growth inhibitor LY294002 has been shown to exert effects on cellular proliferation mediated through the PI3K pathway (Vlahos *et al.* 1994) and its effects are, at least in part, mediated by inhibition of the phosphorylation of AKT (Adi *et al.* 2001). Alterations to the levels of phosphorylated components of the PI3K pathway, including AKT, have also been implicated in the upregulated basal growth rate of oestrogen-deprived MCF-7 cells (Yue *et al.* 2003, Sabnis *et al.* 2005). The mechanistic target of rapamycin (MTOR; also known as mammalian target of rapamycin, mTOR) is a serine-threonine kinase of the PI3K signalling pathway, which integrates growth factor stimulation with metabolic responses and can act through the formation of two distinct complexes MTORC1 and MTORC2 (Wullschlegel *et al.* 2006). Rapamycin is a specific inhibitor of MTORC1, which is formed in response to mitogens and nutrient availability, and can act to regulate

ribosome biogenesis and mRNA translation through phosphorylation of S6 kinase (S6K1) and 4E-binding protein (4E-BP1; Wullschlegel *et al.* 2006). Rapamycin and analogues have been shown to inhibit the proliferation of ER-positive human breast cancer cells (Chang *et al.* 2007), to enhance the inhibitory action of antioestrogens in MCF-7 cells (Sadler *et al.* 2006, Treeck *et al.* 2006) and to inhibit the growth of MCF-7 cells, which have developed resistance to tamoxifen and fulvestrant (Ghayad *et al.* 2008). However, despite the inhibitory actions in cell culture, outcomes from clinical trials have been disappointing (Baselga *et al.* 2009, Ellard *et al.* 2009) and studies are now needed to identify patient groups that might give better response rates to rapamycin treatment. In an attempt to bridge the information gap, we have compared here the effects on cell growth of rapamycin with PD98059 and LY294002 in oestrogen-maintained versus long-term oestrogen-deprived human breast cancer cells. Using three separate cell line models, MCF-7, T-47-D and ZR-75-1, we report an enhanced inhibitory effect of rapamycin following long-term oestrogen deprivation.

Materials and Methods

Stock culture of oestrogen-maintained MCF-7 cells

MCF-7 McGrath human breast cancer cells were kindly provided by Osborne *et al.* (1987) at passage number 390 and these cells are dependent on oestrogen for growth (Darbre & Daly 1989). These MCF-7 cells were grown in our laboratory as monolayer cultures in DMEM (containing phenol red) (Invitrogen) supplemented with 5% (v/v) FCS (Invitrogen), 10 μ g/ml insulin (Sigma) and 10^{-8} M E_2 (Steraloids, Croydon, UK) in a humidified atmosphere of 10% carbon dioxide in air at 37 °C. Cell stocks were subcultured at weekly intervals by suspension with 0.06% (w/v) trypsin and 0.02% (w/v) EDTA pH 7.3.

Stock culture of oestrogen-maintained T-47-D and ZR-75-1 cells

T-47-D human breast cancer cells (Keydar *et al.* 1979) and ZR-75-1 human breast cancer cells (Engel *et al.* 1978) were kindly provided by the originators in 1980. The ZR-75-1 cells are dependent on oestrogen for their growth (Darbre *et al.* 1983, Darbre & Daly 1989). The T-47-D cells are responsive rather than dependent on oestrogen for their growth in that they can proliferate in the absence of oestrogen but their growth rate can be increased by addition of E_2 (Darbre & Daly 1989). These cells were grown as described above for MCF-7 cells but the medium lacked any insulin. The ZR-75-1 cells were not selected originally in the presence of insulin (Engel *et al.* 1978). Although the T-47-D cells were selected with insulin (Keydar *et al.* 1979), they have never been maintained with insulin since receipt in our laboratory.

Long-term oestrogen deprivation of MCF-7 cells

MCF-7 cells (starting from passage number 413) were maintained in phenol red-free RPMI 1640 medium (Invitrogen) with 5% (v/v) dextran-charcoal stripped FCS (DC-FCS; Darbre *et al.* 1983), subculturing every 2–3 weeks during the initial period of slow growth, and increasing to subculturing at weekly intervals as the growth rate increased (Stephen *et al.* 2001, Shaw *et al.* 2006). In this paper, these oestrogen-deprived cells are written as ‘MCF-7-ED cells’ and were used between 52 and 65 weeks of growth in this medium.

Long-term oestrogen deprivation of T-47-D and ZR-75-1 cells

The long-term oestrogen deprivation model for these cells has been described in detail previously (Daly & Darbre 1990a). In brief, the cells were maintained as for the MCF-7-ED cells using phenol red-free RPMI 1640 medium with 5% (v/v) DC-FCS. In this paper, the T-47-D oestrogen-deprived cells are written as ‘T-47-D-ED cells’ and were used after 118–120 weeks in this medium. Long-term growth of ZR-75-1 cells in oestrogen-depleted medium resulted in several clones of cells being isolated which had become independent of oestrogen for their growth and the cells used in this paper were the clone 11A cells (Daly & Darbre 1990a). They were used after 98–102 weeks in the oestrogen deprivation medium and are referred to in this paper as ‘ZR-75-1-ED cells’.

Cell proliferation experiments

Cells (0.2×10^5 cells/ml) were plated in phenol red-free RPMI 1640 medium with 5% (v/v) DC-FCS in monolayer in 0.5 ml aliquots into 24 well plastic tissue culture dishes (Nunc, Roskilde, Denmark) and after 24 h, the medium was changed to the same medium but supplemented with or without E_2 , or the inhibitors PD98059, LY294002, rapamycin or PI3K α inhibitor VIII (Calbiochem, Merck). Inhibitors were prepared in dimethyl sulphoxide (DMSO) under dim lighting conditions, diluted 1/10 000 in culture medium and controls performed with the same volume of DMSO. Culture medium was changed routinely every 3–4 days in all experiments. Cell counts were performed by counting released nuclei on a model ZBI Coulter Counter, as described previously, except that zapoglobin replaced zaponin (Daly & Darbre 1990a). The number of cell doublings in 7 days was calculated as the mean $\pm$ S.E.M. of all nine values for triplicate estimates of cell numbers at time 0 and after 7 days. IC_{50} values were calculated as the concentration of inhibitor needed to reduce the number of doublings by 50%.

Results

The oestrogen deprivation cell culture model

MCF-7 McGrath human breast cancer cells (Osborne *et al.* 1987) are dependent on oestrogen for growth such that if they are grown in phenol red-free medium with DC-FCS, then cell proliferation is low in the absence of added oestrogen and

is stimulated by E_2 (Darbre & Daly 1989). The T-47-D and ZR-75-1 human breast cancer cells are similarly responsive to oestrogen for growth (Darbre & Daly 1989). However, after long-term maintenance under oestrogen-deprived conditions in the phenol red-free medium with DC-FCS, these cells can all adapt and learn to grow such that their growth rate eventually rises to equal that of the previous growth rate only in the presence of E_2 . Models where the growth is no longer influenced by further addition of E_2 have already been published for MCF-7-ED cells (Stephen *et al.* 2001, Shaw *et al.* 2006), T-47-D-ED cells and ZR-75-1-ED cells (Daly & Darbre 1990a).

Effect of inhibitor PD98059 on cell proliferation

The inhibitor PD98059 (Alessi *et al.* 1995) has been shown to exert its effects on cellular proliferation by inhibiting

Figure 1 Effect of the inhibitor PD98059 on growth of oestrogen-maintained MCF-7 cells (A), T-47-D cells (C) and ZR-75-1 cells (E) compared with long-term oestrogen-deprived MCF-7-ED cells (B), T-47-D-ED cells (D) and ZR-75-1-ED cells (F). Cells were plated and grown in phenol red-free RPMI 1640 medium with 5% DC-FCS $\pm 10^{-8}$ M 17β -oestradiol (E_2). The number of cell doublings in 7 days was calculated as the mean $\pm$ S.E.M. for three separate experiments. Where no bars are seen, error was too small for visual display. IC_{50} values were calculated as the concentration of inhibitor needed to reduce the number of doublings by 50%.

the phosphorylation of components of the MAPK pathway. Dose–response curves in Fig. 1A show that growth of the oestrogen-maintained MCF-7 cells was inhibited by PD98059 in a dose-dependent manner with IC_{50} values of $9.1 \mu\text{g/ml}$ ($34.0 \mu\text{M}$) and $7.0 \mu\text{g/ml}$ ($26.2 \mu\text{M}$) in the presence and absence of E_2 respectively. Addition of PD98059 had little effect on the growth of the oestrogen-deprived MCF-7-ED cells irrespective of the presence or absence of E_2 and even up to concentrations of $20 \mu\text{g/ml}$ ($74.8 \mu\text{M}$; Fig. 1B). The inhibitor, PD98059, was found to inhibit the growth of oestrogen-maintained T-47-D cells in a dose-dependent manner with IC_{50} values of $10 \mu\text{g/ml}$ ($37.4 \mu\text{M}$) and $7.5 \mu\text{g/ml}$ ($28.1 \mu\text{M}$) in the presence and absence of E_2 respectively (Fig. 1C). It also inhibited growth of oestrogen-maintained ZR-75-1 cells in a dose-dependent manner with IC_{50} values of $9.5 \mu\text{g/ml}$ ($35.5 \mu\text{M}$) and $8.6 \mu\text{g/ml}$ ($32.2 \mu\text{M}$) in the presence and absence of E_2 respectively (Fig. 1E). However, as for the MCF-7-ED cells, PD98059 had little effect on growth of the oestrogen-deprived T-47-D-ED (Fig. 1D) or ZR-75-1-ED (Fig. 1F) cells irrespective of the presence or absence of E_2 .

Effect of inhibitor LY294002 on cell proliferation

The cell growth inhibitor LY294002 has been shown to exert effects on cellular proliferation mediated through the PI3K pathway (Vlahos *et al.* 1994) and its effects are, at least in part, mediated by inhibiting the phosphorylation of AKT (Adi *et al.* 2001). Dose–response curves over 7 days showed that growth of MCF-7 cells (Fig. 2A) and growth of MCF-7-ED cells (Fig. 2B) were both inhibited by LY294002 at increasing doses between 2 and $10 \mu\text{M}$ and over a similar concentration range irrespective of the presence or absence of E_2 with IC_{50} values as indicated in Fig. 2A and B. Dose-dependent inhibition of growth was found also to be similar between oestrogen-maintained T-47-D (Fig. 2C) and oestrogen-deprived T-47-D-ED (Fig. 2D) cells, and oestrogen-maintained ZR-75-1 (Fig. 2E) and oestrogen-deprived ZR-75-1-ED (Fig. 2F) cells and IC_{50} values are given in Fig. 2. The ZR-75-1 cells were inhibited at lower concentrations of the inhibitor than the MCF-7 or T-47-D cells, but the lack of any major alteration to the dose–response following long-term oestrogen deprivation paralleled the results with the other two cell lines.

Effect of rapamycin on cell proliferation

MTOR is a major downstream effector in the PI3K pathway, which can be inhibited by rapamycin (Wullschlegel *et al.* 2006). Dose–response experiments showed that while growth of the oestrogen-maintained MCF-7 cells was only inhibited to a small extent by rapamycin in the presence of E_2 ($IC_{50} > 100 \text{ nM}$), the more limited growth in the absence of E_2 could be inhibited with an IC_{50} value of 0.1 nM (Fig. 3A) and growth of oestrogen-deprived MCF-7-ED cells could be inhibited with an IC_{50} value of 2 nM rapamycin irrespective

of the presence or absence of E_2 (Fig. 3B). Growth of the MCF-7-ED cells could be reduced to one doubling or less in 7 days at 10 nM rapamycin and irrespective of the presence or absence of E_2 (Fig. 3B), but by contrast, minimal growth inhibition was found in the MCF-7 cells in the presence of E_2 even with 100 nM rapamycin (Fig. 3A). Similarly, in the oestrogen-maintained T-47-D cells, rapamycin had only a small inhibitory effect on cell growth in the presence of E_2 ($IC_{50} > 100 \text{ nM}$), although the limited growth in the absence of E_2 was inhibited with an IC_{50} in the $0.1\text{--}1 \text{ nM}$ range (Fig. 3C), but had a stronger inhibitory action on the oestrogen-deprived T-47-D-ED cells, which was not influenced by the presence or absence of E_2 (IC_{50} in the range of $3\text{--}10 \text{ nM}$; Fig. 3D). The oestrogen-maintained ZR-75-1 cells were more sensitive to growth inhibition by rapamycin in the presence of E_2 ($IC_{50} 0.1 \text{ nM}$; Fig. 3E) than were either the MCF-7 or the T-47-D cells, but strong

Figure 2 Effect of the inhibitor LY294002 on growth of oestrogen-maintained MCF-7 cells (A), T-47-D cells (C) and ZR-75-1 cells (E) compared with long-term oestrogen-deprived MCF-7-ED cells (B), T-47-D-ED cells (D) and ZR-75-1-ED cells (F). Cells were plated and grown in phenol red-free RPMI 1640 medium with 5% DC-FCS $\pm 10^{-8} \text{ M}$ 17β -oestradiol (E_2). The number of cell doublings in 7 days was calculated as the mean of all nine values for triplicate estimates of cell numbers per well at time zero and after 7 days. Results are presented as the mean $\pm$ s.e.m. for three separate experiments. Where no bars are seen, error was too small for visual display. IC_{50} values were calculated as the concentration of inhibitor needed to reduce the number of doublings by 50%.

Figure 3 Effect of rapamycin on growth of oestrogen-maintained MCF-7 cells (A), T-47-D cells (C) and ZR-75-1 cells (E) compared with long-term oestrogen-deprived MCF-7-ED cells (B), T-47-D-ED cells (D) and ZR-75-1-ED cells (F). Cells were plated and grown in phenol red-free RPMI 1640 medium with 5% DC-FCS $\pm 10^{-8}$ M 17β -oestradiol (E_2). The number of cell doublings in 7 days was calculated as the mean of all nine values for triplicate estimates of cell numbers per well at time zero and after 7 days. Results are presented as the mean $\pm$ s.e.m. for three separate experiments. Where no bars are seen, error was too small for visual display. IC_{50} values were calculated as the concentration of inhibitor needed to reduce the number of doublings by 50%.

growth inhibition was found also for the oestrogen-deprived ZR-75-1-ED cells (Fig. 3F). Growth inhibition with rapamycin of both ZR-75-1 and ZR-75-1-ED cells was found to have an IC_{50} of 0.1 nM and growth was reduced to less than one doubling by 1 nM irrespective of the presence or absence of E_2 (Fig. 3E and F).

Effect of rapamycin in combination with LY294002 on cell proliferation

In order to determine whether inhibition of growth of the oestrogen-deprived MCF-7-ED cells could be achieved at lower concentrations of rapamycin by combining with a different PI3K pathway inhibitor, we investigated the effect of combining rapamycin with LY294002 using concentrations at which each alone had little effect. Figure 4B shows that concentrations of 1 nM rapamycin alone or 0.1–1.0 μ M

LY294002 alone had little effect on growth of the MCF-7-ED cells, but combining 1 nM rapamycin with 0.1, 0.5 or 1.0 μ M LY294002 had an increasingly inhibitory effect and the largest effect was noted by combining 1 nM rapamycin with 2.5 μ M LY294002 (Fig. 4B). A concentration of 1 nM rapamycin alone reduced cell doublings only to a small extent from 5.00 ± 0.04 to 4.69 ± 0.05 while 2.5 μ M LY294002 reduced the doublings down to 3.67 ± 0.04 , but combination of 1 nM rapamycin with 2.5 μ M LY294002 reduced the number of doublings to 1.534 ± 0.043 (Fig. 4B). No such effect was observed in the oestrogen-maintained MCF-7 cells where similar concentrations of rapamycin and LY294002 had little effect on cell proliferation either alone or in combination (Fig. 4A).

The effects of combining rapamycin with LY294002 on growth of T-47-D and ZR-75-1 cell lines are shown in Fig. 4C–F. Rapamycin concentration was reduced from 1 nM used for the MCF-7 cells (Fig. 4A and B) to 0.1 nM for the

Figure 4 Effect of combining rapamycin with LY294002 on growth of oestrogen-maintained MCF-7 cells (A), T-47-D cells (C) and ZR-75-1 cells (E) compared with long-term oestrogen-deprived MCF-7-ED cells (B), T-47-D-ED cells (D) and ZR-75-1-ED cells (F). Cells were grown in their respective stock media with increasing concentrations of LY294002 with or without 1 nM (A and B) or 0.1 nM (C–F) rapamycin. The number of cell doublings in 7 days was calculated as the mean of all nine values for triplicate estimates of cell numbers per well at time zero and after 7 days. Results are presented as the mean $\pm$ s.e.m. for three separate experiments. Where no bars are seen, error was too small for visual display.

T-47-D cells (Fig. 4C and D) and ZR-75-1 cells (Fig. 4E and F) because these cell lines were more sensitive to growth inhibition with rapamycin than the MCF-7 cells (Fig. 3). Concentrations of 0.1 nM rapamycin alone or 0.1–2.5 µM LY294002 alone had only a small increasing inhibitory action on growth of the T-47-D-ED cells (Fig. 4D) but combining 0.1 nM rapamycin with 1.0 or 2.5 µM LY294002 had a strong inhibitory effect (Fig. 4D). No such effect was observed in the oestrogen-maintained T-47-D cells where similar concentrations of rapamycin and LY294002 had little effect on cell proliferation either alone or in combination (Fig. 4C). This paralleled the strong combinatorial effect also specifically found only in the MCF-7-ED cells (Fig. 4B).

Figure 5 Effect of combining rapamycin with PI3K α inhibitor VIII on growth of oestrogen-maintained MCF-7 cells (A), T-47-D cells (C) and ZR-75-1 cells (E) compared with long-term oestrogen-deprived MCF-7-ED cells (B), T-47-D-ED cells (D) and ZR-75-1-ED cells (F). Cells were grown in their respective stock media with increasing concentrations of the PI3K α inhibitor VIII with or without 1 nM (A and B) or 0.1 nM (C–F) rapamycin. The number of cell doublings in 7 days is presented as the mean $\pm$ S.E.M. of all nine values for triplicate estimates of cell numbers per well at time zero and after 7 days. Where no bars are seen, error was too small for visual display. Results are shown from one experiment but reproducibility was confirmed from a duplicate experiment. IC₅₀ values were calculated as the concentration of inhibitor needed to reduce the number of doublings by 50%.

The contrasting strong combinatorial effects of rapamycin and LY294002 in the ZR-75-1 (Fig. 4E) as well as in the ZR-75-1-ED cells (Fig. 4F) may reflect the greater initial sensitivity of the ZR-75-1 cell line to rapamycin (Fig. 3).

Effect of rapamycin in combination with PI3K α inhibitor VIII on cell proliferation

Since the class 1 PI3K isoform, PI3K α has been shown to play a role in breast cancer cell growth (Isakoff *et al.* 2005), the combination experiments were repeated with an inhibitor to PI3K α . In contrast to the results using LY294002, the MCF-7 cells were more sensitive than the MCF-7-ED cells to growth inhibition using the PI3K α inhibitor VIII (Fig. 5A and B). Dose–response curves showed that the PI3K α inhibitor VIII had little effect below 1 nM and was cytotoxic at 33 nM in both MCF-7 and MCF-7-ED cells. However, at 3.3, 6.6 and 10 nM of the PI3K α inhibitor VIII, growth of the MCF-7 cells (Fig. 5A) was inhibited but not the MCF-7-ED cells (Fig. 5B), and further addition of 1 nM rapamycin with the PI3K α inhibitor VIII had a greater inhibitory effect on the MCF-7 cells (Fig. 5A) than on the MCF-7-ED cells (Fig. 5B).

The effects of combining rapamycin with the PI3K α inhibitor VIII on growth of T-47-D and ZR-75-1 cell lines are shown in Fig. 5C–F. Rapamycin concentration was reduced from 1 nM used for the MCF-7 cells (Fig. 5A and B) to 0.1 nM for the T-47-D cells (Fig. 5C and D) and ZR-75-1 cells (Fig. 5E and F) because these cell lines were more sensitive to growth inhibition with rapamycin than the MCF-7 cells (Fig. 3). Dose–response curves showed that the PI3K α inhibitor VIII had little effect below 1 nM and was cytotoxic at 66 nM in T-47-D, T-47-D-ED, ZR-75-1 and ZR-75-1-ED cells. Any additional growth inhibitory effect through combining the PI3K α inhibitor VIII at concentrations from 0.1 to 33 nM with 0.1 nM rapamycin was small in any of these cell lines (Fig. 5C–F).

Discussion

The results presented here show that in oestrogen-responsive human breast cancer cells, there are substantial alterations in the sensitivity of cell growth to inhibitors of the PI3K/AKT/MTOR and MAPK pathways following adaptation of growth to long-term oestrogen deprivation and that the results are consistent across three independent cell line models. A reduced dependence on the MAPK pathway for growth was noted in all three cell lines after oestrogen deprivation since MCF-7-ED, T-47-D-ED and ZR-75-1-ED cells were all inhibited to a lesser extent by PD98059 than were the corresponding stock oestrogen-maintained cells. Blockade of the MAPK pathway with PD98059 at the concentrations of 10 µg/ml substantially inhibited growth of all three of the oestrogen-maintained cell lines and with IC₅₀ values in the range 7–10 µg/ml, which is consistent with published IC₅₀ values for the primary targets of this inhibitor

(Alessi *et al.* 1995). However, PD98059 had only marginal effect on growth of any of the three cell lines following long-term oestrogen deprivation irrespective of the presence or absence of E_2 . By contrast, long-term oestrogen deprivation of MCF-7 and T-47-D cells resulted in increased sensitivity to growth inhibition by rapamycin. The limited growth of MCF-7 and T-47-D cells without oestrogen could be inhibited by rapamycin with IC_{50} values of <1 nM, which is consistent with other reports (Akcanat *et al.* 2009). However, growth with E_2 was little affected by rapamycin ($IC_{50} > 100$ nM) and yet growth of the long-term oestrogen-deprived cells was inhibited with IC_{50} values within the 2–10 nM range irrespective of the presence or absence of E_2 . Rapamycin sensitivity of the long-term oestrogen-deprived ZR-75-1-ED cells was also evident but since the stock oestrogen-maintained ZR-75-1 cells were more sensitive to rapamycin, difference between stock and oestrogen-deprived cells was not obvious and IC_{50} values were all 0.1 nM. These reproducible observations across three separate cell line models have clinical implications for assessing the efficacy of inhibitors of signalling pathways in different ER-positive breast cancer subtypes that are oestrogen-responsive or oestrogen-unresponsive for growth.

The finding of enhanced sensitivity to growth inhibition with rapamycin following long-term oestrogen deprivation in all three cell line models has potential translational implications for the ongoing clinical trials of rapamycin and analogues in breast cancer (Di Cosimo & Baselga 2008, Baselga *et al.* 2009, Ellard *et al.* 2009). Since aromatase inhibitors such as letrozole act by blockade of oestrogen synthesis (Lonning 2004), and if long-term oestrogen deprivation of cells in culture provides a valid preclinical model for acquired oestrogen-resistant growth, the results presented here would suggest that perhaps the response rate for rapamycin in clinical breast cancer might be greater in ER-positive tumours with acquired endocrine resistance (Di Cosimo & Baselga 2008, Baselga *et al.* 2009, Ellard *et al.* 2009). The finding of greater sensitivity to growth inhibition in ZR-75-1 cells compared with MCF-7 or T-47-D cells demonstrates a variation in rapamycin sensitivity of ER-positive breast cancer cell lines but the finding of greater sensitivity in all three lines following adaptation to long-term oestrogen deprivation, which gives rise to oestrogen-independent growth, implies a more universal sensitivity to growth inhibition in ER-positive breast cancer cells that have lost growth response to E_2 . This suggests that it could be worthwhile investigating the clinical benefit of rapamycin in patient groups with acquired endocrine resistance rather than simply analysing effects of rapamycin across all patients with or without letrozole from the start (Di Cosimo & Baselga 2008, Baselga *et al.* 2009, Ellard *et al.* 2009).

Although the growth of all three oestrogen-maintained cell lines could be inhibited by blockade of the PI3K pathway with LY294002, no major differences in the dose-response curves for cell growth inhibition were apparent following long-term oestrogen deprivation in any of the three cell

models. This confirms the central importance of the PI3K pathway for growth of all three of these human breast cancer cell lines (Dufourney *et al.* 1997) but does not provide evidence of any alterations during the growth adaptation process. However, it is interesting that combining rapamycin with LY294002 inhibited growth of both MCF-7-ED and T-47-D-ED cells at lower concentrations than when each was added alone. MTORC1 is known to exert a negative feedback on PI3K signalling and the effectiveness of rapamycin can be compromised by release of the negative feedback and activation of AKT (O'Reilly *et al.* 2006). In breast cancer cells, MTOR/S6K has been shown to down-regulate IGF1R signalling, at least in part, through loss of insulin receptor substrate-1 expression and consequent inhibition of the PI3K pathway and rapamycin may reverse this feedback loop (O'Reilly *et al.* 2006). Other studies have shown that rapamycin can further lead to MAPK activation by a PI3K-dependent feedback loop when MTORC1 is inhibited (Carracedo *et al.* 2008). Although the oestrogen-deprived cells may have increased sensitivity to rapamycin through the development of an addiction to the MTOR pathway (Weinstein & Joe 2008), loss of the negative feedback may limit the sensitivity of the cells to rapamycin unless combined with another PI3K inhibitor.

The question then arises as to whether more selective PI3K inhibitors might be even more effective than LY294002 in combination with rapamycin. We chose to investigate the p110 α -selective PI3K inhibitor VIII on the basis that all three cell lines MCF-7, T-47-D and ZR-75-1 are known to express p110 α and p110 β proteins in varying ratios (Crowder *et al.* 2009) and that in MCF-7 cells protein levels for both the p85 regulatory subunits and p110 catalytic subunits are influenced by E_2 (Bernard *et al.* 2006). The dose-responses with this inhibitor were found over a rather small concentration range but the IC_{50} values of 17–23 nM are consistent with a previous report of 19 nM as IC_{50} for this inhibitor on growth of MCF-7 cells (Hayakawa *et al.* 2007). The reduced effect in any of the oestrogen-deprived cells with the PI3K α inhibitor VIII rather than LY294002 in combination with rapamycin suggests that more than one PI3K pathway can be used in the MCF-7-ED, T-47-D-ED and ZR-75-1-ED cells. Although the class 1 PI3K isoform, PI3K α has been shown to be important for breast cancer cell growth (Isakoff *et al.* 2005, Crowder *et al.* 2009), blockade of this pathway was less effective in combination with rapamycin than was the more general PI3K blockade with LY294002. Although the ability of human breast cancer cells to evade long-term growth inhibition by rapamycin remains unknown, these short-term exposure studies imply that it might be more effective to use drug combinations that knock out multiple crosstalking PI3K pathways simultaneously and rapamycin might be more efficacious in combination with a second PI3K inhibitor. It could be good news if appropriate drug combinations would allow for lower concentrations of each to be used.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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