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Evidence that low-dose, long-term genistein treatment inhibits oestradiol-stimulated growth in MCF-7 cells by down-regulation of the PI3-kinase/Akt signalling pathway

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ABSTRACT

The reduced incidence of breast cancer in certain Eastern countries has been attributed to high soy diets although this evidence is simply epidemiological. One of the major constituents of soy is genistein, but paradoxically this phytoestrogen binds to oestrogen receptors and stimulates growth at concentrations that would be achieved by a high soy diet, but inhibits growth at high experimental concentrations. To determine the effects of low-dose, long-term genistein exposure we have cultured MCF-7 breast cancer cells in 10 nM genistein for 10–12 weeks and investigated whether or not this long-term genistein treatment (LTGT) altered the expression of oestrogen receptor α (ERα) and the activity of the PI3-K/Akt signalling pathway. This is known to be pivotal in the signalling of mitogens such as oestradiol (E_2), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF). LTGT significantly reduced the growth promoting effects of E_2 and increased the dose-dependent growth-inhibitory effect of the PI3-K inhibitor, LY 294002, compared to untreated control MCF-7 cells. This was associated with a significant decreased protein expression of total Akt and phosphorylated Akt but not ERα. Rapamycin, an inhibitor of one of the down-stream targets of Akt, mammalian target of rapamycin (mTOR), also dose-dependently inhibited growth but the response to this drug was similar in LTGT and control MCF-7 cells. The protein expression of liver receptor homologue-1 (LRH1), an orphan nuclear receptor implicated in tumourigenesis was not affected by LTGT. The results show that LTGT results in a down-regulation of the PI3-K/Akt signalling pathway and may be a mechanism through which genistein could offer protection against breast cancer.

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1. Introduction

Epidemiological evidence suggests that diets rich in soy products protects against the incidence of breast cancer [1,2] and yet both in vitro and in vivo experiments have shown controversial evidence as to whether ingredients of soy, such as genistein (a weak oestrogenic compound), promote tumour growth or are protective [3]. In vitro studies generally show that low levels of genistein ($\leq 10^{-6}$ M) are stimulatory to growth whereas higher concentrations inhibit growth–an action presumed to be mediated by inhibition of tyrosine kinase [4,5]. In vivo studies have shown that genistein can either stimulate, have no effect or inhibit the growth of MCF-7 xenografts or chemically induced mammary tumours [6–8] and similarly in human studies short-term dietary soy supplements showed no consistent effects of proliferative activity in breast tissue [9–11].

Studies have typically investigated the acute effects of genistein on the growth of breast cancer cell lines which does not reflect the long-term effects that diets rich in soy may induce. We have thus developed an *in vitro* model of long-term exposure to low-dose (10 nM) genistein in MCF-7 cells and investigated whether or not this interferes with oestrogen signalling and alters the PI3-kinase/Akt/mTOR (mammalian target of rapamycin) cell signalling pathway. This pathway is activated not only by the growth factors, epidermal growth factor (EGF) and insulin-like growth factor (IGF), but also by oestrogen, all of which are mitogenic [12,13]. Experiments have shown that this pathway is pivotal in the growth-promoting actions of mitogens and that increased signalling of this pathway is associated with breast cancer and can confer resistance to anti-tumourigenic agents such as tamoxifen.

Liver receptor homologue 1 (LRH1/NR5A2) is a monomeric nuclear receptor and is present in human breast carcinomas and breast cancer cell lines expressing oestrogen receptor (ER)α [14,15]. Whilst LRH1 has been linked to a plethora of developmental and cellular functions [16], with regard to breast cancer its expression is induced by oestradiol through direct binding of ER to the LRH1 promoter [14]. It also induces expression of aromatase/CYP 19 and

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increases the expression of cyclin D1 [17]. Thus LRH1 may play an important role in tumourigenesis.

We hypothesized that long-term genistein treatment (LTGT) may alter the PI3-kinase/Akt/mTOR cell signalling pathway and to alter the expression of LRH1 through binding to ER. To test this hypothesis we compared the effects of oestrogen and inhibitors of PI3-K and mTOR, with and without oestrogen, on the growth of LTGT and control MCF-7 cells and compared these responses to the ER negative breast cancer cell line MDA-MB-123. The protein expression of ERα, Akt, phosphorylated Akt and LRH1 in control and LTGT cells was investigated in parallel.

2. Materials and methods

2.1. Chemicals and drugs

Genistein, oestradiol (E_2), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma (Poole, UK), insulin from Roche Diagnostics GmbH (Mannheim, Germany) and LY 294002 and rapamycin from Axxora Ltd (Nottingham, UK). All drugs were made up as stock solutions in DMSO and stored at −20 or −80 °C. On the day of the experiments they were appropriately diluted with culture medium such that 10 μl of drug solution was added to each well. Doses of genistein ranged from 10^{-8} to 10^{-6} M, LY294002 from 10^{-6} to 2.5×10^{-5} M, rapamycin 10^{-10}–10^{-8} M and E_2 and insulin at 10^{-8} and 10 ngs/ml respectively. After initial dilution of the stock solutions the highest concentration of each drug used contained 0.1 μl DMSO in 10 μl medium. Thus when 10 μl drug solution was added to the cultures (240 μl) the highest concentration of DMSO that the cells were exposed to was 0.04%. Control wells were treated with the same percentage concentration of DMSO.

2.2. Cell culture

Oestrogen receptor positive (ER^+) MCF-7 cells were purchased from the European Collection of Cell Cultures (Salisbury, UK) and the MDA-MB-123 cells were a kind gift from Dr. Kay Colston. They were cultured in 75 cm^2 flasks (Corning) in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), 2 mM glutamate, 100 IU/ml penicillin and 100 μg streptomycin (Sigma) at 37 °C in a fully humidified atmosphere containing 5% CO_2. The initial stock of cells was of passage 5–6. Stock cells were then divided, half being used for controls and the other half for long-term genistein treatment (LTGT). At this point cells were cultured in 10% charcoal-stripped FCS and phenol-free DMEM, the LTGT cells being treated with 10^{-8} M genistein and controls with the dimethyl sulphoxide (DMSO) diluent alone. Flasks were passaged when approximately 90% confluence was reached and medium was changed twice weekly. Experiments were performed after cells had been grown in genistein for 60–80 days although genistein was not present during the experimental protocols outlined below.

2.3. Cell growth

Previous experiments had determined the optimal plating density of MCF-7 cells and the period of linear growth. In these experiments cells were plated (day 1) in 96-well plates at a density of 5000 cells/well in 250 μl medium. To investigate whether there was any difference in the basal growth rates between control and LTGT MCF-7 cells, cells were plated on day 1 and an MTT assay [19] was performed on days 2, 3, 4, 5 and 8. The media and drugs were replaced on day 4. For all other experiments an MTT assay was performed on 8 control wells on day 2 and to the rest of the plate appropriately diluted drugs were added to triplicate wells in a standard 10 μl volume. On day 5 growth of the cells was measured by the MTT assay. For this assay 25 μl MTT (5 mg/ml) was added to each well and after 4 h the medium was removed and 250 μl DMSO was added. The amount of formazan formed by the mitochondrial reduction of MTT was measured by optical density on a plate reader (BioTek Northstar EL808) at a wavelength of 595 nM and reference wavelength of 690 nM. Growth was quantified by taking the ratio of the optical densities measured on day 5 to the mean optical density measured on day 2 and all data were normalized to control values.

2.4. Western blotting

Cells were cultured in 24 well plates at a concentration of 1.5×10^5 cells in 500 μl. After seeding they were cultured for 24 h in the presence of 5% charcoal-stripped FCS which was then replaced with serum-free medium and appropriate drugs were added to triplicate wells. 72 h later proteins were extracted from triplicate wells but for experiments investigating the expression of phosphorylated Akt the cells were exposed to 10 ng/ml insulin for 30 min prior to extraction. Proteins were extracted using NP-40 lysis buffer containing a protease inhibitor (Sigma) plus a fast freeze/thaw cycle. The final concentration of NP-40 was 0.013% and this concentration was compatible with the Bradford reagent (Sigma) used for measuring protein concentrations. 20 μg protein samples were resolved on 4–12% Tris-acetate Nupage gels (Invitrogen) prior to transfer onto a nitrocellulose membrane (Invitrogen). Membranes were blocked with 5% milk/PBS/Tween-20 and then incubated with primary antibodies overnight at 4 °C. Membranes were then exposed to the appropriate secondary antibodies prior to visualization with a chemiluminescent reagent (ECL, General Electric Healthcare, Amersham). The following antibodies were used: 1:1000 dilution ERα (monoclonal 62A3, Cell Signalling Technology), 0.5 μg/ml pan Akt (1,2,3) and phosphorylated Akt (S 473, 474, 472) (monoclonal, R&D Systems) and 1 μg/ml LRH1 (monoclonal, Abcam).

2.5. Statistical analyses

Growth data represent the mean ± S.E.M. of triplicate observations from at least 3 independent experiments and are expressed as a percentage of the control value. Basal growth rates, however, were determined form quadruplet observations from two experiments. Data from the Western blots represent the mean ± S.E.M. densitometry measurements taken from 3–5 individual experiments and all data were normalized to the control value. Statistical significance was determined by an analysis of variance followed by Gabriel's test when more that two groups were compared or with an unpaired Student's *t*-test when two groups were compared.

3. Results

An initial dose response showed that genistein at a dose of 10^{-8} M had a small but significant growth promoting effect in both MCF-7 cells and MDA-MB-231 cells, but only MCF-7 cells showed a dose response to genistein at increasing doses of the phytoestrogen (Fig. 1). The small effect of 10^{-8} M genistein on the growth of MCF-7 cells coupled with the fact that this circulating concentration can be achieved easily by dietary means [20] provided the rationale for using a dose of 10^{-8} M genistein for the LTGT MCF-7 cells. The basal growth rate observed between LTGT and untreated MCF-7 cells showed that growth rate in the absence of oestradiol was similar in both groups although by day 8 growth in the LTGT MCF-7 cells was lower than that observed in the untreated MCF-7 cells but not significantly (Fig. 2).

In untreated MCF-7 cells oestrogen stimulated growth by approximately 60% of control values whilst in LTGT cells stimulation was less than 20% and significantly lower ($p < 0.001$) than that seen in untreated cells. MDA-MB-231 cells showed no response to

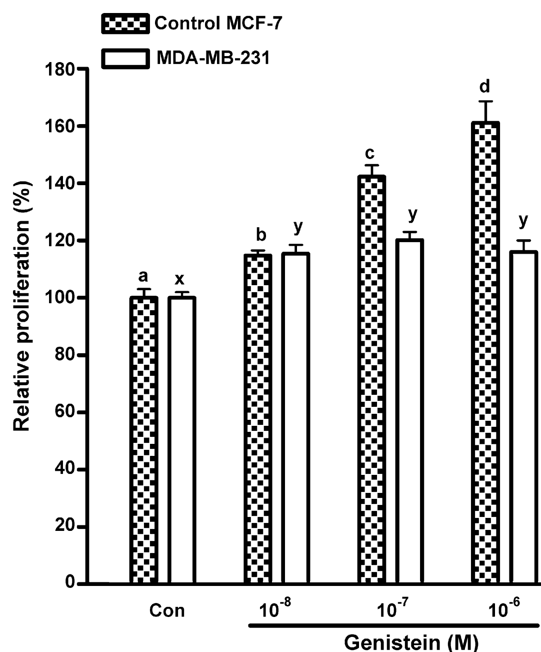

Fig. 1. Dose response of MCF-7 and MDA-MB-231 cells to increasing doses of genistein. Cell lines were cultured for 4 days in the presence of genistein and cell growth determined by the MTT assay. All optical density measurements were normalized to control values and expressed as a % increase or decrease to controls. Data represent the mean ± S.E.M. of triplicate observations from 3 independent experiments ($n = 9$). Different letters, a–d, denote statistical differences between the MCF-7 cells in control and treated groups and *x*, *y* statistical differences between control and treated MDA-231 cells; $p < 0.05$, Gabriel's test.

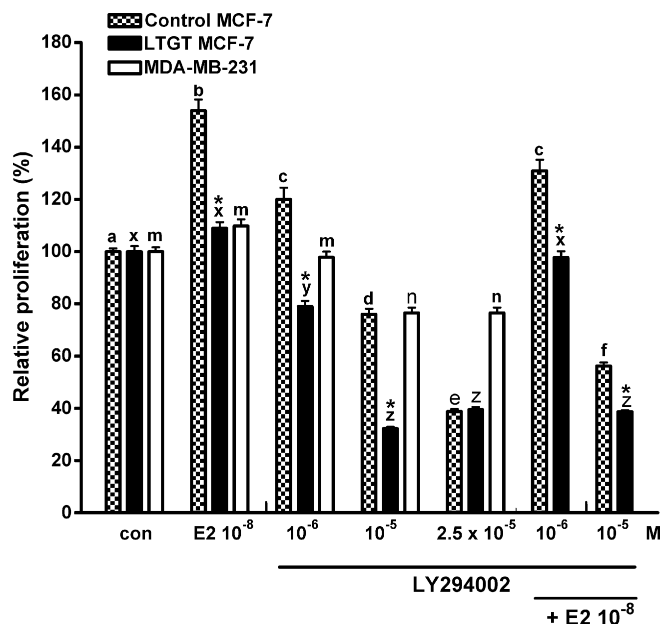

Fig. 3. Long-term genistein treatment (LTGT) impairs the growth promoting effect of 10^{-8} M oestradiol (E_2) and increases the growth retarding effect of the PI3-kinase inhibitor, LY 294002. Control and LTGT MCF-7 cells and MDA-MB-231 cells were cultured in the presence of drugs for 4 days and the relative increase in growth, as determined by the MTT assay, was calculated. All optical density measurements were normalized to control values and expressed as a % increase or decrease to controls. Data represent the mean ± S.E.M. of triplicate observations from 3 independent experiments ($n = 9$). Different letters, a–e, x–z and m, n, denote statistical significance ($p < 0.05$) between control and treatment groups within each cell line (Gabriel's test). $^*p < 0.001$ compared with MCF-7 control cells in the same treatment group (Student's *t*-test).

E_2 (Fig. 3). The inhibitor of PI3-kinase, LY 294002 induced a small increase in growth at the lowest dose tested (10^{-6} M) in control MCF-7 cells but at the two higher doses (10^{-5} and 2.5×10^{-5} M) LY 294002 significantly inhibited growth. In LTGT cells there was no stimulation of growth at the lowest dose and the inhibition of growth observed with 10^{-5} M was significantly greater ($p < 0.001$) than that observed in control cells. At the highest dose the inhibition

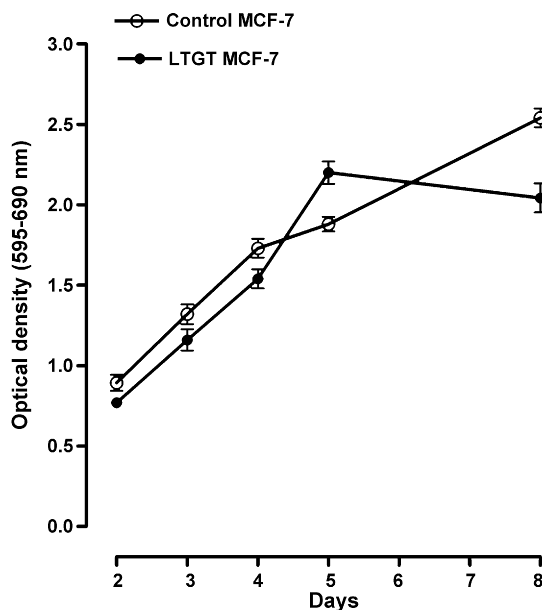

Fig. 2. Basal growth rate of LTGT and control MCF-7 cells. Cells were plated on day 1 and cell growth, measured by the MTT assay, was assessed on days 2, 3, 4, 5, 6 and 8. Data represent the mean ± S.E.M. of quadruplate observations from two independent experiments ($n = 8$).

was similar in both control and LTGT cells (Fig. 3). In MDA-MB-123 cells there was no effect of oestradiol and the inhibitory effect of LY 294002 was significantly less at the highest dose tested compared with the MCF-7 cells. LY 294002 at 10^{-5} M significantly inhibited the stimulatory effect of oestradiol in control MCF-7 cells and this inhibition was significantly greater that that achieved with LY294002 alone (Fig. 3). In LTGT MCF-7 cells the inhibitory effects of LY 294002 were the same irrespective of the presence of oestradiol (Fig. 3).

The expression of ERα was similar in control and LTGT MCF-7 cells and after oestradiol treatment ERα expression was similarly reduced in both groups (Fig. 4). However, the differences in growth rates in response to oestradiol and LY 294002 were reflected in the expression of total Akt. In control MCF-7 cells E_2 increased Akt expression by approximately 50% but in LTGT cells Akt expression was unchanged by E_2 treatment and significantly lower than that observed in control MCF-7 cells treated with E_2 (Figs. 5 and 6). There was a dose related increase in Akt expression associated with an inhibition of PI3-kinase and this effect was potentiated by oestradiol. In contrast LTGT cells showed reduced expression of Akt under all treatments compared with control MCF-7 cells although the same trends with LY 294002 and LY 294002 plus oestradiol were observed. Insulin treatment stimulated AKT phosphorylation in untreated and E_2-treated control MCF-7 cells and this was dose-dependently inhibited by the PI3-kinase inhibitor LY 294002 (Fig. 5). In contrast phosporylated AKT was barely detectable in LTGT cells under all conditions. There was no significant differences in the expression of LRH1 between control MCF-7 and LTGT cells although mean LRH1 expression (pooled data not shown) increased in the presence of LY 294002 in both control and LTGT cells but not in the presence of oestradiol (Fig. 5). There was no difference in the dose-dependent inhibition of growth with rapamycin between

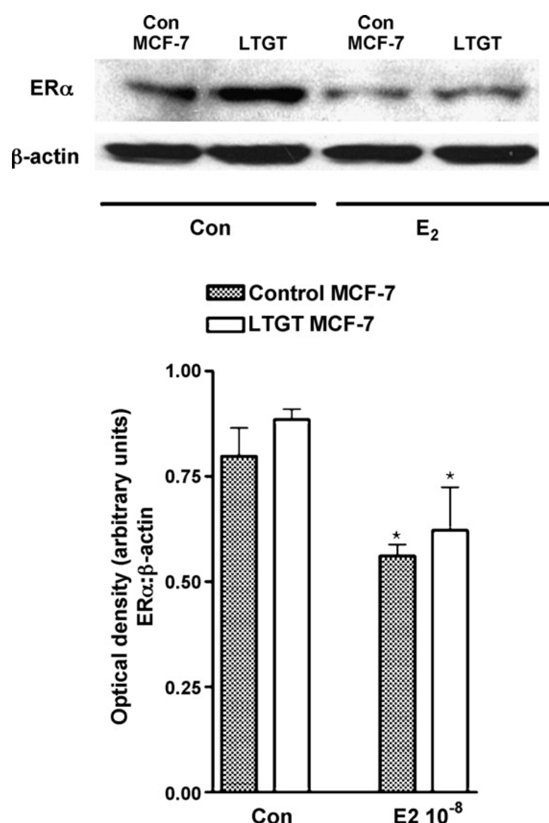

Fig. 4. Expression of ERα in control (Con MCF-7) and long-term genistein treated (LTGT) MCF-7 cells. Protein lysates from control MCF-7 and LTGT cells with or without 72 h exposure to oestradiol (E_2) were subjected to immunodetection. (a) Representative Western blot and (b) pooled data from three Western blots which represent the mean ± S.E.M. of the band intensities which were normalized to β-actin. $^{*}p < 0.01$ versus the corresponding control value in the absence of E_2 (Student's *t*-test).

control and LTGT cells Furthermore maximum inhibition of mTOR only inhibited growth by about 30% compared with around 60% observed with inhibition of PI3-kinase at the highest dose tested (Figs. 7 and 3). Like LY 294002, rapamycin inhibited the mitogenic effect of oestradiol.

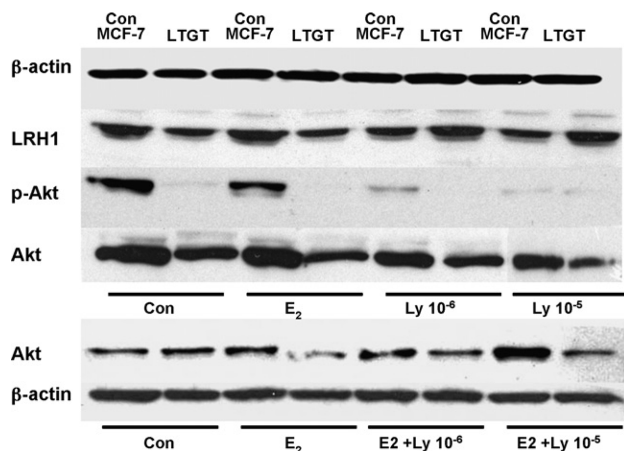

Fig. 5. Representative Western blots of the effects of long-term genistein treatment (LTGT) on the expression of total Akt, phosphorylated Akt (p-Akt) induced by a 30 min exposure to 10 ngs/ml insulin, liver receptor homologue1 (LRH1) and β-actin (upper panels). Protein lysates from control (Con) MCF-7 and LTGT cells exposed to oestradiol (E_2) or LY 294002 (LY) for 72 h were subjected to immunodetection. Lower panels show total Akt and β-actin when cells were cultured for 72 h in the presence of E_2 with or without different doses of LY 294002.

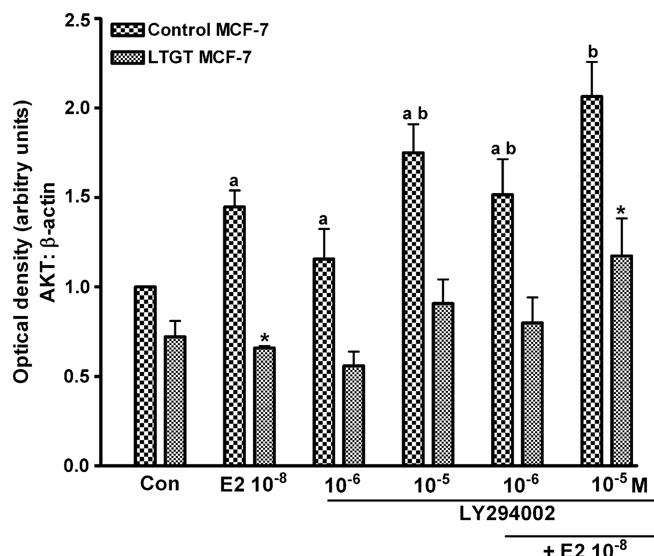

Fig. 6. The expression of Akt in the untreated control MCF-7 and long-term genistein treated (LTGT) MCF-7 cells. Cells were exposed to oestradiol (E_2), LY 294002 (LY) or E_2 plus LY for 72 h and then subjected to immunodetection for expression of total Akt and β-actin. Data represent the mean ± S.E.M. of 4 Western blots and are expressed as a ratio to β-actin and normalized to the control value of the untreated MCF-7 cells for each experiment. Different letters denote statistical significances within the control MCF-7 group (Gabriel's test) and $^{*}p < 0.01$ versus the corresponding value for the control MCF-7 cells (Student's *t*-test).

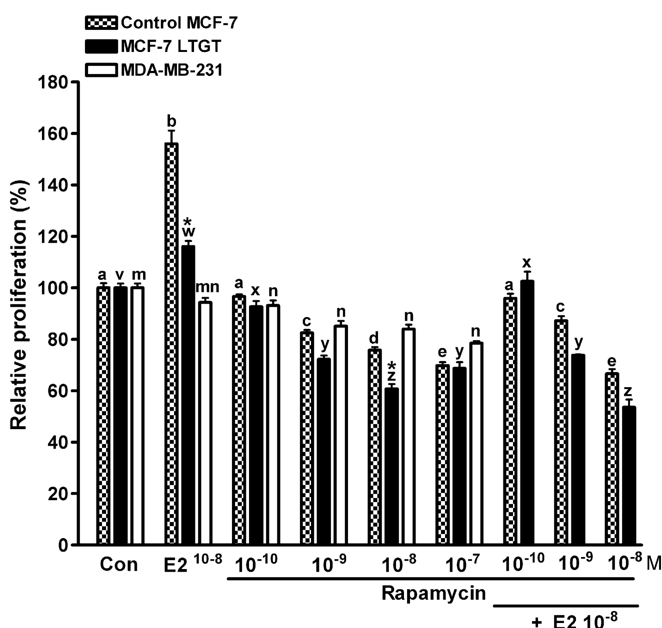

Fig. 7. Dose response of control MCF-7 and LTGT MCF-7 cells and MDA-MB-231 cells to 72 h rapamycin with or without 10^{-8} M oestradiol (E_2). For details see Fig. 3 legend. Data represent the mean ± S.E.M. of triplicate observations from 3 independent experiments ($n = 9$). Different letters, a–e, v–z and m, n, denote statistical significance ($p < 0.05$) between treatment groups within each cell line (Gabriel's test). $^{*}p < 0.001$ compared with MCF-7 control cells (Student's *t*-test).

4. Discussion

LTGT inhibited the growth promoting activity of oestradiol in MCF-7 cells despite the fact that ERα expression was similar in control MCF-7 cells and LTGT cells. It also increased the ability of the PI3-kinase inhibitor, LY 294002, to inhibit growth of these breast cancer cells. Cell proliferation induced by oestrogenic substances in vitro (the E-SCREEN) has been assessed by several different meth-

ods including the MTT assay [19,21,22] and we, like others, have shown a linear correlation between cell number and absorbance validating the use of the MTT assay to discriminate small variations in cellular densities [19,22].

The inhibition of E_2 stimulated growth and increased efficacy of LY 294002 to inhibit growth in LTGT cells at submaximal doses was correlated with a reduction in total Akt expression that was not significantly altered by oestradiol or LY 294002 treatment. This contrasts control MCF-7 cells in which both oestradiol and LY 294002 increased expression of Akt. Oestradiol may directly increase Akt expression through induction of gene transcription whilst the increased expression of Akt in the presence of LY 294002 is less easy to interpret. This inhibitor may in some way alter the turnover rate of Akt thereby increasing its cellular concentration; hence the dose related increase in Akt in the presence of LY 294002. Thus data show that long-term genistein treatment down-regulates the Akt signalling pathway in the absence of any changes in ERα expression.

The PI3-K/Akt signalling pathway is known to be activated by E_2, IGF and EGF in MCF-7 cells and there is considerable cross-talk and synergistic effects between downstream cell signalling pathways stimulated by these mitogens [12,13,23]. Additionally evidence suggests that anti-oestrogen resistant breast cancer cells require an active PI3-kinase/Akt for continued growth [24–26]. Together studies suggest a pivotal role of an active PI3-kinase/Akt signalling pathway for the growth of breast cancer cells.

The E_2-stimulated growth and expression of Akt in control MCF-7 cells measured in these experiments are in agreement with another study showing that E_2 could induce up-regulation of the PI3-K/Akt signalling pathway in $ER\alpha^+$ MCF-7 cells but not in $ER\alpha^-$ MDA-MB-231 cells [27]. In LTGT cells this effect was not observed. The mitogenic effects of oestradiol are considered to be mediated via ERα and studies have shown that IGF-1 stimulation of Akt phosphorylation or PI3-kinase expression is dependent on ERα [12,28]. This suggests that oestrogen-stimulated mitogenesis in MCF-7 cells could be mediated by enhancement of the IGF-1 signalling pathways. This is supported by the finding that E_2 increased the expression of the IGF-1 receptor (IGF-1R) and insulin receptor substrate (IRS-1) proteins [29].

Genistein has also been shown to increase the expression of IGF-1R and IRS-1 in MCF-7 cells at a dose (10^{-6} M) that also stimulated cell growth. This effect was abolished by co-treatment with the oestrogen antagonist, ICI 182,780 or an IGF-1R antagonist [30]. Together this evidence would suggest that oestradiol and genistein stimulates growth by interacting with ERα and up-regulating the PI3-K/Akt signalling pathway; in turn this could enhance the activity of the IGF-1 cell signalling pathway which may be the dominant growth stimulatory pathway in human breast cancer cells [31]. However, these experiments did not investigate the effects of LTGT in MCF-7 cells.

Although it is known that high doses of genistein ($\geq 10^{-5}$ M) induce apoptosis and inhibit growth in breast cancer cells [32] the dose of genistein used in the present experiments (10^{-8} M) for LTGT is below or at the threshold at which it induces significant growth in MCF-7 cells but is within the range of circulating genistein concentrations measured in men and women on a high soy diet [20].

Genistein has a greater affinity for ERβ than ERα and there is evidence that activation of ERβ can silence the growth promoting effects of ERα [3]. Since MCF-7 cells express both ERα and β it is suggested that the present findings could reflect a preferential ability to bind to ERβ at a low dose and thus down-regulate the PI3-K/Akt signalling pathway. At higher doses (e.g. 10^{-6} M) genistein may interact with ERα, stimulate growth and up-regulate the PI3-K/Akt pathway as observed by Chen and Wong [33]. Whether such an effect is mediated through nuclear receptors or extranuclear receptors is high speculative. Alternatively genistein is known to be an inhibitor of tyrosine kinase, although inhibition of this enzyme is only observed at high doses [34]. However, long-term exposure of cells to genistein may induce desensitization or down-regulation of this enzyme and hence cell signalling pathways such as those stimulated by oestrogen and IGF-1.

mTOR is activated both by mitogens that activate the PI3K/Akt pathway and nutrient/energy availability [35] and studies have shown that inhibition of mTOR inhibits growth in ERα+ breast cancer cell lines and also inhibits E_2-stimulated growth in these cells [36,37]. Our studies confirmed these reports although the dose-dependent inhibitory action of rapamycin was not different between control and LTGT cells. Studies have suggested that rapamycin may reduce tumour growth by inhibiting ER-dependent transcriptional activity or through a more generalized effect by inhibiting phosphorylation of S6K1 and 4E-BP1 proteins and hence translation of ribosomal proteins [38,39]. Thus whilst LTGT affected the PI3-K/Akt signalling it had no observable effect on mTOR signalling with respect to growth. However, PI3-K/Akt has numerous other down-stream targets including Bad and Foxo transcription factors and glycogen synthase kinase-3 all of which are involved in cell growth [40].

The contribution of LRH1 to tumourigenesis remains unclear although it can induce the expression of cyclins D1 and E1 [18] and its mRNA is up-regulated in MCF-7 cells by both E_2 [15] and sphingosine-1-phosphate, a second messenger in mitogenesis [41]. These experiments did not show an increase in the protein expression of LRH1 although Annicotte et al. [15] showed that maximal LRH1 mRNA expression was reached after 6 h of E_2 treatment and had markedly declined after 24 h. Whether or not there is any significance in the increased levels of LRH1 in the presence of LY 294002 remains to be determined.

Whatever the mechanisms through which LTGT induces resistance to the growth promoting effects of E_2, these studies have shown that a down-regulation of the PI3-K/Akt pathway could be involved. Furthermore they support epidemiological evidence that diets rich in soy products could provide some protection against the mitogenic effects of oestrogens.

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