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8-Prenylnaringenin inhibits epidermal growth factor-induced MCF-7 breast cancer cell proliferation by targeting phosphatidylinositol-3-OH kinase activity

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ABSTRACT

8-Prenylnaringenin (8PN), one of the strongest plant-derived oestrogen receptors (ERs) ligand, has been suggested to have potential cancer chemo-preventive activities and anti-angiogenic properties. Because published data suggest that ERs serve as nodal point that allows interactions between hormones and growth factors mediated pathways, we decided to investigate the effects exerted by 8PN on Epidermal growth factor (EGF)-elicited pathways in breast cancer cells. Here we show that in ER positive MCF-7 cells, 8PN interferes with EGF induced cell proliferation by strongly inhibiting activation of PI(3)K/Akt pathway, without affecting EGFR expression or tyrosine phosphorylation, and exerting a synergistic activation of Erk1/2 phosphorylation. Moreover, we demonstrate that 8PN is a direct inhibitor of PI(3)K activity as it is shown by *in vitro* experiments with the purified enzyme and by its inability to impair serine phosphorylation of a constitutive active form of Akt. These findings suggest that inhibition of PI(3)K is a novel mechanism which contributes to 8PN activity to inhibit cancer cell survival and EGF induced proliferation.

1. Introduction

Accumulated evidence in the last few years indicates chemo-prevention as a valuable strategy to either prevent or impair cancer progression [1]. Among chemo-preventive chemicals, natural non-toxic products (or their synthetic analogs), in particular dietary components, have been under scrutiny for their potential to halt or reverse the development or progression of cancer [2]. Studies of several compounds of plant origin, especially the flavonoids, suggest that these molecules can harbour cancer chemo-preventive and/or therapeutic activities. Flavonoids are a group of constituents of food and drinks commonly consumed by humans, that includes compounds of different chemical classes such as flavones (7,8-benzoflavone), flavonols (quercetin), flavanol (cathechin), flavanones (naringenin), isoflavones (genistein), and chalcones (xanthohumol) [3]. Among flavonoids, 8-prenylnaringenin (8PN) has been identified as the estrogenic principle of the hop plant (*Humulus lupulus* L.), that is largely used in the brewing industry as a preservative and flavouring agent to add bitterness and aroma to beer [4]. While the biological effects of the dietary exposure to 8PN are substantially unknown, several intriguing observations have been done regarding its activity at cellular level [5–7]. 8PN is one of the strongest plant-derived estrogenic compounds identified to date. 8PN has been shown to compete strongly with oestradiol for binding to both oestrogen receptor (ER) α and β with a relative binding affinity of about 0.01 (oestradiol = 1), ten times higher than that of the archetypal phytoestrogen genistein [8,9]. ERα and ERβ, belong to the steroid/thyroid hormone family of transcription factors and mediate most of the biological effects of estrogens, by interacting with the transcription machinery [10–13]. Moreover, a small fraction of ERs is associated with the plasma membrane and through concerted activation of the MAP kinase and the PI(3)K/Akt signalling pathways, contribute to regulate cell proliferation and prevent apoptosis [14–20]. In particular, the activation of PI(3)K and the generation of $PI(3,4,5)P_3$ are both necessary for the phosphorylation on Thr-308 and Ser-473 of Akt/PKB, a downstream mediator of PI(3)K signalling [21–24]. In different cell types, Akt/PKB has been

Abbreviations: PI, phosphatidylinositol; PI(4,5)P2, phosphatidylinositol-(4,5)-bis-phosphate; PI(3,4,5)P3, phosphatidylinositol-(3,4,5)-tris-phosphate; PI(3)K, phosphatidylinositol-3-OH kinase; PS, phosphatidylserine.

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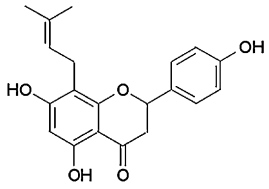

Fig. 1. Structural formula of 8-prenylnaringenin.

shown to promote cell survival and suppress apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of adhesion, but has been also described to be involved in the control of cell proliferation [25–27].

Increasing evidence suggests that ERs serve as nodal points allowing multiple interactions and crosstalk between hormones and growth factors mediated pathways. For instance, ERs are functionally involved in Epidermal Growth Factor Receptor (EGFR)-induced MAP kinase activation in MCF-7 cells [28]. Interestingly, long term blockade of ERs function with tamoxifen irreversibly causes cells to over-express members of the EGFR family [29] and administration of the antiestrogen ICI 182780 reduces the response to EGF in the mammary gland [29,30]. Finally, deletion of ERα in ER-knockout mice, does not allow EGF-induced DNA synthesis in the uterus, without affecting EGF and EGFR expression [29,31]. These observations indicate that there is a true requirement for the presence of ERs for EGF-mediated biological functions, at least in some cell types, and that the combination of inhibitors of EGFR and oestrogen deprivation is more effective in inhibiting ER-positive breast cancer growth than either agent alone [29,32]. In a recently published paper we have demonstrated that 8PN significantly disrupted the ER mediated PI(3)K/Akt signalling pathway and induced apoptosis in a dose dependent manner in MCF-7 cells [33]. In this study, we examine if 8PN features inhibitory effects on EGFR function in breast cancer cells.

2. Results

2.1. 8PN inhibits EGF dependent MCF-7 cell proliferation

In order to determine if 8PN (a structural formula is shown in Fig. 1) might interfere with EGF induced proliferation, serum starved MCF-7 cells were exposed to different concentrations (1–100 μM) of 8PN in the absence or in the presence of human recombinant EGF (10 ng/ml). Proliferation was scored by counting cell number after 24 h of treatment. As previously reported 8PN significantly impaired cell survival in the absence of EGF (Fig. 2, left panel). Moreover, 8PN reduced EGF induced proliferation in a concentration dependent manner (Fig. 2, right panel). At doses between 1 and 20 μM, 8PN significantly inhibited EGF induced cell proliferation. The further cell number decrease, observed upon EGF stimulation of cells treated with 8PN at doses higher than 20 μM, likely reflected the cytotoxic activity observed at similar concentrations in the absence of EGF. Moreover, 20 μM 8PN did not affect neither expression nor tyrosine phosphorylation of EGFR (Fig. 3A, panels 1 and 2), suggesting that 8PN acts on a more distal event in the EGFR-mediated signal transduction cascade

2.2. 8PN interferes with Erk 1/2 and PI(3)K/Akt pathways in MCF-7 cells

As mentioned above both estrogens and growth factors promote c-Src/MAPK and PI(3)K/Akt signal transduction pathways which are both involved in the control of cell proliferation [24]. We therefore examined if 8PN could interfere with EGF induced activation of these pathways in MCF-7 cells. As described in our previous work [37], upon 10 min of treatment, 20 μM 8PN, like 17β-oestradiol, stimulated Erk 1/2 phosphorylation in ER dependent manner. Moreover, when cells were co-treated with EGF (10 ng/ml), 8PN synergized with growth factor resulting in a higher Erk 1/2 phosphorylation, as compared with each treatment alone (Fig. 3A, panels 3 and 4). On the contrary and differently from 17β-oestradiol, 8PN did not induce Akt phosphorylation and abolished that induced by EGF, without affecting Akt protein content (Fig. 3A, panels 5 and 6). In conclusion these data show that 8PN increases EGF induced Erk 1/2 activity, likely through ER activation, while it does not induce and inhibits the EGF-mediated Akt activation, through an unknown mechanism.

2.3. 8PN inhibits the EGF induced expression of cyclin D1

It is well documented that cyclin D1, which regulates the cell-cycle progression through CDK4/6, is induced by hormones and growth factors treatments. Moreover it has been described that both Erk 1/2 and PI(3)K/Akt activated pathways are required for its induction in MCF-7 cells [24]. Based on the opposite effects exerted by 8PN on Erk 1/2 and Akt pathways, we examined if 8PN could inhibit EGF induced cyclin D1 expression. As shown in Fig. 3B, EGF (10 ng/ml) induced cyclin D1 expression, with maximal extent at 2 h of treatment. Interestingly, cyclin D1 expression in response to EGF was abolished by co-treatment with 20 μM 8PN. Thus we can raise the hypothesis that 8PN inhibits EGF-induced MCF-7 cell cycle progression through cyclin D1down-regulation.

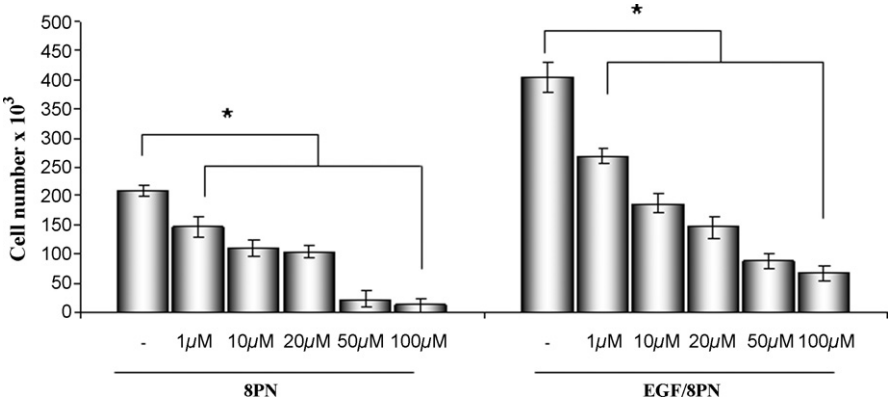

Fig. 2. Effects of 8PN on MCF-7 cell viability and EGF-mediated proliferation. MCF-7 cells made quiescent as indicated in Section 4 for 24 h, were incubated with different concentrations of 8PN in the absence or the presence of EGF (10 ng/ml), the effect on cell growth was examined after 24 h of treatment. The results are means converted to fold induction above the control value. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control).

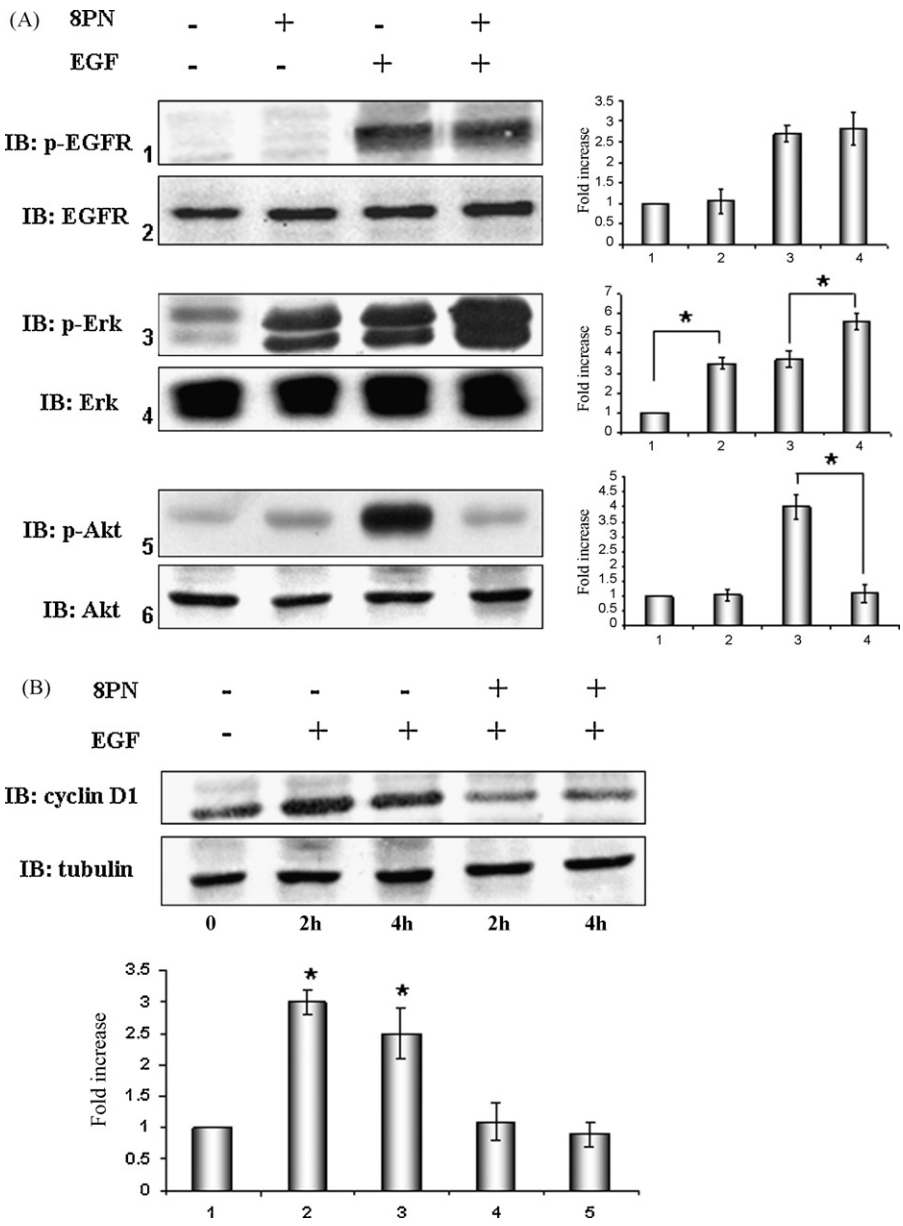

Fig. 3. Effects of 8PN on EGFR-mediated signalling in MCF-7 cells. (A) MCF-7 cells made quiescent as indicated in Section 4 for 24 h, were treated with 20 μM 8PN in the presence of EGF (10 ng/ml) for 10 min and detergent extracted. Levels of phosphorylated EGFR (pY 1086), Erk 1 (Thr 202 and Tyr 204), Erk2 (Thr 185 and Tyr 187) MAP kinases, and Akt (Ser 473 and Thr 308) were analyzed by immunoblotting. Membranes were also blotted with antibodies to EGFR, Erk 1/2 and Akt to show an equal amount of loading. The relative phosphorylation levels normalized with the total protein and converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control). (B) MCF-7 cells in the same conditions as in A) were treated with 20 μM 8PN in the presence of EGF for 2 or 4 h. Cell extracts were run on 10% SDS–PAGE, transferred to nitrocellulose, blotted with anti-cyclin D1. The bands were normalized to tubulin. The normalized cyclin D1 expression levels converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control).

2.4. 8PN inhibits EGF induced PI(3)kinase activation

PI(3)K is central to the coordinated control of multiple cell-signalling pathways regulating cell survival, proliferation and migration. Upon activation, PI(3)K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) that recruits at the cell membrane and activates serine-protein kinases such as Akt/PKB and PDK1 [29,30]. Thus we tested the hypothesis that 8PN might inhibit Akt/PKB phosphorylation by direct inhibition of PI(3)K activity. In order to test this hypothesis we assayed *in vitro* PI(3)K activity, in the presence of different doses of 8PN. PI(3)K assay was performed on proteins immunoprecipitated with anti-phosphotyrosine antibodies from lysates of MCF-7 cells stimulated 10 min with EGF (10 ng/ml). Indeed, 8PN inhibited PI(3)K activity in a dose-dependent manner, being 1 μM the minimal effective concentration (Fig. 4). These results strongly suggest that 8PN abrogate Akt/PKB activation by direct inhibition of PI(3)K activity. It must be underlined that in these *in vitro* conditions 8PN acted at concentration ten times lower then those that were effective on live cells, probably due to different availability of the molecule.

2.5. Expression of constitutively activated Akt restores EGFR-mediated cell proliferation

In order to provide further support to the tenet that 8PN, by direct inhibition of PI(3)K activity, blocks Akt signalling and EGF-

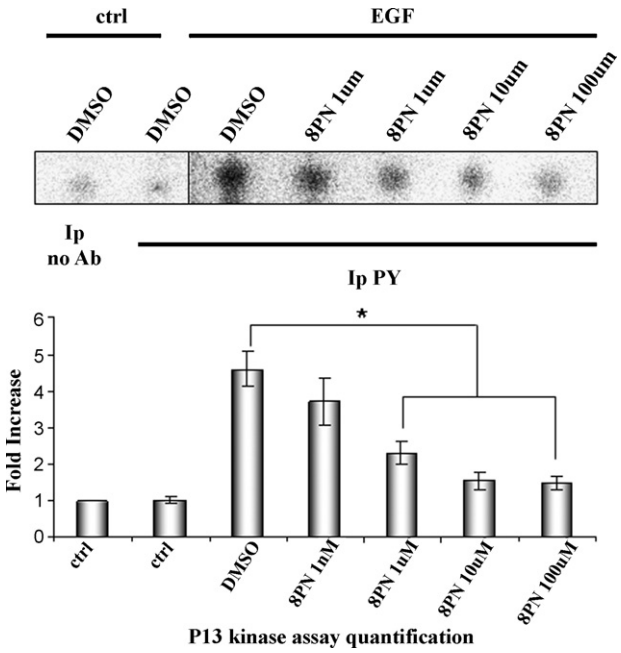

Fig. 4. Effects of 8PN on EGF-induced PI(3)K activation. After treatment with or without EGF (10 ng/mL) for 10 min MCF-7 cells were lysed and subsequently tyrosine phosphorylated proteins were immunoprecipitated from cell extracts. PI(3)K activity was determined as described in Section 4 *in vitro* after addition of the indicated doses of 8PN. The relative PIP_3 levels converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control).

sustained cell proliferation, we assayed whether expression of a constitutively active form of Akt (MyrAkt), which does not require PI(3)K contribution for activation, could rescue EGF-dependent cell proliferation from 8PN-mediated inhibition. Myr-Akt expressed in MCF-7 cells was maximally activated, measured as both Ser-473 and Thr-308 sites phosphorylation, even in the absence of EGF. As expected, Akt/PKB activation, without affecting both basal and EGF-induced Erk 1/2 phosphorylation (Fig. 5A), resulted in cyclin D1 induction and enhanced MCF-7 cell proliferation, even in the absence of EGF (Fig. 5B and C). Importantly, in Myr-Akt expressing cells 20 μM 8PN did not inhibit both basal and EGF induced cyclin D1 expression and cell proliferation (Fig. 5B and C), demonstrating that its inhibitory activity lies indeed in its ability to directly affect PI(3)K activity.

2.6. 8PN inhibits Akt phosphorylation in MDA-MB231 cells

Because we demonstrated a direct inhibition of 8PN on PI(3)K activity, we tested its effects on ER negative MDA-MB231 cells. Cells were exposed to different concentrations (1–100 μM) of 8PN in the absence or in the presence of human recombinant EGF (10 ng/ml) and proliferation was assayed after 24 h of treatment. As shown in Fig. 6A, 8PN reduced the viability of MDA-MB231 cells only at the highest dose employed (100 μM) in both basal and stimulated conditions. Moreover, as reported in literature, EGF did not significantly induce MDA-MB231 cell proliferation. We therefore examined if 8PN could interfere with basal and EGF induced Erk 12 and Akt/PKB phosphorylation in MDA-MB231 cells. Upon 10 min of treatment, 100 μM 8PN did not stimulate Erk 1/2 phosphorylation but reduced basal and EGF induced Akt phosphorylation, without affecting Akt protein content (Fig. 6B). In conclusion these data indicate that ER positive cells are more sensitive to 8PN and that this molecule can interfere with EGFR pathway either modulating ER mediated signals or directly inhibiting, in a ER independent manner, PI(3)K activity.

3. Discussion

Dietary phytochemicals are plant-derived food components suspected to be involved in chemo-preventive actions. Identification of increasing numbers of pharmacologically active compounds from natural products and examination of their molecular mechanisms are opening new perspectives. Several flavonoids have been shown to inhibit cancer cells proliferation and tumour growth in preclinical studies and are now being tested in phase II trials of prevention. Phytoestrogens which structurally resemble mammalian estrogens may contribute to lower risks for breast, prostate and colon tumours by modulating molecular events involved in tumour progression. These compounds may bind to ERs and thus inhibit cell proliferation in ERs positive cells, although adverse, pro-estrogenic effects may also occur. The ERs are quite promiscuous in ligand binding, and many non-steroidal compounds of dietary and environmental origin have been found to possess either estrogenic or anti-estrogenic activities [34,35]. The mechanism by which E2 exert proliferative effects is assumed to be exclusively mediated by rapid membrane-starting actions [20,36,37]. The ability of membrane ER and or growth factor receptor tyrosine kinases to signal through multiple kinases to the nucleus undoubtedly has an impact on different aspects of cellular functions (cell proliferation, survival, transcriptional co-activator recruitment) [16]. Recently Migliaccio et al. have reported evidence of interplay between the EGF-activated EGFR, and steroidal receptors. They have described that the EGF-activated Src, which is associated with ER/Androgen Receptor (AR) dimer, acts strongly on EGFR phosphorylation. Conversely, when ER and/or AR are locked in an inactive conformation, their action on Src and EGFR is missing or heavily impaired. Interestingly, in MCF-7 cells, steroid antagonists and silencing of steroid receptor genes abolish the EGF-elicited DNA synthesis, thus indicating that such an effect requires steroid receptors [28]. In a recent study, we have analyzed rapidly activated signal transduction pathways and biological effects exerted by 8PN on breast cancer cells [33]. 8PN biological profile appeared well differentiated from that of E2 and genistein-type phytoestrogens. Because of the established relevance of EGFR in breast cancer and its documented crosstalk with ER, in this study we have examined the effects exerted by 8PN on signals elicited by this tyrosine kinase receptor. The detection of upstream EGF receptor activation using specific antibodies that recognize phospho-tyrosine residues showed that 20 μM 8PN did not reduce the ligand induced phosphorylation of EGFR and in ER positive cells synergized with EGF to induce Erk 1/2 MAP kinase activation. Conversely, we describe that 8PN did not induce and significantly inhibited EGF-mediated Akt phosphorylation at Ser-473 and Thr-308 sites, without affecting the protein level. It has been reported that some flavonoids inhibit PI(3)K activity through competition with ATP at its binding site or inhibit PI(3)K activation through suppression of the binding of PI(3)K to the EGFR. Herein, we show that 8PN directly inhibited the PI(3)K activity immunoprecipitated with anti-phosphotyrosine antibodies form EGF-stimulated MCF-7 cells lysates. The 8PN mediated inhibition of PI(3)K was dose dependent and quite effective at 1 μM, concentration of 8PN that resulted in a 50% inhibition of PI(3)K activity. We propose that part of 8PN cellular activities derived from direct inhibition of PI(3)K kinase resulting in the impairment of Akt activation and Akt-mediated cell proliferation and inhibition of apoptosis. In live-cell studies we observed effects only when we used ten times higher concentrations that are clearly non-dietary but pharmacological. Serum concentration of free 8PN in the range of 0.1 μM has been observed in pharmacokinetics experiments with single, well tolerated therapeutic doses of this compound [33]. Though well over one to ten orders of magnitude higher, the concentrations we employed might be achieved intracellularly in sensitive tissues by selective uptake, parentheral administration, or repeated administrations of 8PN.

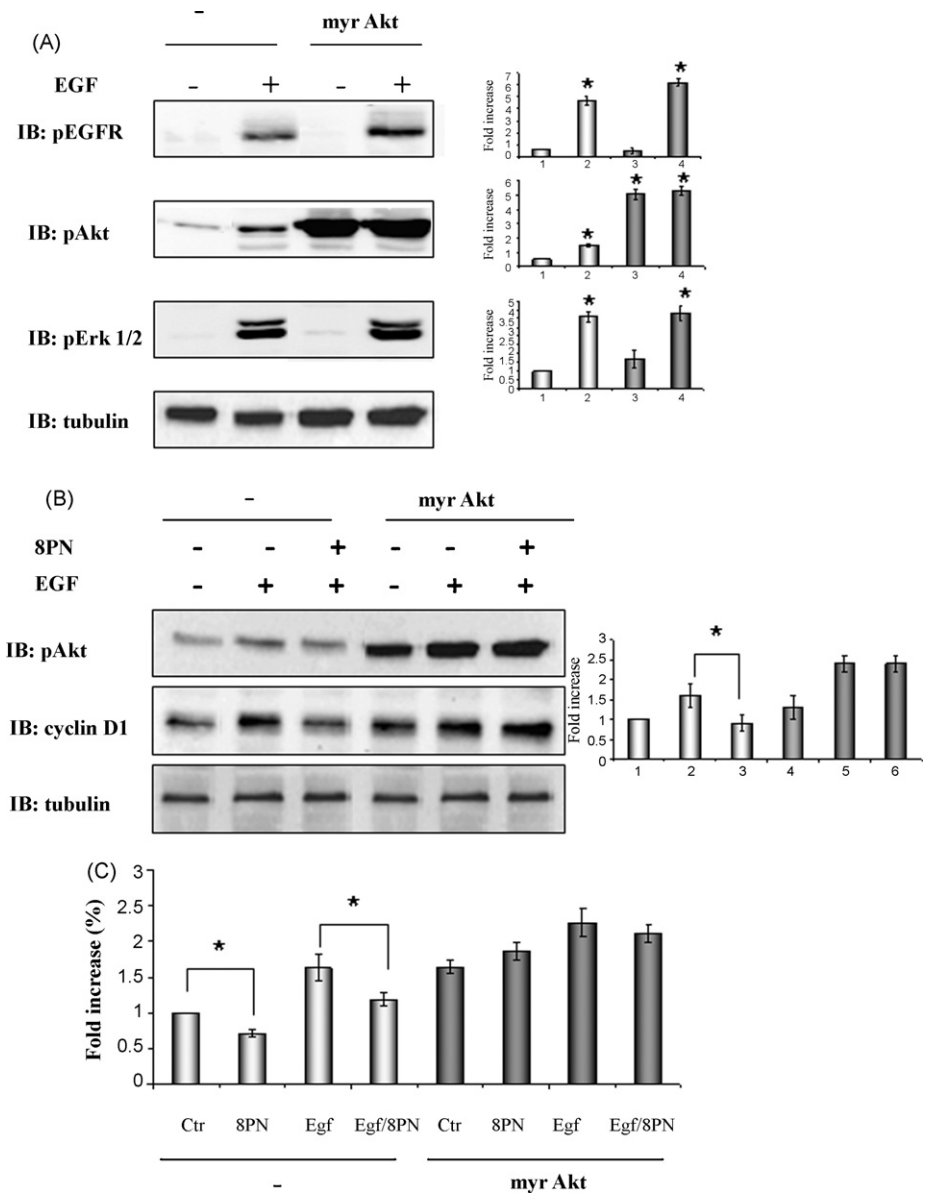

Fig. 5. Effects of a constitutively activated Akt on EGFR-mediated cyclin D1 expression and cell proliferation. (A) MCF-7 cells were transiently transfected with a constitutive form of Akt (Myr Akt) then were cultured for 48 h in double stripped phenol red-free medium and treated with 10 ng/ml of EGF for 10 min in the presence or not of 20 μM 8PN. Cell lysates were separated by 10% SDS–PAGE and immunoblot analysis was done using antibodies against pEGFR, pAkt and pErk.1/2 (upper panels) and re-blotted with polyclonal antibodies to tubulin as a control. The relative phosphorylation levels normalized with the total protein and converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control). (B) Transfected cells cultured for 48 h in double stripped phenol red-free medium were treated with 10 ng/ml of EGF for 2 h. Cell lysates were separated by 10% SDS–PAGE and immunoblot analysis was done using antibodies against cyclin D1 and with anti pAkt and anti tubulin as a control. The relative cyclin D1 levels converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control). (C) MCF-7 cells were transiently transfected with a constitutive form of Akt (Myr Akt) then treated 48 h with 10 ng/ml of EGF in the absence or in the presence of 20 μM 8PN. Cells were tripsinized and counted, data are reported as increase over the control. The results are means converted to fold induction above the control value. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control).

Induction of cyclin D1 gene transcription by ER plays an important role in oestrogen-mediated proliferation. Furthermore, cyclin D1 is over expressed in about 50% of all breast cancers. The mammary glands of cyclin-D1 over expressing transgenic mice develop mammary adenocarcinomas, while cyclin-D1 deficient mice have a defect in proliferation of breast epithelium during pregnancy [38,39]. Although the cyclin D1 promoter includes no classical oestrogen response element, the induction by ER has been linked to the cyclic AMP response element (CRE), the activating protein-1 (AP-1) site, and the Sp1 site on it. To aim at this point, we examined the suppression properties of 8PN on cyclin D1 in MCF-7 cells. Strikingly, the inhibition profile showed that 8PN down regulates the cyclin D1. This was due to the ability of 8PN to block PI(3)K/Akt signalling, which plays an important role in regulating D-type cyclins in fact it was rescued by reintroducing a constitutively activated form of Akt. In conclusion, this is the first report showing that 8PN acts as an ER ligand and in hormone sensitive cells modulates EGFR induced signals, on the other hand data suggest that this molecule can exert a direct inhibitory effect on PI3K activation. It remain to be elucidated if 8PN is a specific inhibitor of PI(3)K and, being a potent ER ligand, if, *in vivo* treatments it can interfere with ER/EGFR cross-talk. However, we retain that the results of the present study may

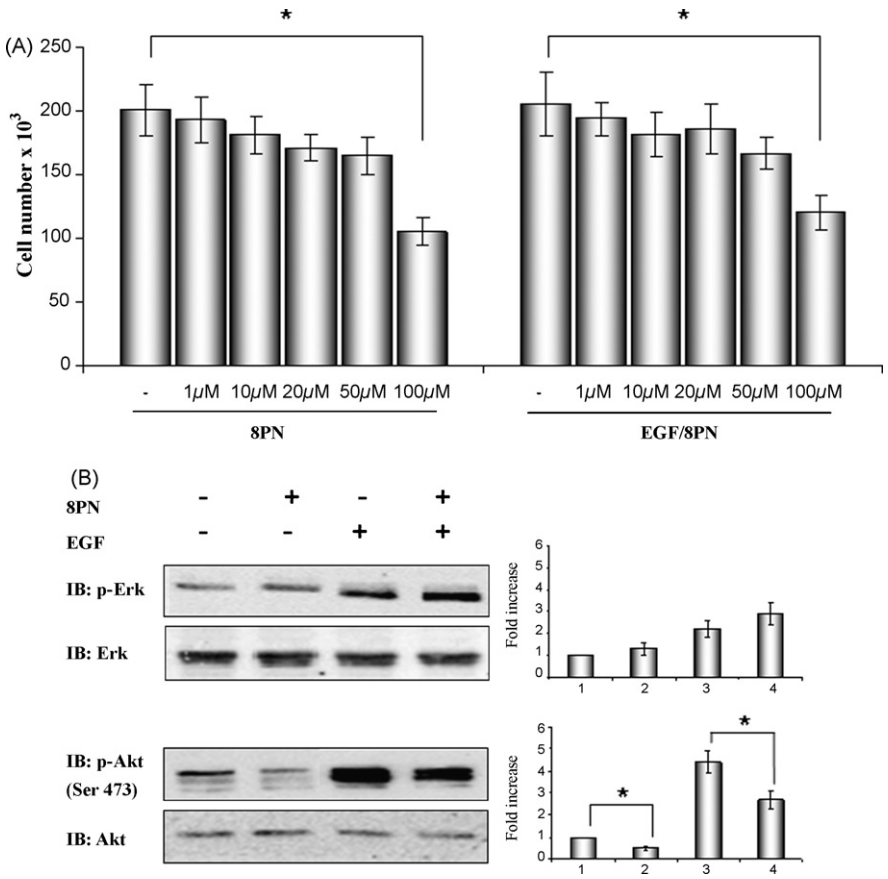

Fig. 6. Effects of 8PN on viability and EGFR-mediated signalling in MDA-MB-231 cells. (A) MDA-MB-231 cells made quiescent as indicated in Section 4 for 24 h, were incubated with different concentrations of 8PN at 37 °C for 24 h in the absence or the presence of 10 ng/ml EGF, the effect on cell viability was examined by proliferation assay. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. This experiment was performed three times. Bars represent fold increase in means over the control ± SEM (*$P < 0.05$, compared with control). (B) MDA-MB-231 cells grown at made quiescent as indicated in Section 4 for 24 h, were treated with 20 μM 8PN in the presence of 10 ng/ml EGF for 10 min and detergent extracted. Levels of phosphorylated Erk 1 (Thr 202 and Tyr 204), Erk2 (Thr 185 and Tyr 187) MAP kinases, and Akt (Ser 473 and Thr 308) were analyzed by immunoblotting. Membranes were also blotted with antibodies to Erk 1/2 and Akt to show an equal amount of loading. The relative phosphorylation levels normalized with the total protein and converted to fold induction above the control value are shown in the adjacent bar graphs. Each column represents the mean ± SEM of triplicate determinations (*$P < 0.05$, compared with control).

help to provide a rational basis for the design of novel strategies for treatment of breast cancers.

4. Materials and methods

4.1. Reagents and antibodies

Monoclonal antibodies to phospho-tyrosine (PY20) and tubulin and polyclonal antibodies to Erk-1/2 MAP kinase and cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to Akt and phospho-specific polyclonal antibodies to Erk 1 (Thr 202 and Tyr 204), Erk2 (Thr 185 and Tyr 187) MAP kinases, and to Akt (Ser 473 and Thr 308) were from Cell Signalling Technology (Beverly, MA). Protein A-Sepharose and ECL reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose membranes and protein assay kits were from Bio-Rad (Hercules, CA). Anti mouse and anti rabbit IgG peroxidase conjugated antibodies, human recombinant EGF, phospholipids and chemical reagents were from Sigma–Aldrich (St Louis, MO). All reagents were of analytical grade. Racemic 8-Prenylnaringenin (8PN) (99% purity), was synthesized according to the literature [4]. The purity of the samples was calculated from the relative magnitude of the phytoestrogen peak and any minor peaks due to impurities as revealed by high pressure liquid chromatography (HPLC).

Culture media, sera and antibiotics, LipofectAMINE and the polyclonal antibody to pEGFR (pY1086) were from Invitrogen (Karlsruhe, Germany).

4.2. Cell cultures treatments and transfection

MCF-7 and MDA-MB-231, human breast cancer derived cell lines, were purchased from ATCC. MCF-7 cells were grown at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentanamicin (50 μg/ml), insulin (6 ng/ml), hydrocortisone (3.75 ng/ml) and 5% fetal calf serum (FCS). MDA-MB-231 cells were grown in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentanamicin (50 μg/ml), insulin (0.02 U/ml) and 10% FCS. Prior to the experiments, cells were maintained in the same media lacking phenol red supplemented with charcoal-stripped FCS, prepared as previously described [40]. 8PN was dissolved in ethanol and added to cell cultures at the indicated concentrations; the final EtOH vehicle concentration was maintained at 0.1%. Samples indicated as controls received vehicle (0.1% EtOH). Human MCF-7 cells grown to 80% confluence in tissue culture dishes were transiently transfected with the pcDNA3 Myr HA Akt1 plasmid encoding a constitutively activated form of Akt (Addgene, Cambridge, USA) by the LipofectAMINE reagent as described by the manufacturer.

4.3. Cell lysis and immunoblotting

Cells were extracted with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 10 mM NaF,

10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin). Cell lysates were centrifuged at 13,000 × *g* for 10 min and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad). Proteins were separated by SDS–PAGE under reducing conditions. Following SDS–PAGE, proteins were transferred to nitrocellulose, reacted with specific antibodies and then detected with peroxidase-conjugate secondary antibodies and chemioluminescent ECL reagent. When appropriate, the nitrocellulose membranes were stripped according to manufacturer's recommendations and re-probed. Densitometric analysis was performed using the GS 250 Molecular Imager (Bio-Rad). For Cyclin D1 expression, cells were extracted in RIPA Buffer (1% Triton X-100, 0.1% SDS, 1% Na-deoxycholate, 150 mM NaCl, 50 mM Tris–HCl pH 7, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin) and analyzed as indicated above.

4.4. Proliferation assay by cell count

Breast cancer cells were seeded at a density of 10×10^4 cells/well on 6-well plates in growth medium with FCS and incubated overnight at 37 °C in a humidified environment containing 5% CO_2 to allow adherence. MCF-7 cells were transiently transfected with the pcDNA3 Myr HA Akt1 plasmid or with control vector by the LipofectAMINE reagent as described by the manufacturer and after 48 h treated with 10 ng/ml human recombinant EGF and/or 20 μM 8PN diluted in FCS-free growth medium. After 48 h treatment, cells were trypsinized and stained with Trypan blue, by mixing (1:1) with 4% Trypan blue (diluted in 1 × phosphate-buffered saline). The number of cells which excluded Trypan blue (considered viable) was counted in a Burker chamber within 5 min after staining.

4.5. Anti-phosphotyrosine immunoprecipitation and PI(3)K kinase assay

Anti-phosphotyrosine immunoprecipitation (PY20 mAbs, Santa Cruz) was carried out from pre-cleared cell lysates from either control or EGF stimulated MCF-7 cells. Immunoprecipitates were washed three times with lysis buffer, twice with 0.5 M LiCl/25 mM Tris pH8 and three times TNE (25 mM Tris, 150 mM NaCl, 1 mM EDTA, pH8), and PI(3)kinase assay was carried out in immunoprecipitates by measuring phosphorylation of PI(4,5)P_2 to PI(3,4,5)P_3 [41]. Immunoprecipitates were pre-incubated 10 min with different doses of 8PN and then incubated for 5 min with pre-prepared lipid mix (sonicated PI(4,5)P_2 and PS 1:1 to 0.3 mg/ml total, 25 mM HEPES pH 7.4 and 1 mM EDTA) and ATP mix (10 mM MgCl2, 50 mM Tris pH 7.4, 60 μM ATP, 0.2 mCi/ml [^{32}P]-gamma-ATP). The reaction was stopped by addition of 0.1 ml of 1N HCl and 0.16 ml of CHCl3/MeOH (1:1). Samples were vigorously stirred for 30 s, spinned for 1 min at 13,000 × *g* and the organic phase extracted. Organic extracts were washed with 80 μl of MeOH/100 mM HCl, 2 mM EDTA (1:1) to discard ATP contamination from the aqueous phase. Them 10 μl of each sample were then spotted on the pre-coated TLC plate allowing the plate to dry for 20 min. TLC plate was run in the TLC chamber saturated with n-propanol/2 M acetic acid (65:35) and let run up to 15 cm, air-dried, wrapped in plastic wrap and film was exposed overnight at −80 °C. The radiolabelled spot of PIP_3 was quantified using a Typhoon 9410 image analyzer (Amersham Biosciences) and NIH Image 1.61 software.

4.6. Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA was performed to analyze experiments. Post hoc tests were used to determine where statistically significant differences were located among the groups (Tukey's test). The level of significance was $P < 0.05$.

Acknowledgements

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