Targeting protein kinase C by Enzastaurin restrains proliferation and secretion in human pancreatic endocrine tumors

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Abstract

Dysregulation of the protein kinase C (PKC) signaling pathway has been implicated in tumor progression. In this study, we investigate the effects of a PKC inhibitor, Enzastaurin, in human pancreatic neuroendocrine neoplasms (PNN) primary cultures and in the human pancreatic endocrine cancer cell line, BON1. To this aim six human PNN dispersed in primary cultures and BON1 cells were treated without or with 1–10 μM Enzastaurin and/or 100 nM IGF1 in the presence or absence of serum. Cell viability and apoptosis were evaluated after 48–72 h; Chromogranin A (CgA) and/or insulin secretion was assessed after 6 h of incubation. PKC expression was investigated by immunofluorescence and western blot. We found that Enzastaurin significantly reduced human PNN primary culture cell viability, as well as CgA and insulin secretion. Moreover, in the BON1 cell line Enzastaurin inhibited cell proliferation at 5 and 10 μM by inducing caspase-mediated apoptosis, and reduced phosphorylation of glycogen synthetase kinase 3β (GSK3β) and of Akt, both downstream targets of PKC pathway and pharmacodynamic markers for Enzastaurin. In addition, Enzastaurin blocked the stimulatory effect of IGF1 on cell proliferation, and reduced CgA expression and secretion in BON1 cells. Two different PKC isoforms are expressed at different levels and have partially different subcellular localization in BON1 cells. In conclusion, Enzastaurin reduces cell proliferation by inducing apoptosis, with a mechanism likely involving GSK3β signaling, and inhibits secretory activity in PNN in vitro models, suggesting that Enzastaurin might represent a possible medical treatment of human PNN.

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Introduction

Pancreatic neuroendocrine neoplasms (PNN) account for <3% of pancreatic tumors (Eehalt et al. 2009). Current therapy is complete surgical resection (Fendrich et al. 2006), which is however achieved in the minority of cases, with high recurrence rates and a 5-year survival rate of ~40% (Chamberlain et al. 2000). Most tumors are diagnosed late, especially in endocrine-inactive forms, prompting the need for further medical therapy. Chemotherapy is of limited value for the treatment of low-proliferating endocrine tumors, while it might attain 30–50% response rates in high grade PNN (Oberg et al. 2010). Biological therapy, such as somatostatin analogs and α-interferons, is effective in controlling hormone production and release and may have cytostatic effects, as demonstrated in the PROMID study (Rinke et al. 2009, Oberg et al. 2010). However, an effective treatment for PNN is still to be found, indicating that understanding the molecular pathways regulating neuroendocrine tumor cell proliferation is crucial for future drug development.
The serine–threonine protein kinase C (PKC) family is composed of at least 11 members that play central regulatory roles in a multitude of cellular processes including proliferation, cell cycle progression, differentiation, tumorigenesis, apoptosis, and secretion (Hug & Sarre 1993, Musashi et al. 2000). Dysregulation of PKC signaling pathways is implicated in the progression of several tumors (Podar et al. 2001). Enzastaurin, an acyclic bisindolylmaleimide developed as a PKCβ-selective inhibitor, suppresses not only PKC signaling but also the PI3 kinase (PI3K)/Akt pathway, cascades that mediate tumor-induced angiogenesis, as well as tumor cell survival and proliferation. These pathways have been indicated as the most dysregulated in PNN (Capurso et al. 2009), suggesting a possible application for PKC inhibitors in medical therapy of unresectable disease.

The aim of this study, performed in human PNN primary cultures and in a human PNN cell line, the BON1 cell line, is therefore to explore whether targeting PKC by Enzastaurin might represent a new approach for controlling PNN cell proliferation.

Materials and methods

Human pancreatic neuroendocrine tumors

Samples derived from six patients (Fig. 1, lower panel), diagnosed and operated on for PNN at the University of Ferrara (Section of Endocrinology and Section of Clinical Surgery), and at the University of Padova (Department of Medical and Surgical Sciences).

Tissue collection and primary culture

Tissues were collected and immediately minced in RPMI 1640 medium under sterile conditions. Primary cultures were prepared as described previously (Mergler et al. 2005, Zatelli et al. 2005), with minor modifications. Informed consent of the patients was obtained for disclosing clinical investigation and performing the in vitro study.

Cell culture

BON1 cells (a kind gift from Dr C Auernhammer, Medizinische Klinik II, University of Munich, Germany) were grown in 1:1 mixture of F12K and DMEM (Euroclone, Milano, Italy) medium, supplemented with 5% fetal bovine serum (FBS), at 37 °C in a humidified atmosphere with 5.0% CO₂ (Parekh et al. 1994).

Figure 1 Effects of Enzastaurin on PNN primary cultures. (Upper panel) Six PNN primary cultures were incubated in 96-well plates in culture medium supplemented with Enzastaurin from 1 to 10 µM in the presence of serum; control cells were treated with vehicle solution. Cell viability was measured in each culture (#1 to #6) as absorbance at 560 nm after 48 h (white columns) with eight replicates each. CgA (black columns) concentration in the conditioned media from each culture (#1 to #6) was assessed as absorbance after 6 h with three replicates each. Insulin (gray columns) concentration in the conditioned media from culture #1 and #3 was assessed as absorbance after 6 h with three replicates each. Data are expressed as the mean value ± S.E.M. percent versus vehicle control cells. *P<0.05 versus vehicle control cells; **P<0.05 versus IGF1 treated cells. (Lower panel) Patients characteristics.

Compounds

Enzastaurin was provided by Eli Lilly. Staurosporine was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and IGF1 from PeproTek, Inc. (Rocky Hill, NJ, USA). All other reagents, if not otherwise specified, were purchased from Sigma.

Viable cell number assessment

Variations in cell number were assessed by the ATPLite kit (Perkin Elmer Life Sciences, Waltham, MA, USA) by seeding 2×10^4 cells/well in 96-well plates, exposed to Enzastaurin 1–10 µM without or with IGF1 100 nM for 48 and 72 h, in the presence or absence of FBS. Control cells were treated with vehicle alone (dimethyl sulfoxide, DMSO). Staurosporine 100 nM was used as positive control for cell viability.
inhibition. After incubation, the revealing solution was added and the luminescent output (relative light units, RLU) was recorded by the Wallac Victor TM 1420 Multilabel Counter (Perkin Elmer Life Sciences). Results were obtained by determining the mean value of six replicates in three different experiments.

**Chromogranin A and insulin ELISA**

Cells were plated in 6-well plates at $10^6$/well and exposed the next day to Enzastaurin 1–10 μM for 6 h. Chromogranin A (CgA) and/or insulin levels were determined in conditioned media by the CgA ELISA kit (DakoCytomation, Glostrup, Denmark) and, in selected cases, the Insulin ELISA kit (Calbiotech, Sring Vally, CA, USA) respectively, following the manufacturer’s instructions. Samples were analyzed in triplicate by the Wallac Victor 1420 multilabel counter (Perkin Elmer).

**DNA synthesis**

Variations in DNA synthesis were assessed as $[^3]$H]thymidine ($[^3]$H]thy) incorporation rates, as described previously (Zatelli et al. 2002). Cells were seeded at $5 \times 10^4$ cells/well in 24-well plates and exposed to Enzastaurin 1–10 μM without or with IGF1 100 nM for 48 and 72 h, without or with FBS in the presence of $[^3]$H]thy (1.5 μCi/ml; 87 Ci/mmol, Amersham–Pharmacia Biotech Italia). Staurosporine 100 nM was used as positive control for DNA synthesis inhibition. Cell-associated radioactivity was determined after harvesting cells on glass fibers and liquid scintillation counting of quadruplicate wells in at least three separate experiments. Results are calculated as percent $[^3]$H]thy incorporation compared with control untreated cells.

**Apoptosis assay**

Caspase activity was measured by the Caspase-Glo 3/7 assay (Promega). Cells were seeded at $10^4$ cells/well in 96-well, white-walled plates and then exposed to Enzastaurin 1–10 μM, for 48 and 72 h without or with IGF1 100 nM, without or with FBS. Staurosporine 100 nM was used as a positive control for apoptosis induction. Control cells received vehicle alone (DMSO). After 48 and 72 h, an equal volume of Caspase-Glo 3/7 reagent was added and RLU were recorded by the Wallac Victor 1420 multilabel counter (Perkin Elmer). Results are expressed as mean value ± S.E.M. percent RLU versus control cells in six replicates.

**Glycogen synthetase kinase 3β (Ser9) activity assay**

The phosphorylation of glycogen synthetase kinase 3β (GSK3β) (Ser9) was measured by AlphaScreen SureFire p-GSK3β (Ser9) Assay Kits (Perkin Elmer). Briefly, cells were seeded at $2 \times 10^4$ cells/well in 96-well plates, and, after overnight attachment, cells were incubated with or without Enzastaurin 1–10 μM without or with IGF1 100 nM for 48 h and evaluated as per the manufacturer’s protocol. The plates were measured in Read plate on AlphaScreen plate reader (Perkin Elmer), using standard AlphaScreen settings.

**Immunofluorescence microscopy**

PKCβII and PKCδ localization in BON1 cells was determined by immunofluorescence. Briefly, BON1 cells ($2 \times 10^5$/well) were seeded in chamber slides (Lab-Tek, Christchurch, New Zealand), and fixed in methanol and acetone (1:1) for 10 min at −20 °C. Slides were incubated with blocking buffer and then with a mouse monoclonal anti-human PKCβII antibody (1:100) (Santa Cruz Biotechnology) or a rabbit monoclonal anti-human PKCδ antibody (1:100) (Santa Cruz Biotechnology). Cells were then incubated for 45 min at room temperature with a secondary tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse antibody (1:200; Santa Cruz Biotechnology) and fixed in methanol containing 4′,6′-diamino-2-phenylindole (DAPI). Slides were mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing 4′,6′-diamino-2-phenylindole (DAPI) under glass coverslips (M enzel-Glaser, Freiburg, Germany), and examined with the TRITC and FITC filters (Nikon, Tokyo, Japan). Nuclear staining with DAPI was detected with the Nikon u.v. filter. Images were acquired using the DS-5M Nikon color charge-coupled device digital camera and cell fluorescence was analyzed with the Multi-Analyst software (Bio-Rad).

A similar procedure was followed for CgA immunofluorescence, by a mouse monoclonal anti-human CgA antibody (1:100; Novus Biologicals LLC, Littleton, CO, USA) and a secondary FITC-conjugated goat anti-rabbit antibody (1:200; Santa Cruz Biotechnology) for PKCδ. Chamber slides were mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing 4′,6′-diamino-2-phenylindole (DAPI) under glass coverslips (Menzel-Glaser, Freiburg, Germany), and examined with the TRITC and FITC filters (Nikon, Tokyo, Japan). Nuclear staining with DAPI was detected with the Nikon u.v. filter. Images were acquired using the DS-5M Nikon color charge-coupled device digital camera and cell fluorescence was analyzed with the Multi-Analyst software (Bio-Rad).

**Western blot analysis**

Protein isolation was performed as described previously (Tagliati et al. 2006). Total protein cell extracts were analyzed by Western blotting.
were measured by the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal protein amounts were fractionated on 8% SDS–PAGE and transferred by electrophoresis to Nitrocellulose membranes (Schleicher & Schuell Italia SRL, Milano, Italy).

For Akt phosphorylation level assessment, the membranes were incubated with 1:2000 rabbit monoclonal anti total human phospho Akt (Ser 473) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) or with 1:1000 rabbit polyclonal anti human total Akt antibody (Millipore, Billerica, MA, USA). HRP-conjugated goat anti-rabbit IgG (Pierce) secondary antibody was used at 1:2000 and binding was revealed using ECLTM Western Blotting Detection Reagents (Amersham Biosciences).

For PKC isoform expression, the membranes were incubated with 1:200 rabbit polyclonal anti-human PKCβII antibody (Santa Cruz Biotechnology) or with 1:100 rabbit polyclonal anti-human PKCδ antibody (Santa Cruz Biotechnology). HRP-conjugated goat anti-rabbit IgG (Pierce) secondary antibody was used at 1:2000 and binding was revealed as described earlier.

Statistical analysis

Results are expressed as the mean ± S.E.M. Student’s paired or unpaired *t*-test was used to evaluate individual differences between means. *P* values <0.05 were considered significant.

Results

Effects of Enzastaurin on primary cultures

Since Enzastaurin was shown to have a direct antiproliferative effects on human tumor cells (Wiegand & Hipler 2008), we evaluated its ability to influence basal and IGF1 induced PNN cell viability first of all on dispersed human PNN cells. Therefore, six PNN primary cultures were treated for up to 48 h with Enzastaurin 1–10 μM in the presence of serum, without or with IGF1 100 nM. Enzastaurin and IGF1 did not significantly influence cell viability of the six evaluated PNN primary cultures after 6 h (data not shown). As shown in Fig. 1, Enzastaurin significantly reduced primary PNN cell viability at 5 and 10 μM (mean reduction: −37 and −41% respectively; *P* <0.05 versus control; IC$_{50}$ = 11 μM), while IGF1 enhanced PNN primary culture cell viability (mean induction: +32%; *P* <0.05), an effect completely blocked by Enzastaurin at 5 and 10 μM. At the same concentrations Enzastaurin inhibited both basal (mean reduction: −25 and −30% respectively; *P* <0.05 versus control; IC$_{50}$ = 15.2 μM) and IGF1 stimulated CgA secretion (from 54 to 4%) after 6 h. More strikingly, a sharp reduction in insulin levels was observed in the conditioned media from the insulinoma PNN primary cultures after treatment with Enzastaurin 5 and 10 μM (mean reduction: −65 and −72% respectively; *P* <0.05 versus control; IC$_{50}$ = 5.9 μM) for 6 h, even in the presence of IGF1.

Effects of Enzastaurin on BON1 cell proliferation

To further explore the mechanisms by which Enzastaurin might influence human PNN cell proliferation, we employed the BON1 cell line model. In Fig. 2A, after 48 h in the presence of serum BON1 cell viability was significantly reduced by Enzastaurin at 10 μM (−15%; *P* <0.05 versus control; IC$_{50}$ = 19.4 μM), and after 72 h a greater inhibitory effect was observed at 5 and 10 μM (−40 and −56% respectively; *P* <0.01 versus control; IC$_{50}$ = 8.1 μM). IGF1 significantly stimulated cell viability at both 48 and 72 h (+12 and +16% respectively; *P* <0.05 versus control),
an effect completely blocked by co-incubation with Enzastaurin at 5 and 10 μM.

We then explored the effects in the absence of serum, to avoid growth factors interference. Basal cell viability was indeed from 30% to threefold lower in samples grown in the absence versus in the presence of serum. As shown in Fig. 2B, after 48 h BON1 cell viability was significantly reduced by Enzastaurin at 5 and 10 μM (−12 and −18% respectively; \( P < 0.05 \) versus control; IC\(_{50}\) = 26.8 μM) and after 72 h a greater inhibitory effect was observed (−33 and −44% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 10.1 μM). IGF1 significantly reduced cell viability at both 48 and 72 h (+26%; \( P < 0.05 \) versus control), an effect that was counteracted by co-incubation with Enzastaurin at 5 and 10 μM. Staurosporine significantly reduced cell viability at both 48 and 72 h, either in the absence or in the presence of serum (−70 to −85%; \( P < 0.01 \) versus control) (data not shown).

To confirm the antiproliferative effects of Enzastaurin, DNA synthesis was also evaluated. In Fig. 3A, after 48 h in the presence of serum DNA synthesis was significantly reduced by Enzastaurin at 5 and 10 μM (−30 and −50% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 9.6 μM), and a greater inhibitory effect was observed after 72 h (−40 and −75% respectively; \( P < 0.01 \) versus control IC\(_{50}\) = 6.9 μM). IGF1 significantly stimulated DNA synthesis at both 48 and 72 h (+25%; \( P < 0.01 \) versus control), an effect completely blocked by co-incubation with Enzastaurin at 5 and 10 μM. As shown in Fig. 3B, after 48 h in the absence of serum DNA synthesis was significantly reduced by Enzastaurin at 5 and 10 μM (−50 and −65% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 6.9 μM), and after 72 h a much greater effect was observed (−70 and −80% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 5.4 μM). IGF1 significantly stimulated DNA synthesis at both 48 and 72 h (+15 and +45% respectively; \( P < 0.05 \) and \( P < 0.01 \) versus control), an effect that was completely counteracted by co-incubation with Enzastaurin at 5 and 10 μM. Staurosporine significantly reduced DNA synthesis at both 48 and 72 h, either in the absence or in the presence of serum (−80 to −93%; \( P < 0.01 \) versus control) (data not shown).

**Effects of Enzastaurin on BON1 apoptosis**

To investigate whether the antiproliferative effects of Enzastaurin on BON1 cells are due to apoptosis, caspase 3/7 activity was measured. In Fig. 4A, after 48 h in the presence of serum Enzastaurin significantly induced apoptosis at 5 and 10 μM (+40 and +100% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 5.4 μM), with a similar effect after 72 h (+55% and +85% respectively; \( P < 0.01 \) versus control; IC\(_{50}\) = 4.7 μM). IGF1 significantly reduced caspase activity at both 48 and 72 h (−25%; \( P < 0.01 \) versus control), an effect strongly counteracted by co-incubation with Enzastaurin at 5 and 10 μM. As shown in Fig. 4B, after 48 h in the absence of serum Enzastaurin strongly and significantly induced apoptosis at both 5 and 10 μM (approximately fivefold; \( P < 0.01 \) versus control; IC\(_{50}\) = 4.2 μM), with an even stronger effect after 72 h (approximately tenfold; \( P < 0.01 \) versus control; IC\(_{50}\) = 4.1 μM). IGF1 did not significantly modify caspase activity at both 48 and 72 h, but counteracted the stimulatory effects of Enzastaurin. Staurosporine significantly stimulated apoptosis at both 48 and 72 h, either in the absence or in the presence of serum (two- to three-fold; \( P < 0.01 \) versus control) (data not shown).

**Effects of Enzastaurin on GSK3β (Ser9) and Akt phosphorylation**

Phosphorylation of GSK3β (Ser9) is triggered by both PKC (Goode *et al.* 1992, Fang *et al.* 2002) and Akt
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Figure 4 Effects of Enzastaurin on BON1 apoptosis. (A) BON1 cells were incubated in 96-well plates for 48 (white columns) or 72 h (black columns) in culture medium supplemented with Enzastaurin from 1 to 10 μM in the presence of serum (with FBS), with or without IGF1 100 ng/ml; control cells were treated with vehicle solution. Apoptosis was measured as caspase 3/7 activity. (B) BON1 cells were incubated in 96-well plates for 48 (white columns) or 72 h (black columns) in culture medium supplemented with Enzastaurin from 1 to 10 μM in the absence of serum (w/o FBS), with or without IGF1 100 ng/ml; control cells were treated with vehicle solution. Apoptosis was measured as caspase 3/7 activity. Data were evaluated in at least six independent experiments with eight replicates each, and are expressed as the mean value ±S.E.M. percent caspase 3/7 activity versus vehicle control cells. *P<0.05 versus vehicle control cells; **P<0.01 versus vehicle control cells; #P<0.05 versus IGF1 treated cells.

pathways (Cross et al. 1995), therefore its downregulation may reflect PKC and Akt pathways inhibition. Therefore, we tested GSK3β (Ser9) phosphorylation after incubation of BON1 cells with Enzastaurin 1–10 μM. In Fig. 5A, in the presence of serum Enzastaurin significantly reduced GSK3β (Ser9) phosphorylation at both 5 and 10 μM (−26 and 34% respectively; P<0.01 versus control; IC50 =13.55 μM), with a lower effect in the absence of serum (−16 and −26%; P<0.05 versus control; IC50 =18.6 μM). No effect was observed after treatment with Enzastaurin 1 μM, either in the presence or in the absence of serum. On the contrary, IGF1 significantly stimulated GSK3β (Ser9) phosphorylation both in the presence and in the absence of serum (14 and 27% respectively; P<0.01 versus control), an effect that was dose-dependently counteracted by co-incubation with Enzastaurin.

Akt phosphorylation was also tested after incubation of BON1 cells with Enzastaurin 1–10 μM. As shown in Fig. 5B, in the presence of serum Enzastaurin significantly and dose-dependently reduced Akt phosphorylation, with a lower effect in the absence of serum (data not shown). On the contrary, IGF1 significantly stimulated Akt phosphorylation both in the presence (Fig. 5B) and in the absence (not shown) of serum, an effect that was counteracted by Enzastaurin.

Effects of Enzastaurin on CgA secretion and expression

To test whether Enzastaurin influences CgA secretion in BON1 cells, CgA concentration in the conditioned media was measured after 6 h of incubation. As shown in Fig. 5C, in the presence of serum Enzastaurin significantly reduced CgA secretion at both 5 and 10 μM (−40 and −50% respectively; P<0.01 versus control; IC50 =9 μM). A greater inhibition was observed in the absence of serum at both concentrations (−50 and −60% respectively; P<0.01 versus control; IC50 =7.4 μM). On the contrary, IGF1 significantly stimulated CgA secretion both in the presence and in the absence of serum (64 and 77% respectively; P<0.01 versus control), an effect that was again dose-dependently counteracted by co-incubation with Enzastaurin. Staurosporin, used as negative control, did not influence CgA secretion (data not shown).

To assess whether the inhibitory effect of Enzastaurin on CgA secretion was due to a reduced production, CgA immunofluorescence was evaluated, being markedly reduced after treatment with Enzastaurin from 6 to 24 h in the presence of serum (Fig. 6A). Similar results were obtained in the absence of serum (data not shown).

PKC expression

BON1 cells were analyzed by immunofluorescence and by western blot for the PKCβII and the PKCδ isoforms. As shown in Fig. 6B, both isoforms are expressed in BON1 cells, with a different subcellular localization. Indeed, PKCβII dotted immunofluorescence was mainly nuclear and cytoplasmic, while PKCδ immunofluorescence was observed at the plasma membrane, in intracytoplasmic dots and in the perinuclear region. After treatment with Enzastaurin 5 μM, PKCβII was almost completely localized to the perinuclear region and PKCδ localization did not change significantly. As shown in the right panel of Fig. 6B, in a human PNN primary culture PKCβII immunofluorescence was mainly located at the plasma membrane, while PKCδ immunofluorescence was also distributed in the perinuclear region. After treatment with Enzastaurin 5 μM, PKCβII was almost...
versus vehicle control cells.

Cells were treated with Enzastaurin (1–10 μM) in the presence (With FBS) or in the absence (w/o FBS) of serum, with or without IGF1 100 ng/ml; control cells were treated with vehicle solution. GSK3β phosphorylation was measured as described in Materials and methods section. Data were evaluated in at least three independent experiments with three replicates each, and are expressed as the mean value ± S.E.M. percent GSK3β (Ser9) phosphorylation versus vehicle control cells. *P < 0.05 and **P < 0.01 versus vehicle control cells; *P < 0.05 versus IGF1 treated cells. (B) BON1 cells were grown in culture medium for 48 h in the presence of serum. Total and phosphorlated (Ser473) Akt expression levels were assessed by western blot as described in Materials and methods section. The top panel corresponds to phosphorlated Akt, the middle panel to total Akt, and the bottom panel to β-actin, evaluated for normalization purposes. Arrows indicate the respective molecular weights. (C) BON1 cells were plated in 6-well plates and then exposed for 6 h to Enzastaurin 1–10 μM in the presence (With FBS) or in the absence (w/o FBS) of serum, with or without IGF1 100 ng/ml. CgA levels were determined as described in Materials and methods section. Data were evaluated in at least three independent experiments with three replicates each, and are expressed as the mean value ± S.E.M. percent CgA secretion versus vehicle control cells. *P < 0.05 and **P < 0.01 versus vehicle control cells.

Figure 5 Effects of Enzastaurin on BON1 GSK3β (Ser9) phosphorylation, Akt phosphorylation, and CgA secretion. (A) BON1 cells were incubated in 96-well plates for 48 h in culture medium supplemented with Enzastaurin from 1 to 10 μM in the presence (with FBS) or in the absence (w/o FBS) of serum, with or without IGF1 100 ng/ml; control cells were treated with vehicle solution. GSK3β (Ser9) phosphorylation was measured as described in Materials and methods section. Data were evaluated in at least three independent experiments with three replicates each, and are expressed as the mean value ± S.E.M. percent GSK3β (Ser9) phosphorylation versus vehicle control cells. *P < 0.05 and **P < 0.01 versus vehicle control cells; *P < 0.05 versus IGF1 treated cells. (B) BON1 cells were grown in culture medium for 48 h in the presence of serum. Total and phosphorlated (Ser473) Akt expression levels were assessed by western blot as described in Materials and methods section. The top panel corresponds to phosphorlated Akt, the middle panel to total Akt, and the bottom panel to β-actin, evaluated for normalization purposes. Arrows indicate the respective molecular weights. (C) BON1 cells were plated in 6-well plates and then exposed for 6 h to Enzastaurin 1–10 μM in the presence (with FBS) or in the absence (w/o FBS) of serum, with or without IGF1 100 ng/ml. CgA levels were determined as described in Materials and methods section. Data were evaluated in at least three independent experiments with three replicates each, and are expressed as the mean value ± S.E.M. percent CgA secretion versus vehicle control cells. *P < 0.05 and **P < 0.01 versus vehicle control cells.

completely delocalized in cytoplasmic dots, while PKCδ localization did not change strikingly.

In addition, as shown in Fig. 6C, PKCδ was expressed at higher levels compared with PKCβII. Similar protein levels of both isoforms were detected in BON1 cells treated with Enzastaurin and/or IGF1 for up to 72 h.

Discussion
PKC activity has been implicated in the regulation of tumor-induced angiogenesis, cell proliferation, apoptosis, and invasiveness (Goekjian & Jirousek 2001), representing an attractive and promising target for cancer treatment. Enzastaurin has been developed as an ATP-competitive, PKCβ-selective inhibitor, but following studies demonstrated that it inhibits several other PKC isoforms (including γ, δ, θ, ξ and ε) at the concentrations reached in clinical trials, corresponding to 2–8 μM with substantial interpatient variability (Herbst et al. 2002, Graff et al. 2005, Carducci et al. 2006).

Enzastaurin has been advanced into clinical trials, such as phase II clinical trials in pancreatic, colonic, and non-small-cell lung carcinoma, as well as phase III clinical trials for the treatment of refractory glioblastoma and diffuse large B-cell lymphoma (Moreau et al. 2007).

In this study, we show that Enzastaurin has antiproliferative activity also toward human PNN primary cultures and a human PNN cell line. To obtain homogeneous and straightforward results, primary cultures derived from primary PNN were selected for this study. Despite the different TNM, stage and grading of the original PNN, the primary cultures displayed a similar response to Enzastaurin treatment, which determined a clear-cut reduction in cell viability at drug concentrations similar to the circulating levels observed in clinical trials (Graff et al. 2005). Carducci et al. (2006) reported that Enzastaurin reaches lower plasma concentrations (2.2 μM) in patients treated with Enzastaurin 525 mg/day per os. However, it should also be taken into account that circulating Enzastaurin is 95% bound to plasma proteins, therefore the drug concentration at tumor site might be very different from that recorded at plasma level. Moreover, no objective response was recorded, with stabilization of disease in 45% of the patients in phase I trials treating the patients with Enzastaurin 525 mg/day per os (Carducci et al. 2006). These results, together with our data, might suggest that Enzastaurin plasma concentrations higher than 2.2 μM might be necessary to observe biologically relevant results. Since these plasma concentrations have not been achieved with higher doses (700 mg/die), delivery at tumor site might be a suitable alternative.

In addition, an antisercretory effect was observed, since Enzastaurin was capable of reducing both basal and IGF1 stimulated CgA and insulin secretion. These data are in line with previous reports, showing that PKC plays a crucial role in secretory processes in both
normal and neoplastic pancreatic insulin-secreting cells (Miura et al. 1998, Mendez et al. 2003) and indicate that PKC inhibition may represent a suitable pharmacological therapy also for PNNs.

It has been previously demonstrated that Enzastaurin, at concentrations reached in clinical trials, directly inhibits proliferation and induces apoptosis of tumor cells in culture and xenografts by suppressing phosphorylation of Akt and its downstream effectors GSK3β and ribosomal protein S6 (Graff et al. 2005). Our data are in keeping with this evidence, since Enzastaurin in our experimental conditions down-regulates Akt phosphorylation, both basally and after IGF1 treatment. Previous studies have underlined the importance of the PI3K/Akt signaling pathway in mediating the effects of PKC inhibition, showing that phosphorylation of Akt and GSK3β, one of the Akt downstream targets, is decreased by Enzastaurin treatment (Querfeld et al. 2006, Rizvi et al. 2006).

Our results are in line with the reported studies, since we found that Enzastaurin in the same concentration range (5–10 μM) is capable of blocking the proliferative effects of IGF1, which activates the PI3K/Akt signaling pathway. This evidence is further strengthened by the demonstration that basal and IGF1 stimulated Akt and GSK3β phosphorylation was decreased by Enzastaurin treatment, suggesting that Enzastaurin might control PNN cell proliferation by hampering IGF1 downstream signaling. In addition, these data suggest that PKC may be important for transducing IGF1 proliferative signals.

The important role of IGF1 in the control of neuroendocrine tumor proliferation is underlined by a high or constitutive expression of IGF1, IGF2 and IGF receptors in gastroenteropancreatic neuroendocrine tumors (GEP-NET; Wulbrand et al. 2000). In addition, IGF1 dependent signaling promotes tumorigenesis and increases hormonal secretion in a GEP-NET cell line.
(Grozinsky-Glasberg et al. 2008). Our data confirm that PNN cell proliferation is promoted by IGF1, as already demonstrated by von Wichert et al. (2000), and indicate that IGF1 proliferative effects are hampered by a PKC inhibitor, reducing both cell viability and DNA synthesis. Indeed, the latter was reduced to a greater extent compared with cell viability, suggesting an important effect on DNA replication. This evidence is in line with previous findings indicating that Enzastaurin downregulates the expression of cell cycle genes (Kuo et al. 2010), including cyclin D, which controls the transition through the G1/S phase. This effect was more apparent in the absence of serum, indicating that serum growth factors (including IGF1 and insulin) may exert a protective action toward the antiproliferative effects of Enzastaurin. This hypothesis is further strengthened by the evidence that pro-apoptotic effects of Enzastaurin are more apparent in the absence of serum. Indeed, our data show that Enzastaurin exerts its antiproliferative effects also by inducing apoptosis, in keeping with previous studies on different cellular models (Teicher et al. 2001, Gong et al. 2007). This effect was more apparent in the absence of serum, indicating that serum growth factors (including IGF1 and insulin) may exert a protective action toward the antiproliferative effects of Enzastaurin. This hypothesis is further strengthened by the evidence that pro-apoptotic effects of Enzastaurin are more apparent in the absence of serum. Indeed, our data show that Enzastaurin exerts its antiproliferative effects also by inducing apoptosis, in keeping with previous studies on different cellular models (Teicher et al. 2001a,b, 2002). Better antiproliferative effects may be obtained by Enzastaurin in combination, since phase II trials reported limited results in terms of objective response, even if combination with pemetrexed, carboplatin, and bevacizumab in non-small-cell lung cancer (Casey et al. 2010) or with gemcitabine in exocrine pancreatic cancer did not show improved efficacy (Richards et al. 2011).

Our results support the hypothesis that PKC pathway may play an important role in promoting PNN cell survival, since its inhibition by Enzastaurin potently triggers apoptotic phenomena, while its stimulation by IGF1 has anti-apoptotic effects. In this study, however, the IGF1 anti-apoptotic effects are not apparent in the absence of serum in untreated cells, but they can be observed in cells treated with Enzastaurin. These results suggest that IGF1 promotes PNN cell proliferation not only by protecting from apoptosis, but also directly stimulating DNA replication and cell division. However, IGF1 was not capable of completely reversing the antiproliferative effects of PKC inhibition, suggesting that Enzastaurin action may be transduced also by IGF1 independent pathways.

It has been previously demonstrated that IGF1 induces CgA secretion, a marker protein for neuroendocrine secretion (Zatelli et al. 2007), in BON1 cells by PI3-kinase activation (von Wichert et al. 2000). Our results show that Enzastaurin significantly reduces both CgA secretion and protein expression, an effect in part counteracted by IGF1, suggesting that this drug may be effective also in controlling secretory activity of PNN. In addition, in prostate neuroendocrine cells CgA induces the overexpression of the anti-apoptotic protein survivin with a mechanism implicating Akt phosphorylation (Xing et al. 2001, Gong et al. 2007), indicating that CgA has anti-apoptotic effects. Despite our data do not provide direct evidence for an anti-apoptotic effect of CgA in BON1 cells, we can argue that its reduction might mediate part of the observed antiproliferative effects of Enzastaurin, since the latter inhibits CgA secretion and production.

Most cells co-express multiple PKC isoforms whose substrate specificity has been attributed to isoform-specific interactions with various anchoring proteins that localize individual PKC isoforms to specific membrane domains (Jaken & Parker 2000). Previous studies have shown that PKCβII translocates in different subcellular compartments, depending on different stimuli (Hoccevar et al. 1993). Our data show that PKCβII is localized mainly in the nuclear and cytoplasmic regions in BON1 cells, translocating to the perinuclear region after treatment with Enzastaurin, suggesting that the enzyme is completely inactivated (Murray & Fields 1998). On the contrary, PKCδ was localized at the plasma membrane, in cytoplasmic dots and in the nuclear region, with no significant changes after treatment with Enzastaurin. These data may suggest that PKCδ is active (at the plasma membrane) both in the absence and in the presence of the PKC inhibitor, possibly indicating that this isoform is only slightly influenced by Enzastaurin. This hypothesis is further strengthened by the findings reported for the PNN primary cultures. However, localization of PKC isoforms is not constantly linked to the activation status of the enzyme; therefore, further studies are needed to fully elucidate this issue in PNN. Our data also point out that at least two isoforms are expressed by BON1 cells, even if at different levels, and that their expression levels do not change under treatment with either stimulatory (IGF1) or inhibitory (Enzastaurin) agents. This evidence indicates that Enzastaurin reduces the activity of PKC isoforms (in terms of PKC pathway downstream targets inhibition) and not their expression.

In summary, our results demonstrate that Enzastaurin reduces cell growth, at least in part by inducing apoptosis, as well as CgA secretion and protein expression in human PNN primary cultures and cell lines, with a mechanism that implicates PKC inhibition, suggesting that PKC may represent a new pharmacological target for PNN medical therapy.
**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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**Author contribution statement**

D Molè performed the cell line studies, immunofluorescence, cell viability, apoptosis, and GSK3β (Ser9) activity assays; T Gagliano performed the primary culture studies; E Gentilin performed the hormone assays and helped providing the biochemical information; G Ansini provided surgical and biochemical information for patients from Padova; M R Ambrosio provided patients clinical and surgical information; F Tagliati supervised the Molecular Biology studies and performed western blot analysis; C Pasquali provided surgical and biochemical information for patients from Ferrara; E C degli Uberti supervised and coordinated the medical aspects and provided part of the related funding; M C Zatelli wrote the manuscript, collected, re-elaborated and matched in vitro and clinical data, provided part of the related funding and revised the manuscript.

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**References**


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