Mechanism of action of potato carboxypeptidase inhibitor (PCI) as an EGF blocker

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Abstract

The epidermal growth factor receptor (EGFR) signal transduction pathway plays a prominent role in the development of carcinomas, and is an interesting target for antitumoral therapy. We have previously described how potato carboxypeptidase inhibitor (PCI), a 39-amino acid protease inhibitor with a T-Knot motif, binds to EGFR receptor and inhibits the activation of receptor protein tyrosine kinase. In this paper it is shown that PCI interferes with EGFR activation through inhibition of receptor dimerization and receptor transphosphorylation induced by epidermal growth factor (EGF) and by transforming growth factor alpha (TGF-α). Moreover, PCI blocks the formation and activation of ErbB1/ErbB-2 heterodimers that have a prominent role in carcinoma development. As a result of these effects, PCI interferes in the EGFR signal transduction pathway by reversing the effects of EGF on the growth of two tumoral cell lines, A431 and MDA-MB-453, and promotes EGFR down-regulation. These results show that PCI acts as an EGF/TGF-α antagonist, which suggests its therapeutic potential in the treatment of carcinomas.

Keywords: EGF; EGF antagonist; EGFR; ErbB-2; PCI; Signal transduction; TGF-α

Abbreviations

BSA, bovine serum albumin; EGF, epidermal growth factor; DMEM, Dulbecco modified Eagle’s medium; dNTP, deoxynucleotides; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCI, potato carboxypeptidase inhibitor; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RITC, rhodamine isothiocyanate; RMSD, root mean square deviation; RT-PCR, retrotranscriptase and polymerase chain reaction; SDS-PAGE, sodium dithionate polyacrilamide gel; TGF-α, transforming growth factor alpha.

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1. Introduction

The epidermal growth factor (EGF) signal transduction pathway is a significant mediator of several cell functions and processes such as survival, motility, differentiation, proliferation and death (for reviews see [1–5]). The epidermal growth factor receptor (EGFR) subfamily consists of four closely related tyrosine kinase receptors: the ErbB-1 (also known as EGFR), ErbB-2 (or HER2/neu) for which no ligand has been described so far, ErbB-3 (or HER3) which is characterized by an impaired kinase function, and ErbB-4 (or HER-4) [6–9]. The ErbB’s receptors are transmembrane glycoproteins and signal transduction is initiated by ligand binding to the surface ectodomain that induces receptor homo or heterodimerization [10]. This dimerization activates the receptor intrinsic tyrosine kinase activity leading to receptor transphosphorylation that induces the recruitment of intermediary effectors [11]. These events initiate signaling pathways that form a complex cellular network.

The EGF family of ligands can be divided into three groups based on their ability to bind and activate distinct ErbB receptor homo- and hetero-dimers. EGF and transforming growth factor alpha (TGF-\(\alpha\)) are highly homologous growth factors. Both are EGFR ligands but they have different abilities in inducing EGFR receptor heterodimerization and down-regulation [12]. TGF-\(\alpha\) induces greater mitogenic potency than EGF in vitro [13] and in vivo [14]. Moreover, ErbB-2 plays a prominent role as it is the preferred partner for heterodimerization with the other three ErbBs, creating high-affinity heterodimers with extremely high signaling potency and mitogenic superiority [15,16]. The complexity of the receptor signal depends on the various heterodimers formed between ErbB family members upon activation by ligands of the EGF family and, at least in some aspects, on the activating ligand [17,18].

The important role of ErbB pathway in many tumors provides an excellent potential target for cancer therapy, several strategies aimed at disrupting this pathway have been explored: EGF antagonists [19–21], tyrosine kinase inhibitors [22], antibodies directed to the ErbB extracellular domain [15,23] and toxin conjugates to ErbB ligands [24].

We have previously described how potato carboxypeptidase inhibitor (PCI) competes with EGF for binding to EGFR and has antitumoral properties [21]. The molecular basis of these effects lies in that PCI shows three-dimensional structural similarities with EGF [25] and seems to act as an antagonistic analogue of human EGF, the first reported example.

As an approach to better understanding of the PCI mechanism of action, in the present study we examined at the molecular level: (a) if PCI can block EGFR dimerization and transphosphorylation induced by EGF and TGF-\(\alpha\), (b) if PCI can interfere with the activation of ErbB-2, and, finally, (c) if PCI can lead to EGFR internalization and modify protein and mRNA EGFR levels. The results show that PCI blocks receptor dimerization induced by EGF and TGF-\(\alpha\) in A431 cells and inhibits EGFR transphosphorylation induced by both ligands. The inhibitor also reduces ErbB-2 phosphorylation promoted by EGF. In addition, PCI treatment induces the internalization of EGFR, reduces EGFR phosphorylation and alters EGFR mRNA levels in MDA-MB-453, Capan-1 and A431 cell lines.

2. Experimental procedures

2.1. Materials

Human recombinant EGF and TGF-\(\alpha\) were purchased from R & D Systems (Abingdon, UK), human recombinant insulin from Roche Diagnostics (Basel, Switzerland), monoclonal antibody against EGFR and anti-actin antibody from Amersham (Little Chafont, UK), polyclonal antibody against EGFR or ErbB-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), recombinant anti-phosphotyrosine antibody (RC20) from Transduction Laboratories (Lexington, KY, USA), protein molecular weight standards from Bio-Rad (Hercules, CA, USA), and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-phenylcarbamoyl-2H-tetrazoliumhydroxide (EZ4U) reduction assay kit from Biomedica Corp. (Vienna, Austria). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Badford, MA, USA), chemiluminiscence blotting substrate (POD) was from Roche Diagnostics or Pierce (Basel, Switzerland and Rockford, IL, USA, respectively).
Pansorbin from Calbiochem (La Jolla, CA, USA) and Sulfo-NHS-LC-biotin and Ultra Link Immobilized NeutrAvidin were from Pierce (Rockford, IL, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St Louis, MO, USA).

PCI was obtained as a recombinant protein. The construction of a synthetic gene for PCI, its expression in \textit{E. coli} and a procedure to purify recombinant PCI secreted into the culture medium have been previously reported [26,27]. PCI was detected and quantified by inhibitory activity assays according to Hass and Ryan [28], and by enzyme-linked immunosorbent assay using a rabbit polyclonal antibody raised against PCI. The concentration of the purified solutions of recombinant PCI was determined from the A280 of the final solution (PCI extinction coefficient: $E_{0.1\%}$ is 3).

2.2. Cell culture

The human vulvar epidermoid cell line A431, the pancreatic adenocarcinoma cell line Capan-1, and the human breast cancer cell lines MDA-MB453 and BT474, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 20 µg/ml gentamicine, unless otherwise indicated. All of these products were purchased from Gibco BRL LifeTechnologies.

2.3. Covalent cross-linking experiments

For each experiment, a 50% confluent A431 cell culture was used, growing in 100-mm dishes. Cells were harvested in 0.5 ml RIPA B lysis buffer (20 mM sodium phosphate pH 7.4; 150 mM NaCl; 1% Triton X-100; 5 mM EDTA; 5 mM PMSF; 10 µg/ml apro- tinin and leupeptin; 250 µg/ml sodium deoxycholate) and lysed using a glass homogenizer. Protein concentration in cell lysates was determined by Bradford [29] or Lowry [30] assay. Aliquots of 10 µg of protein were incubated with PCI, insulin or BSA at room temperature for 30 min. Subsequently, EGF (at 1 or 10 µg/ml) or TGF-α (at 5 µg/ml) was added. After a 30 min incubation at room temperature, cross-linking was initiated by addition of 40 mM glutaraldehyde. After 1 min, reaction was stopped with 0.2 M glycine (pH 9). Samples were then added of SDS-PAGE loading buffer with 5% β-mercaptoethanol, heated for 5 min at 100 °C and loaded in a 5% polyacrilamide gel. Electrophoretic transfer to PVDF membranes was followed by immunoblotting with anti-EGFR antibody and detection via chemiluminiscence.

2.4. Receptor phosphorylation analysis of EGFR or ErbB-2 immunoprecipitated

Serum-starved MDA-MB-453 and A431 cells growing in 100 mm dishes were treated with various concentrations of PCI or insulin (in DMEM) for 10 min and immediately stimulated with EGF or TGF-α for 10 min. The cells were then lysed as described and protein concentration was determined by Bradford or Lowry assay. The EGFR was immunoprecipitated from cell extracts, using equal amounts of proteins of each sample, with an anti-EGFR antibody for 1 h on ice. Pansorbin (a suspension 1:1 (v:v) in 20 mM Tris–HCl pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.02% sodium azide; 0.5% sodium deoxycholate; 0.5% NonidetP-40; 1 mg/ml BSA) was then added and mixed with gentle agitation, followed by 30 min of incubation on ice. The immune complexes were washed thoroughly three times with ICKA buffer (10 mM sodium phosphate pH 7.4; 150 mM NaCl; 0.1% Triton X-100), and resuspended in Hepes–vanadate buffer (20 mM Hepes pH 7.4; 100 mM sodium vanadate). Samples were then added to SDS-PAGE loading buffer with 5% β-mercaptoethanol, heated for 5 min at 100 °C and electrophoresed in a 8% gel. The level of tyrosine phosphorylation of the immunoprecipitated receptor was assessed by immunoblotting with anti-phosphotyrosine (PTyr) antibody RC-20. The blots were visualized by enhanced chemiluminiscence.

For ErbB-2 phosphorylation analysis the protocol was the same as for EGFR but BT474 cells were incubated for 15 min with PCI and/or EGF and anti-erbB-2 antibodies were used to immunoprecipitate the receptor. After visualization of phosphotyrosine level by enhanced chemiluminiscence, antibodies were removed incubating the membranes for 30 min at 50 °C with 10 mM Tris (pH 7.5), 100 mM NaCl, 2% SDS and 100 mM β-mercaptoethanol. Membranes
were then immunoblotted with anti-ErbB-2 antibody, and visualized by enhanced chemiluminescence. The average ratio between tyrosine phosphorylation level versus ErbB-2 was assessed using the Quantity One Software (Bio-Rad, USA).

2.5. Receptor phosphorylation analysis of A431 lysates

Cells were grown, treated and lysed as described in the previous paragraph, and protein concentrations determined. Equal amounts of protein of each sample were subjected to SDS-PAGE and the level of tyrosine phosphorylation assessed by immunoblotting with anti-phosphotyrosine (PTyr) antibody RC-20. After visualization by enhanced chemiluminescence, antibodies were removed as described above. Membranes were then immunoblotted with anti-EGFR antibody, and visualized by enhanced chemiluminescence.

2.6. Biotin-labeling of EGFR for internalization analysis

A431 serum starved cells were treated for different times with 50 μg/ml PCI at 37 °C. Cells were labeled with NHS-sulfo-biotin for 30 min at 4 °C, the reaction was stopped with glycine 10 mM. Cells were lysed and protein concentration was determined by Lowry assay. Equal amounts of protein were precipitated with Neutravidin for 30 min at 4 °C and the precipitates were washed with 0.1% Triton X-100, 0.1% SDS in PBS. Equal amounts of precipitates (membrane protein), supernatants (intracellular protein) and cell lysates (total protein) were loaded to 8% SDS-PAGE and analyzed by immunoblotting with an anti-EGFR antibody. After visualization by enhanced chemiluminescence, the bands were quantified by Quantity One software and the ratio between treated and control band were plotted.

2.7. PCI internalization assays

RITC (rhodamine B isothiocyanate) labeling of PCI was performed according to Billings et al. [31]. The RITC-labeled PCI was used for fluorescence internalization assays. Cells were cultured in chamber slides (Nunc, Kamstrup, Denmark) in DMEM supplemented with 10% serum, and RITC-labeled PCI was added at a concentration of 10 μg/ml. After 2 h 30 min, the medium was removed, and the cell monolayers were washed with PBS, fixed with 1:1 methanol:acetic acid and observed under a fluorescence microscope.

2.8. Western blot analyses of EGFR levels

A431, MDA-MB-453 and Capan-1 cells were grown in 6-well plates in DMEM+10% FBS with 50 or 10 μg PCI/ml. Control, untreated cells were grown simultaneously in absence of PCI. Cells were split when they were about to reach confluence, and equal numbers of control and treated cells seed in new wells and in 100-mm culture dishes. At the indicated time of treatment, cells growing in 100 mm dishes were washed twice with PBS, lysed and protein concentration was determined by Bradford or Lowry assay. Equal amounts of protein of control and treated samples were subjected to SDS-PAGE and immunoblotted with an anti-EGFR antibody. After visualization by enhanced chemiluminescence, antibodies were removed as described above, and the membranes were then immunoblotted with anti-actin antibody and visualized.

2.9. Semiquantitative RT-PCR analyses of EGFR mRNA levels

Fifty-percent confluent, serum-starved MDA-MB-453, Capan-1 and A431 cells growing in T/25 flasks were washed twice with PBS, treated with 1 ng/ml EGF or PCI (in DMEM) for 4, 8 or 24 h. The concentration of PCI was 10 μg/ml for MDA-MB-453 and Capan-1 cells and 50 μg/ml for A431 cells. In the case of MDA-MB-453 cells, they were also treated for 4 and 12 days with 10 μg PCI/ml in DMEM 10%FBS.

RNA purification was performed by the acid guanidium thiocyanate–phenol–chloroform method [32]. The RNA was quantified by determining absorbance at 260 nm, and its quality assessed by subjecting it to electrophoresis in citric acid urea gels. 2 μg of total RNA extract was converted to cDNA by incubation at 37 °C during 1 h 30 min, and 70 °C for 10 min, with 200 units of Superscript RNase H− Reverse Transcriptase in a 30 μL reaction volume containing 6 mM DTT, 0.4 mM dNTP and 0.5 μg OligodT. All reagents for cDNA synthesis were
obtained from Gibco BRL Life Technologies. EGFR expression was examined by semiquantitative RT-PCR using the β-actin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression as internal reference. The RT-PCR was performed using 1.3 μL of cDNA, 18 pmol of the forward (F) and reverse (R) primers and 18 μL of PCR SuperMix (Gibco BRL Life Technologies). After a polymerase activation step (94 °C for 10 min), samples were amplified for 20-35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min, and extension at 72 °C for 2 min, followed by an additional extension step (72 °C for 5 min). The primer sequences, temperature of annealing and PCR product size were: for EGFR, 5′-GCT GCC AAA AGT GTG ATC CAA G-3′ (F) and 5′-CAT GGA GGT CCG TCC TGT TTT C-3′ (R), 54 °C, 599 pb [33]; for GAPDH, 5′-TGT TCG CTC TGG GTA TTG-3′ (F) and 5′-TGA TAA GGA CAG CCA-3′ (R), 60 °C, 368 pb; and for β-actin 5′-CGA GCG GGA AAT CGT GCG TGA CAT TAA GGA GA-3′ (F) and 5′-CGT CAT ACT CCT GCT TGC TGA ACA TCT GC-3′ (R), 54 °C, 479 pb.

Amplified cDNAs were run on 2% agarose gels with 0.5 μg/ml ethidium bromide, and visualized under UV light. The average ratio between EGFR transcript levels in treated versus untreated cells was assessed, following normalization by GAPDH or β-actin levels, using the Quantity One Software (Bio-Rad, USA).

2.10. Cell-proliferation assays

To measure the effect of PCI on EGF induced cell proliferation, A431 and MDA-MB-453 cells were seeded at a density of 5×10² and 2×10³ per well, respectively, in 96-well plates in the presence of DMEM supplemented with 10% FBS. After 72 h, the cells were washed with PBS twice, and grown in DMEM plus 0.5% FBS with EGF (0.1–25 ng/ml in the case of A431, 0.01–100 ng/ml for MDA-MB-453), PCI (50 or 10 μg/ml, respectively), or both. After 48 h in the case of A431 or 96 h in the case of MDA-MB-453, the cells were washed with PBS, and the EZ4U reduction assay was performed to estimate the number of cells according to the manufacturer’s instructions.

3. Results

3.1. PCI inhibits EGFR dimerization induced by EGF and TGF-α in A431 cells

The first step in ligand-induced activation of EGFR is the dimerization of the receptor. To analyze whether PCI could block this process, covalent cross-linking experiments were performed on samples from A431 detergent extracts.

To determine the concentration of EGF necessary to induce dimerization, the samples were mixed with concentrations of EGF ranging from 1 ng/ml to 10 μg/ml, and after 30 min were cross-linked with glutaraldehyde. No significant increase in the amount of dimer formed was observed at concentrations of EGF above 1 μg/ml EGF (not shown). Nor did PCI alone at concentrations ranging from 1 μg/ml to 5 mg/ml cause any detectable dimerization of the receptor (Fig. 1a).

When cell extract samples were incubated with PCI for 30 min and 1 μg EGF/ml added for 30 more minutes, inhibition of receptor dimerization by PCI was observed. The inhibition was complete at 5 mg/ml PCI. Thus, at 5 mg/ml of PCI, EGF was unable to induce any detectable dimerization of the receptor (Fig. 1a).

TGF-α is another ligand of EGFR and both form one of the best-defined autocrine loops in human tumors [36]. To analyze whether PCI could also block the dimerization of EGFR induced by TGF-α, similar covalent cross-linking experiments were performed. Concentrations of TGF-α ranging from 1 ng/ml to 10 μg/ml were tested. Although a significant level of dimerization can be observed at 1 μg/ml, a concentration of 5 μg/ml TGF-α was required to reach saturation (not shown). PCI showed again a dose-dependent inhibitory effect on TGF-α induced receptor dimerization. The effect was apparent at concentrations of PCI greater than 0.5 μg/ml, and 2.5 μg/ml PCI were sufficient to block completely any detectable dimerization of EGFR induced by TGF-α at 5 μg/ml (Fig. 1b). In both experiments bovine serum albumin (BSA) and insulin were used as negative controls. They had no effect on EGF-induced dimerization, indicating that the effect of PCI was specific.
3.1.1. PCI inhibits EGFR transphosphorylation induced by EGF and TGF-α

The second step in the ligand-induced activation of EGFR is the transphosphorylation of both receptor molecules of the dimer. This led us to investigate whether PCI was able to inhibit EGF and TGF-α-induced EGFR transphosphorylation in A431 and MDA-MB-453 cells.

MDA-MB-453 cells have been reported to express levels of EGFR undetectable by Northern or Western blots [37] although they have low levels of EGF binding sites [38]. However, in our hands they showed significant receptor levels. The reason for this unexpected result is probably that during laboratory culture (high passage) a sub-population of cells expressing higher receptor levels was selected. Some examples of selection of sub-populations of a cell line during culture have been reported, including an A431 sub-population with lower EGFR levels and resistant to EGF-mediated growth inhibition [39].

Fig. 1. Inhibition of EGFR dimerization induced by (a) EGF or (b) TGF-α in A431 cells by PCI. Cell lysates (10 μg) from A431 cells were treated with various concentrations of PCI, insulin or BSA for 30 min and immediately stimulated with EGF (1 μg/ml) or TGF-α (5 μg/ml) for 30 min. Cell lysates were cross-linked, loaded onto SDS-PAGE gels and immunoblotted with anti-EGFR antibody. The arrows show the position of the receptor monomer and dimer. The results shown are a representative of three different experiments.
or A431 cells with gradual ErbB-2 up-regulation due to prolonged passage [12].

When EGF at 1 or 10 ng/ml was added to serum-starved high-passage MDA-MB-453 cells for 10 min, the level of tyrosine phosphorylation increased significantly. When the cells were treated with 50 μg PCI/ml for 10 min, and then with EGF at 1 ng/ml, the inhibitor completely suppressed the ligand-induced phosphorylation of the receptor. However, if the EGF concentration was raised to 10 ng/ml, PCI at 50 μg/ml failed to have any detectable effect on EGFR phosphorylation (Fig. 2a).

To determine whether PCI inhibits EGFR phosphorylation induced by TGF-α, assays were performed adding PCI to serum-starved A431 cultures for 10 min and TGF-α for 10 more minutes, followed by cell lysis. Detergent extracts were either immunoprecipitated or loaded directly in a SDS-PAGE, electrotransferred and immunoblotted with anti-phosphotyrosine antibodies. The addition of 2 ng/ml

![Fig. 2](image-url)

**Fig. 2.** Inhibition by PCI of EGFR tyrosine phosphorylation induced by EGF in MDA-MB-453 cells (a) and induced by TGF-α in A431 cells (b) and (c). Serum-starved cells were added PCI or insulin for 10 min, and then treated with EGF or TGF-α for 10 more minutes. (a) The activation of the EGF receptor in MDA-MB-453 cells was measured by blotting of the immunoprecipitated receptor (IPαEGFR) with anti-phosphotyrosine antibody. (b) A431 protein lysates were solved by SDS-PAGE, transferred to a membrane, and analyzed using anti-phosphotyrosine antibodies to determine the extent of EGFR tyrosine phosphorylation (PTYR, top panel). To monitor the amount of EGFR, filters were stripped and immunoblotted with anti-EGFR antibodies (bottom panel). (c) A431 protein lysates were immunoprecipitated with anti-EGFR antibodies (IPαEGFR), resolved by SDS-PAGE and immunoblotted using antibodies against phosphotyrosine. The results shown are a representative of two different experiments.
TGF-α to serum-starved A431 cultures markedly stimulated tyrosine phosphorylation of EGFR, and PCI was found to be an effective inhibitor of this process. Preincubation of the cells with 100 or 300 μg/ml PCI for 10 min inhibited TGF-α-induced EGFR tyrosine phosphorylation in a concentration-dependent way, and this effect was observed in both cell extracts and immunoprecipitates (Fig. 2b and c). The density of the bands showed in top and bottom panel was measured by densitometry and the ratio of the Ptyr to ErbB-2 bands was then plotted. The results shown are a representative of three different experiments.

3.1.2. PCI inhibits ErbB-2 transphosphorylation induced by EGF

The important biological role of ErbB-2 in the signaling network that drives epithelial cell proliferation prompted us to analyze if PCI could also block the phosphorylation of ErbB-2 induced by EGF.

BT474 cells, that overexpress erbB-2, were activated with EGF, lysed, immunoprecipitated with anti-ErbB-2 antibodies and immunoblotted with anti-phosphotyrosine antibodies. A significant increase in ErbB-2 phosphorylation was observed when cells were treated with 1–100 ng/ml EGF (not shown). The addition of PCI (10–50 μg/ml) significantly reduced the activation of ErbB-2 in a dose-dependent way (Fig. 3); PCI at 50 μg/ml was able to block EGF stimulation of ErbB-2 tyrosine phosphorylation by 75%.

3.2. PCI induces EGFR internalization in A431 cells

Following EGF or TGF-α binding, the ligand-EGFR complexes are internalized by cells via coated pits [40]. For greater insight into the possible function of PCI as an EGF antagonist, we decided to examine whether the inhibitor induces internalization of EGFR in A431 cells.

Serum-starved cells were treated with 50 μg/ml of PCI for 5, 10, 30 and 60 min, and then were labeled with NHS-sulfo-biotin that only labels extracellular proteins. Equal amounts of cell extracts were precipitated with Neutravidin, and membrane, intracellular and total proteins were subjected to SDS-PAGE and western blot with anti-EGFR antibodies. The quantification of the immunoblots showed that, after 30 min of PCI treatment, A431 cells decrease significantly their amounts of extracellular EGFR. This change was associated with an increase in intracellular EGFR at 60 min of treatment (Fig. 4). At shorter times of treatment there was no significant change. It can be concluded that PCI induces the internalization of EGFR. The time course with PCI is similar to that was observed with EGF (data not shown): the maximal internalization of EGFR induced by this growth factor was observed after 60 min [41].

3.2.1. PCI is internalized by A431, MDA-MB-453 and BT474 cells

The internalization of fluorescent labeled PCI was determined in A431, MDA-MB-453 and BT474 cells. RITC-conjugated PCI was observed in the cells’ cytoplasm 2 h 30 min after its addition to the culture.
medium of exponentially growing cells. The fluorescence was located mainly around the nucleus and appeared to be concentrated in granules (data not shown). It is interesting to note that FITC-EGF has also been reported to cluster around the nucleus after internalization in several human tumor cell lines, including A431 [42] and MCF-7 [43].

3.3. PCI reduces the levels of EGFR in A431, MDA-MB-453, and Capan-1 cells

After the ligand-induced internalization, ligand-EGFR complexes are either subjected to lysosomal degradation or recycled [40]. To find whether PCI has some effect on EGFR expression, we decided to examine the EGFR levels in the A431 and MDA-MB-453 cell lines after PCI treatment.

A431 cells were treated with 50 μg/ml PCI up to 27 days, and MDA-MB-453 and Capan-1 cells with 10 μg/ml PCI up to 49 and 14 days, respectively. Control and treated cells were grown simultaneously and were subjected to detergent extraction at different times. Immunoblotting showed that A431 cells treated with PCI had a lower level of EGFR than control cells, an effect which was observable at all times tested, from 6 to 27 days (Fig. 5a). This reduction of EGFR levels was also observed in the case of MDA-MB-453 and Capan-1 cells treated with PCI for at least 11 or 9 days, respectively, but was not detected at shorter times. On the contrary, the receptor levels were even
higher than control cells after 4 days of treatment in MDA-MB-453 (Fig. 5b and c).

3.3.1. PCI affects EGFR mRNA expression as EGF does

To determine whether the variation in EGFR protein levels, observed in cells treated with PCI, was associated with variation in gene expression, we analyzed the effect of PCI on the levels of EGFR mRNA in A431, MDA-MB-453, and Capan-1 cell lines. We also studied EGFR gene expression in cells treated with EGF, which has been shown to have a cell-type specific effect on ErbB-1 expression [44]. The study was realized at maximum 24 h of treatment because serum free media was used.

Serum-starved MDA-MB-453 and Capan-1 cells were treated with 1 ng/ml EGF and/or 10 μg/ml PCI for 4, 8 or 24 h. For A431 cells, the concentration of PCI used was 50 μg/ml. Levels of EGFR mRNA were determined by the reverse transcriptase polymerase chain reaction (RT-PCR) technique. On analysis of series of PCR cycles to reach non-saturation
concentration of the final RT-PCR product, it was established that the optimal number of cycles to study EGFR expression were 20 for A431, and 30 for MDA-MB-453 and Capan-1 (data not shown).

In A431 cells, EGF decreased the expression of the EGFR mRNA, the effect started at 8 h and after 24 h the expression of treated cells was only 50% of control levels. In the case of PCI, there was no significant change in EGFR mRNA levels at 8 h, but after 24 h a significant decrease in ErbB-1 messenger was apparent. Simultaneous treatment with PCI and EGF for 8 h increased the effect of the ligands, with a reduction to 17% of control EGFR mRNA levels (Fig. 6a).

In MDA-MB-453 cells, EGF induced an increase in the EGFR mRNA levels at 8 h, which levels remained high at 24 h. In the case of PCI, no change in mRNA levels was observed until after 24 h of treatment, at which time a slight increase in EGFR mRNA levels became apparent (Fig. 6b). When the PCI treatment was prolonged to 4 and 12 days, the differences between treated and untreated cells were more significant; there was an important up-regulation of EGFR mRNA levels (Fig. 6c). The results in Capan-1 cells are similar to those in MDA-MB-453. EGF induces an up-regulation of EGFR mRNA levels at 8 h, while it appears in the case of PCI after 24 h of treatment (Fig. 6d). In all three cell lines PCI induced the same change as EGF in the levels of EGFR mRNA, but these changes take longer to appear with PCI.

3.3.2. PCI alters the proliferative response of A431 and MDA-MB-453 cells to EGF
To evaluate the effect of PCI on the regulation of cell growth by EGF, several experiments were run. EGF significantly inhibited the growth of the A431 cell line in a dose-dependent manner, starting at concentrations of 0.1 ng/ml. This inhibition was accompanied by a change in cell morphology. The inhibition of A431 cell growth by EGF has been previously reported [45]. This is a consequence of the high levels of EGFR expression by this cell line [46] and the possible activation of the signal transducers and activators of transcription (STAT) pathway [47]. PCI at 50 μg/ml reduced the inhibitory effect of 10 and 100 ng/ml EGF on cell growth (Fig. 7a), but was unable to prevent the change in cell morphology.

In the case of the cell line MDA-MB-453 the dose-response curve of the cells to increasing concentrations of EGF was altered when PCI was added simultaneously to the cultures (Fig. 7b). Exogenous EGF at concentrations from 1 to 50 ng/ml, with maximum stimulation at 10 ng/ml, stimulated cell growth. However, EGF at lower or higher concentrations failed to have any effect on cell growth. This altered when PCI was present, in which case all EGF concentrations ranging from 0.1 to 50 ng/ml had a modest stimulatory effect on cell proliferation in a dose-independent way (see Fig. 8).

4. Discussion
Signal-transduction pathways are involved in the appearance and maintenance of the transforming phenotype, and many cancer cells express overactive signaling pathways. In the case of carcinomas, tumors of epithelial origin, the EGFR transduction pathway seems to play a prominent role. In consequence, efficient signal interceptors of this pathway could become useful therapeutic agents [22].

PCI is the first human EGF antagonist described. Our results show the mechanism of action of PCI as an EGF antagonist. PCI inhibits all the steps in ligand-induced activation of ErbB-1 by EGF: ligand binding, receptor dimerization, induction of tyrosine kinase activity and tyrosine transphosphorylation. The blockade of EGF-induced receptor activation by PCI was observed in cell lines derived from pancreatic, breast and epidermoid carcinomas, with different levels of EGFR expression.

In addition, PCI can inhibit EGFR dimerization and transphosphorylation induced by TGF-α. The quantity of PCI necessary to inhibit these processes is different from those induced by EGF. This can be due to the different mitogenic action of these molecules although they activate the same receptor [12,49].

Another receptor of the ErbB family, ErbB-2, is overexpressed in a great many human carcinomas and involved in the progression of some malignancies. Several reports indicate that ErbB-2 is the preferred heterodimerization partner for the other family members [17] and that the heterodimers EGFR/ErbB-2 transduce a powerful mitogenic signal [15]. That PCI can block ErbB-2 activation induced by
Fig. 6. Effect of EGF and PCI treatment on EGFR mRNA levels in A431, MDA-MB-453 and Capan-1 cells. Serum-starved cells were treated for 4, 8 or 24 h with EGF (1 ng/ml) and/or PCI. The concentration of PCI was 50 μg/ml for A431 and 10 μg/ml for MDA-MB-453 and Capan-1. Specific transcript levels were assessed by semi-quantitative RTPCR as described in ‘Section 2’ and representative results are showed. (a) EGFR mRNA expression in A431, (b) in MDA-MB-453 and (d) in Capan-1 cells. In the case of MDA-MB-453, (c) cells were also treated with 10 μg/ml PCI for 4 and 12 days in DMEM 10%FBS and was analyzed EGFR mRNA expression. GAPDH and β-actin mRNA expression (bottom panels) was used as a control. The PCR product sizes (in pb) were 699 for EGFR, 368 for GAPDH and 479 for β-actin. The DNA markers (first lane) were 1353, 1078, 872, 603, 310 and 281 pb.
EGF raises its potential as a therapeutic agent, as it could also inhibit specific pathways induced by EGF that depend on ErbB-2 activation [50].

The experiments of EGFR and ErbB-2 activation show that PCI blocks their activation induced by natural ligands. In those experiments its also shown that the binding of PCI to EGFR causes EGFR phosphorylation to rise slightly (Fig. 2a and c), however, PCI binding was unable to induce receptor dimerization or receptor kinase activation because EGFR dimers and erbB-2 phosphorylation levels (Fig. 3) were not increased by PCI treatment.

The binding of PCI to EGFR also induces EGFR internalization. It is probably that receptor internalization was simultaneous to PCI internalization because PCI was located in perinuclear region as EGF does. Phosphorylation of EGFR has been described as essential for internalization [51], so the slight increase of EGFR phosphorylation that induces PCI might be enough to promote EGFR endocytic traffic.

The superposition of PCI and TGF-α tridimensional structures, based on the space distribution of the aminoacid residues, can help to explain the role played by PCI as a TGF-α antagonist (see Appendix). Once a ligand has bound to ErbB receptors, cells internalize and degrade ligand-receptor complexes [40], resulting in a decrease of total receptor levels: a process referred to as “down-regulation”. Treatment with PCI induced a decrease in the levels of EGFR in the three cell lines tested, A431, MDA-MB-453 and Capan-1. This could be due to the internalization of the receptor after binding to PCI, and the subsequent proteolytic degradation in endosomes. In the A431 cell line, EGF causes detectable EGFR down-regulation in 4 h and is persistent in cells treated with EGF over a period of 6 days [12,49]. The presence of PCI in the culture medium of A431 cells reduced the levels of EGFR at all the times tested, ranging from 6 to 27 days. The time-evolution of the receptor levels in MDA-MB-453 and Capan-1 cells treated with PCI was not so simple. A modest increase was detected after 4 days of treatment, but this became a decrease after 9 and 11 days, respectively. This greater delay in the down-regulation of EGFR in MDA-MB-453 and Capan-1 than in A431 could be due to various effects at other regulatory points.

The regulation of the amount of EGFR in cells is complex and occurs at multiple points: EGFR transcription, mRNA decay, and protein synthesis and decay. In addition, it is ligand and cell-type specific [52]. To investigate further PCI’s mode of action we tested whether the inhibitor modifies EGFR mRNA levels in A431, MDA-MB-453 and Capan-1 cells. Semi-quantitative RT-PCR showed that PCI induces an increase in EGFR mRNA levels in
MDA-MB-453 and Capan-1 cells, and a decrease in A431 cells detectable after 24 h. EGF had the same effects as PCI on the EGFR mRNA levels, and promoted a decrease in EGFR gene expression in A431 and an increase in MDA-MB-453 and Capan-1 cells (Fig. 6). It has been reported that addition of the ligand induces synthesis of EGFR in several cell lines [53], including breast cancer cell lines [54]. In short, these results show that the effects of PCI on EGFR mRNA levels are cell type-specific and similar to the effects induced by EGF, although they take longer to appear. In this context, the increase of EGFR mRNA levels induced by PCI can explain the increase in EGFR protein levels observed by Western blot analysis in MDA-MB-453 after 4 days. Longer treatments with PCI induced a downregulation of EGFR protein, what may be due to multiple changes at different levels in the regulation of EGFR expression [12].

In conclusion, our results show that PCI is an EGF antagonist that inhibits all the steps of EGF-induced activation of EGFR, being also able to block receptor dimerization and transphosphorylation induced by TGF-α. In addition, it prevents EGF-induced ErbB-2 activation. To some extent, PCI could be considered a weak partial agonist because it stimulates receptor phosphorylation and internalization. Nevertheless, it should also stimulate EGFR dimerization and we have not detected it in our experimental. This is the reason we prefer using ‘antagonism’ because it is not inducing a mitogenic effect.

Some properties of PCI make it a good potential candidate for a therapeutic agent: it lacked any toxic side-effects when tested in mice [21], and it induces
receptor down-regulation. Combinations of signal interceptors and other anticancer agents and treatments will probably be needed to treat some kinds of tumors. Further research is needed in order to determine if PCI could be one of the signal interceptors with therapeutic applications, and if it could be considered a leader compound to develop powerful EGF/TGF-α antagonists.

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Appendix A. Appendix (useful extra information)

A.1. Superimposition of the Three-dimensional structures of PCI and TGF-α

In a previous work we had superimposed PCI and mouse EGF by means of an automated program, Knot-Match [25]. However in the present work we have improved manually the superimposition between both proteins. PCI and human TGF-α were superimposed by forcing the Cα positions of His 15, Phe 23 and Tyr 37 of PCI upon those of His 12, Phe 17 and Phe 15 from TGF-α. These three key functional residues of TGF-α superimpose with a very good root mean square deviation (RMSD = 2.5 Å) with PCI residues of similar chemical character.

These residues of TGF-α seems to be involved in the interaction with the EGF receptor [34,35]. The distances between the Cα of these residues in PCI were similar to the corresponding residues on TGF-α. In the present version the N-and C-terminals of both proteins run in opposite directions. When His3i of PCI superimposed on His45 of TGF-α, the RMSD was 4.5 Å. His3i is located in the N-terminal of PCI, a particularly non-rigid area which had been studied by molecular dynamics [48].

References
