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Down-Regulation of PAR1 Activity with a pHLIP-based Allosteric Antagonist Induces Cancer Cell Death

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Abstract

Even though abnormal expression of G protein-coupled receptors (GPCRs) and of their ligands is observed in many cancer cells of various origins, only a few anti-cancer compounds directly act on their signaling. One promising approach to modulate their activity consists of targeting the receptor cytoplasmic surfaces interacting with the associated G proteins using peptides mimicking the intracellular loops of the receptor. Thus, to be fully effective, the peptide mimics must be selectively targeted to the tumor while sparing healthy tissues, translocated across the cell membrane and stay anchored to the cytoplasmic leaflet of the plasma membrane. Here, we introduce a novel way to selectively target and inhibit the activity of a GPCR in cancer cells under acidic conditions, such as those found in solid tumors. We find that the conjugation of a peptide fragment derived from the third intracellular loop of the Protease Activated Receptor 1 (PAR1) to a peptide that can selectively target tumors solely based on their acidity (pHLIP), produces a construct capable of effectively down-regulating PAR1 activity in a concentration - and pH-dependent manner, and of inducing a potent cytotoxic effect in a panel of cancer cells that is proportional to the relative level of receptor expression at the cell surface. This strategy not only allows for a more selective targeting and specific intracellular delivery than current approaches, but also offers new possibilities for developing novel anti-cancer drugs targeting GPCRs.

Keywords

targeted drug delivery; peptide; tumor acidity; cancer cytotoxicity; GPCR; allosteric receptor modulation; intracellular loops

INTRODUCTION

G-Protein Coupled Receptors (GPCRs) represent the largest family of cell surface receptors and are involved in almost every aspect of cell biology and physiology (e.g., sensory systems, neurotransmission, inflammation pathways, hormone signaling, cell-cell

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SUPPORTING INFORMATION

The synthesis scheme for pHLIP-P1AP, characterization of peptides, data on growth inhibition of P1pal-7, P1AP and pHLIP, and cell membrane integrity data.

AUTHOR CONTRIBUTION

DT and KEB designed the project, performed the experiments, analyzed the data, and wrote the manuscript. KEB synthesized the constructs.

communication) by mediating a plethora of cellular signals across the cell plasma membrane in response to an array of ligands. Despite exhibiting striking diversity in primary sequence and biological function, GPCRs share the same fundamental architecture, consisting of a hydrophobic core of seven-transmembrane α -helices joined by extracellular and intracellular loops that interact to form a three-dimensional barrel within the cell membrane. They transduce extracellular signals by coupling to hetero-trimeric guanine nucleotide binding proteins (G-proteins), consisting of α , β and γ subunits. G-proteins are associated to the cytoplasmic face of the receptor through interactions with the intracellular loops [1]. Upon ligand binding, the receptor undergoes conformational changes that stimulate exchange of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) on $G\alpha$ subunits, dissociating $G\alpha$ and $G\beta\gamma$ subunits that, in turn, trigger biological responses by binding to effector proteins that regulate processes (e.g., second messenger production, protein kinase cascades, gene transcription and ion channel activity).

Due to their critical role in physiological processes and links to numerous diseases, GPCRs are prime pharmaceutical drug targets. Most drugs developed against GPCRs bind to the extracellular orthosteric ligand domain. However, these binding sites across members within a receptor subfamily are often highly conserved, making it difficult to achieve high selectivity for a specific GPCR [2]. Therefore, the ability to modulate signaling through an allosteric site may provide novel drug target opportunities [2–4]. Allosteric modulation provides greater selectivity as it can perturb the signaling cascade irrespective of the presence or absence of the orthosteric ligand [5]. While most allosteric modulators act extracellularly, intracellular domains of GPCRs are key sites for allosteric regulation due to multiple regions in the cytoplasmic domains of GPCRs to which G proteins can make contact [4]. Mutagenesis studies have demonstrated that the third intracellular loop (i3) mediates a large part of the coupling between receptor and G protein [6–8].

One approach to modulate the interaction of GPCRs with G proteins is to target the intracellular portions of the receptors using pepducins, which are palmitoylated peptide fragments based on the intracellular loops of a GPCR [6]. Biochemical and biophysical studies indicated that the lipid moiety of pepducins facilitates translocation across the lipid bilayer and serves to anchor the pepducin in the cytosolic face of the plasma membrane [9]. Even though the exact mechanism of action remains unclear, it is proposed that the peptide moiety directly interacts with its cognate receptor, and stabilizes the active or inactive conformation of the receptor [3]. Pepducins have been shown to function as allosteric agonists or antagonists of several GPCRs, including protease-activated receptors (PAR1, PAR2 and PAR4), chemokine receptors (CXCR1, CXCR2 and CXCR4), sphingosine 1-phosphate receptor-3 (S1P3), melanocortin-4 receptor, and formyl peptide receptor 2 [3,6,9–13]. Specifically, the PAR receptors are tethered-ligand receptors that are activated through the proteolytic cleavage of their extracellular domains by serine proteases such as thrombin, activated protein C, plasmin and factor Xa [14]. Within the PAR subfamily, PAR1, PAR2 and PAR4 have been shown to be modulated effectively by pepducins [6,13,15,16].

Particularly, PAR1 has proved to be an attractive target for oncogenic treatment because of its involvement in the invasive and metastatic processes of various cancers [11]. For instance, PAR1 has shown to promote tumorigenicity, invasion, and metastasis in breast and

ovarian carcinoma xenograft models [10,11,16–22]. Pepducins have been shown to inhibit the PAR1/G-protein signaling in breast cancer cells. However, the targeting specificity relies solely on the overexpression of PAR1 in cancer cells [6,11], which would likely result in off-target toxicity in in vivo settings if used as a treatment modality.

In the present study, we hypothesized that higher selectivity towards cancer cells and a more controlled intracellular delivery can be achieved by replacing the lipid vehicle with the pH(Low) Insertion Peptide (pHLIP), a tumor-targeting peptide. pHLIP is a unique peptide that can selectively target tumors in mice based solely on their acidity rather than a specific marker, and that is capable of translocating a wide array of cargo molecules across the plasma membrane and into the cytoplasm [23–25]. Importantly, the entry of pHLIP into the membrane and the translocation of molecules is not mediated by endocytosis, cell receptor interactions, or by the formation of pores within the membrane [26,27]. Therefore, upon pHLIP insertion, the modulating peptide will remain membrane anchored, if conjugated to the C-terminal end of pHLIP through a non-releasable linker. Here, we show that a peptide fragment derived from the third intracellular (i3) loop of PAR1, induces cytotoxicity from the down-regulation of PAR1 cell signaling pathway when conjugated to the C-terminus of pHLIP. This study represents not only the first demonstrated example of using pHLIP to modulate a GPCR, but also a new way to selectively modulate the activity of this important class of membrane receptors in cancer cells.

EXPERIMENTAL SECTION

Materials

N-Hydroxybenzotriazole (HOBt), o-benzotriazol-N, N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU), and all N-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids were purchased from GL Biochem Ltd. H-Rink Amide-ChemMatrix solid support resin was purchased from PCAS BioMatrix Inc. Palmitic Acid, Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), Diisopropylethylamine (DIEA), Piperazine, N, N-dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), methanol, acetonitrile, dimethyl sulfoxide (DMSO), Chloroform, and Dulbecco's modified Eagle's medium (DMEM) were all purchased from Thermo Fisher Scientific Inc. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals Inc. Penicillin-Streptomycin and Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids Inc. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from EMD Millipore. Anti-PAR1 mouse and goat anti-mouse IgG-FITC was purchased from Life Technologies. Fluo-4 NW Calcium Assay Kit was purchased from Life Technologies.

Solid-Phase Peptide Synthesis

pHLIP-Cys (pHLIP(WT)) with a cysteine residue at its C terminus ($\text{H}_2\text{N-AAEQNPYYWARYADWLFTTPLLDDLALLVDADEGTCTG-CONH}_2$) and P1AP ($\text{Cl-Ac-H}_2\text{N-KKSRLF-CONH}_2$) peptides were prepared in our laboratory by Fmoc solid-phase synthesis. A non-cleavable linker (chloro-acetylchloride) was coupled to the P1AP peptide

on resin (Scheme S1). The P1AP pepducin (P1pal-7) was obtained by the overnight coupling of the peptide with palmitic acid in the presence of PyBOP in chloroform on resin. Briefly, peptides were purified via reversed-phase high performance liquid chromatography (RP-HPLC) (Phenomenex Luna prep 10 μ 250 $\times$ 21.20 mm C8; flow rate 10 mL/min; phase A: water 0.1% TFA; phase B: acetonitrile 0.1% TFA; gradient 60 min from 95/5 A/B to 0/100 A/B. The purity of the peptides were determined by RP-HPLC as listed, and their identity was confirmed via matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Figure S1).

Preparation of pHLIP-P1AP Construct

The pHLIP peptide was dissolved in DMSO to a concentration of 1 mM, followed by the addition of 1.5 eq. P1AP in DMSO and 50 μ L of 1 M Tris buffer, pH 8.0 was added to the solution and allowed to mix at room temperature for 2–3 hours (Scheme S1). The desired pHLIP conjugate was isolated using the same techniques described for the peptides. The purity of the peptide- conjugate was determined by RP-HPLC as listed, and their identity was confirmed by MALDI-TOF MS (Figure S1). The conjugates were quantified at 280 nm by UV/Vis absorbance spectroscopy using the molar extinction coefficient of pHLIP ($13,940 \text{ M}^{-1} \text{ cm}^{-1}$) and lyophilized to 10^{-8} mole aliquots.

Preparation of POPC Liposomes

Ten milligrams of POPC in chloroform were dried and then held under vacuum overnight. The dried lipid film was rehydrated with 1 ml of 5 mM sodium phosphate, pH 8.0, and mixed by vigorous vortex. The resulting multilamellar vesicle solution was freeze-thawed in liquid nitrogen for seven cycles and were extruded through a polycarbonate membrane with 100 nm diameter pores using a mini extruder (Avanti Polar Lipids) to produce large unilamellar vesicles. Liposomes were used immediately following their preparation. Lipid concentration was checked using the Marshall's assay²⁶ and the size distribution was verified by dynamic light scattering analysis performed at a scattering angle of 90° using an ALV/GSC-3 goniometer system equipped with ALV/ALV-7004 correlator (ALV-GmbH, Langen, Germany).

Sample preparation for Circular Dichroism and Tryptophan Fluorescence Measurements

Prior to CD or fluorescence measurements, lyophilized aliquots of pHLIP constructs were resuspended to 20 μ M with 5 mM sodium phosphate, pH 8.0, and incubated for 1 h at room temperature. This stock solution was diluted to a final concentration of 7 μ M and, when appropriate, incubated with POPC liposomes at a 1:300 peptide-to-lipid molar ratio for 30 minutes at room temperature. Subsequently, samples were adjusted to the desired experimental pH values with the addition of aliquots of a HCl solution to elicit pH-dependent insertion. Samples were allowed to equilibrate at the desired pH for 30 minutes at room temperature before spectroscopic measurements.

Tryptophan Fluorescence Spectroscopy

All measurements were carried out using a Cary Eclipse Fluorescence Spectrophotometer (Varian), and performed at 25 °C with pHLIP construct concentrations equal to 7 μ M.

Samples were excited at 295 nm and the emission spectra were taken from 300 to 500 nm, with the slit widths for emission and excitation both set 5 nm.

Circular Dichroism Spectroscopy

Far-UV CD spectra of pHLIP constructs were recorded on Jasco J-815 CD spectrometer equipped with a Peltier thermal-controlled cuvette holder (Jasco, Inc.). All measurements were performed in 0.1 mm quartz cuvette at 25 °C with pHLIP constructs concentrations equal to 7 µM. CD intensities are expressed in mean residue molar ellipticity $[\theta]$ calculated from the following equation:

$$[\theta] = \frac{\theta_{\text{obs}}}{10 \times l \times c \times n} \text{ (in degrees cm}^2\text{.dmol}^{-1}\text{)}$$

where, θ_{obs} is the observed ellipticity in millidegrees, l is the optical path length in centimeters, c is the final molar concentration of the peptides, and n is the number of amino acid residues. Samples were measured in a 0.1 cm path length quartz cuvette and raw data were acquired from 260 nm to 190 nm at 1 nm intervals with a 100 nm/min scan rate, and at least five scans were averaged for each sample. The spectrum of POPC liposomes was subtracted out from all construct samples.

Cell Culture

Human cervical adenocarcinoma HeLa cells, human breast adenocarcinoma MDA-MB-231 and MCF7 cells (kind gifts from Matthew Robinson, Fox Chase Cancer Center) and MCF7-PAR1/N55 stably transfected to express PAR1 (kind gift from the Lidija Covic, Tufts University) were cultured in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

PAR1 Cell-surface Expression

Cells were detached using trypsin and pelleted, resuspended and washed with 100 µL PBS 3 times. Cells were then incubated at 4 °C for 30 minutes with PAR1 mouse monoclonal antibody (Invitrogen), and washed 3 times with PBS. Cells were then incubated with goat anti-mouse IgG-FITC antibody (Invitrogen) for 30 minutes at 4 °C, and washed 3 times with PBS. Cells were then fixed in 2% formaldehyde and cells were analyzed using a BDFacs Canto II flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and a 530 bandpass filter (FL1). A minimum of 10,000 events were counted for each data point. The data was analyzed using the FACSDiva version 6.1.1 software. Fluorescence data is expressed as mean arbitrary fluorescence units and were gated to include all healthy cells.

Anti-proliferation Assay

Cells were seeded in 96-well plates at a density of 3,000 cells/well and incubated overnight. Before treatment, construct aliquots were solubilized in an appropriate volume of DMEM without FBS (pH 7.4) so that upon pH adjustment the desired treatment concentration is

obtained, and gently sonicated for 30–60 seconds using a bath sonicator (Branson Ultrasonics). After removal of cell media, this treatment solution was added to each well and incubated at 37 °C for 5–10 minutes. The pH was then adjusted to the desired pH using a pre-established volume of DMEM, pH 2.0 buffered with citric acid (final volume = 50 µL) and the plate was incubated at 37 °C for 2 hours. After treatment, the media was removed, cells were washed once with 100 µL of complete DMEM, and 100 µL of complete medium was added to each well before returning the plate to the incubator. Treatment solutions were collected and their pH values measured using a micro-combination pH probe (Microelectrodes, Inc.). For physiologic pH treatments a small down-drift (~0.2 pH unit) was usually observed, whereas an up-drift was observed for low pH treatments (e.g., pH 7.4 → pH 7.2, and pH 5.0 → pH 5.2). Cell viability was determined after 72 hours using the colorimetric MTT assay. Briefly, 10 µL of a 5 mg/mL MTT stock solution was added to the treated cells and incubated for 2 hours at 37 °C. The resulting formazan crystals were solubilized in 200 µL DMSO and the absorbance measured at 580 nm using an Infinite 200 PRO microplate reader (Tecan). Cell viability was calculated against control cells treated with media at physiologic pH.

Statistical Analyses

All error bars were calculated as standard error of the mean ($\pm$ SEM) in GraphPad Prism (version 4.0 for Macintosh) (GraphPad Inc., La Jolla, CA). To calculate statistical significance, two tailed Student's *t*-test analyses were performed with 95% confidence ($p < 0.05$). Asterisks represent statistically significant differences, where * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

Cell Membrane Integrity Assay

Cells were seeded in 96-well plates at a density of 3,000 cells/well and incubated until confluent (~ 72 hours). Before treatment, construct aliquots were solubilized in an appropriate volume of DMEM without FBS (pH 7.4) so that upon pH adjustment 10 µM is obtained. Cell media was removed and the treatment solution was added to each well and incubated at 37 °C for 5–10 minutes. The pH was then adjusted to the desired pH using a pre-established volume of DMEM, pH 2.0 buffered with citric acid (final volume = 50 µL). The plate was incubated at 37 °C for 2 hours. After treatment, the media was removed, cells were detached using trypsin and counted based on trypan blue uptake using an hemacytometer.

PAR1-Mediated Calcium Mobilization Assay

Cells were seeded in 96-well plates at a density of 40,000 cells/well and incubated overnight. Before treatment, construct aliquots were solubilized in an appropriate volume of DMEM without FBS (pH 7.4) so that upon pH adjustment 10 µM is obtained. Cell media was removed and the treatment solution was added to each well and incubated at 37 °C for 5 minutes. The pH was then adjusted to the desired pH using a pre-established volume of DMEM, pH 2.0 buffered with citric acid (final volume 50 µL). The plate was incubated at 37 °C for 10 minutes, shorter pHLIP-P1AP treatment time was implemented to avoid unwanted effects on intracellular Ca^{2+} due to a prolonged exposure to an acidic

environment. After treatment the media was removed and the calcium indicator fura-2, NW (Life Technologies) was added according to the manufacturer's protocols. After incubation for 30 min at 37 °C and then for 30 min at room temperature, 10 nM thrombin was added to each well. Fluorescence emission intensity at 530 nm (Excitation = 485 nm) was immediately measured with an Infinite 200 PRO microplate reader (Tecan) after a brief shaking.

RESULTS AND DISCUSSION

Synthesis of pHLIP-P1AP conjugate

Peptides that mimic the residues of the intracellular loops of GPCRs have been shown to allosterically modulate receptor conformation and signaling. Particularly, the pepducin P1pal-7, which consists of a palmitoyl and a seven-residue peptide (KKSRALF; named in this study, PAR1 Allosteric Peptide or P1AP) that mimics i3 of PAR1, was shown to induce toxicity in breast cancer cells expressing PAR1 by blocking its signaling. It was also shown to suppress breast tumor survival in MD-MB-231 xenografts and to inhibit metastasis to the lungs in mice [6,11,16].

To improve selectivity and cytotoxicity towards cancer cells, we synthesized P1AP, conjugated it to the C-terminus of pHLIP through a non-labile chloro-acetylchloride linker (Scheme S1), and purified the conjugates by HPLC and determined its identity with MALDI-TOF mass spectrometry (see Experimental Section). This linking strategy should anchor P1AP to the cytosolic face of the plasma membrane of cancer cells upon translocation by pHLIP in an acidic environment such as the one found in tumors, allowing for P1AP to stabilize PAR1 in its nonactive conformation and effectively down-regulating its signaling cascade.

Biophysical Characterization of the Interaction of pHLIP-MMAE Conjugates with Lipid Bilayers

Before evaluating pHLIP-mediated translocation of P1AP into cancer cells, the conjugates interaction with lipid bilayers was investigated using far UV circular dichroism (CD) spectroscopy and tryptophan fluorescence to monitor the process of insertion at low pH (Figure 2). CD was used to determine the secondary structure of pHLIP-P1AP in the presence of POPC LUVs at both normal and low pH. Results in Figure 2A show the typical pH-dependent transition of pHLIP from an unstructured conformation to an α -helical structure when the pH is lowered from pH 7.4 (blue line) to 4.0 (red line). Tryptophan fluorescence was employed to determine the insertion of pHLIP into the vesicle bilayers based on the sensitivity of Trp fluorescence emission to the polarity of the environment.

At pH 7.4, the Trp fluorescence emission maximum of pHLIP is centered at 340 nm (Figure 2B, blue line), reflecting the relative exposure of Trp residues to polar, aqueous environments. Lowering the pH to 4.0 results not only in λ_{max} blue shift (albeit a small one, ~ 3 nm), but also in a large increase in fluorescence emission intensity. These observations are characteristic for Trp residues buried in more hydrophobic environments upon peptide folding, and are consistent with the conformational change observed by CD. All together,

these results demonstrate that the presence of P1AP at the C-term of pHLIP, does not disrupt significantly the pH-mediated shape-shifting behavior of pHLIP.

The anti-proliferative effect of pHLIP-P1AP correlates with the level of PAR1 cell surface expression

The efficacy of pHLIP-P1AP to down-regulate PAR1 signaling and inhibit cell proliferation in a concentration- and pH-dependent manner was investigated across a panel of cell lines with varying expression levels of PAR1. Relative PAR1 membrane surface expression levels were determined by flow cytometry in several cell lines: HeLa, MCF7, MCF7-PAR1/N55 and MDA-MB-231. Little PAR1 cell surface expression was observed in the PAR1 'null' MCF7 and HeLa cell lines (Figure 3). The MCF7 breast cancer cell line that was transfected to stably express PAR1, MCF7-PAR1/N55 (MCF7/PAR1+; kind gift of Lidija Covic, Tufts University) [11,13,16], exhibited a 2-fold increase in PAR1 expression compared to MCF7; while MDA-MB-231, a breast cancer cell line that constitutively expresses high levels of PAR1 [11,16,17], showed the highest level of PAR1 surface expression (4-fold increase compared to MCF7). We reasoned that the cell lines with the highest relative level of PAR1 membrane surface expression levels would be those that respond the most to pHLIP-P1AP treatments. Importantly, the invasive MDA-MB-231 cells are classified as triple negative breast cancer (TNBC) cells due to their lack of estrogen receptor, progesterone receptor and HER2 expression, all of which are crucial cellular receptors for targeted cancer therapeutics [28–30]. Thus, in contrast to the other treatment groups, there are no effective targeted therapies against TNBC [31,32].

Cell treatments were conducted from concentrations ranging from 1.25 to 10 μ M at either pH 7.4 or 5.0. After a 2 h incubation at 37 °C, the treatment media was replaced by fresh media, and cells were grown for an additional 72 h at normal pH before assessing cell viability using the MTT assay. This treatment protocol is quite stringent when compared to the typical multi-days exposure treatments used with pepducins [11]. As hypothesized, treatments with pHLIP-P1AP at either normal or low pHs had no significant effect on cell viability of HeLa and MCF7 cells (Figure 4A–B), consistent with their low level of PAR1 membrane expression. On the other hand, when MDA-MB-231 cells were treated with pHLIP-P1AP, cell proliferation was drastically disrupted. Up to 77% growth inhibition is observed (Figure 4C). The antiproliferative effect is concentration-dependent, with cell viability ranging from 65 to 23%, with increasing treatment concentration ranging from 1.25 to 10 μ M, respectively (Figure 4C, grey bars). Noteworthy, similar growth inhibition is seen when MDA-MB-231 cells are treated with the pepducin P1pal-7, albeit for an extended 72 hour treatment and at pH 7.4 (Figure S2). As expected, inhibition of cell growth is also pH-selective: Treatment with pHLIP-P1AP at pH 7.4 under the same conditions had no significant effect on cell proliferation (Figure 5D, black bars). This is consistent with the notion that P1AP delivery is mediated by the pH-dependent insertion of pHLIP across the plasma membrane. When MCF7/PAR1+ cells were treated with pHLIP-P1AP a concentration and pH-dependent disruption of cell proliferation (51% cell death) was also observed (Figure 4D). However, MCF7/PAR1+ cells seem to be less sensitive to pHLIP-P1AP treatment than MDA-MB-231 cells, as shown by a higher cell viability at all treatment concentrations (Figure 4D). These results are consistent with the lower PAR1 surface

expression in MCF7/PAR1+ cells. Notably, the antiproliferative effect of pHLIP-P1AP is directly correlated to the level of PAR1 expression at the cell surface (Figure 5).

The relatively high cytotoxicity observed at higher concentrations is not due to any intrinsic peptide cytotoxicity or to pHLIP insertion itself, as neither pHLIP nor P1AP has any harmful effects on the cell viability of any cell line at 10 μ M (Figure S3). This is consistent with previous observation that pHLIP is minimally toxic [25], and that P1AP is unlikely to cross the plasma membrane on its own based on its physical properties (MW = 924 g.mol⁻¹ and octanol/water partition coefficient [Log Po/w] = -2.93; calculated with QikProp 3.0) [33]. Importantly, our results also showed that the inhibitory effect of P1AP appears to be sequence specific, as a scrambled version of P1AP (AFKCLKSR) does not affect significantly cell growth when conjugated to either pHLIP (Figure 6) or a palmitoyl (Figure S2). It is also important to note that there are no detrimental contribution of the low pH treatment in itself on proliferation of any of the cell lines studied, as shown by treatment without pHLIP-P1AP (Figure 4, 0 μ M treatments).

To ensure that pHLIP-P1AP was not causing cell death through disruption of the plasma membrane, we assessed the percentage of intact membrane after treatments based on the uptake of trypan blue. Membrane disruption was not observed in any of the cell lines after treatment with neither pHLIP-P1AP nor low pH environments (Figure S4). All together, these results suggest that the antiproliferative effect of pHLIP-P1AP is due to the pH-selective translocation of P1AP across the plasma membrane and to the inhibition of PAR1 activity.

pHLIP-P1AP Down-Regulates PAR1-Mediated Calcium Mobilization

To determine whether pHLIP-P1AP inhibits PAR1 activity, we conducted functional activity assays of PAR1 by monitoring changes in intracellular calcium fluxes in response to serine protease activation. Upon activation by thrombin, PAR1 stimulates phospholipase C β via G α q/11 and/or β γ (Gi), which causes production of inositol triphosphate and mobilization of Ca²⁺ from intracellular stores [17]. This mobilization can be monitored by using the calcium sensitive indicator fura-2 (Life Technologies) that fluoresces when bound to calcium. P1AP was previously shown to inhibit Ca²⁺ by specifically blocking PAR1 activity and not another downstream component in the Ca²⁺ flux pathway [6].

As shown in Figures 7, MDA-MB-231 cells showed a sharp increase in fluorescence signal that quickly falls back to baseline levels when cells were treated with thrombin after treatments at both normal (Figure 7A) and low pH (Figure 7B), indicating robust intracellular Ca²⁺ fluxes and that PAR1 is functionally active. Conversely, cells not exposed to thrombin do not exhibit any increase in fluorescence. Treating cells with pHLIP-P1AP at normal pH before thrombin activation elicited a sharp increase in intracellular Ca²⁺ similar to that of the one observed with thrombin alone (Figure 7C). However, as hypothesized, the Ca²⁺ response was greatly reduced (58%) when cells were treated with pHLIP-P1AP at lower pH, indicating that PAR1 activity is down-regulated even after activation with thrombin. It is important to note that a shorter pHLIP-P1AP treatment time was implemented to avoid unwanted effects on intracellular Ca²⁺ due to a prolonged exposure to

an acidic environment. We thus believe that the inhibition of PAR1 is more robust during the 2-hour treatment used in the anti-proliferative assay.

CONCLUSION

In summary, we have demonstrated that pHLIP provides localized delivery and membrane anchorage of a polar, conformationally flexible allosteric peptide inhibitor of a GPCR. In combination, the pHLIP-P1AP conjugate inhibits cancer cell proliferation in a concentration- and pH-dependent manner by specifically down-regulating PAR1 activity, allowing for a more selective targeting and specific intracellular delivery than current approaches. To our knowledge, it is the first study to employ pHLIP to deliver a membrane receptor modulating peptide. While this work focused on PAR1, we believe that this approach can be applied to a broad range of GPCRs. With over 800 members in the human genome, which includes more than a hundred orphan receptors, there is an abundance of opportunities for directly targeting GPCRs expressed on tumor cells. We thus envision that our strategy will enable the discovery and development of a potentially important class of therapeutic peptides in oncology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CD	Circular dichroism
MALDI-TOF	Matrix-assisted laser desorption/ionization Time of Flight
MS	Mass spectroscopy
pHLIP	pH (Low) Insertion Peptide
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RP-HPLC	Reverse-phase high performance chromatography
Trp	Tryptophan

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Figure 1. Schematic representation of the hypothesized antagonistic effect of the pHLIP-PAR1 Inhibitory Peptide conjugate (pHLIP-P1AP) on the activity of the Protease-Activated Receptor 1 (PAR1). The peptide sequences of pHLIP and P1AP are shown.

Figure 2. Interaction of pHLIP-P1AP with lipid bilayers
(A–B) pHLIP-P1AP circular dichroism spectroscopy and Trp Fluorescence, respectively. Spectra were measured in presence of POPC liposomes at pH 7.5 (blue line) and at pH 4.0 (red line). [peptide] = 7 μ M and peptide-lipid ratio =1:300 with POPC liposomes.

cell line	HeLa	MCF7	MCF7/PAR1+	MDA-MB-231
secondary alone	26959 ± 207	21759 ± 1843	13496 ± 817	16633 ± 220
primary + secondary	37747 ± 1190	28484 ± 1927	36981 ± 2191	65767 ± 456
relative expression	1.4 ± 0.045	1.31 ± 0.093	2.7 ± 0.16	3.9 ± 0.029

Figure 3. PAR1 Expression Levels

(A) HeLa, (B) MCF7, (C) MCF7/PAR1⁺ and (D) MDA-MB-231 cells were analyzed for PAR1 surface expression levels employing mouse anti-PAR1 and goat anti-mouse IgG-FITC antibodies. After incubation with antibodies cells were fixed and analyzed using BD FACS Canto II flow cytometer. Cells treated with secondary antibody alone are shown in grey, and primary plus secondary antibodies are shown in black. Results are shown in the table as mean ± SEM (n=3).

Figure 4. Inhibition of cancer cell growth

pHLIP-P1AP treated in (A) HeLa, (B) MCF7, (C) MDA-MB-231 and (D) MCF7/PAR1⁺ cells. Black columns represent cells treated at pH 7.4 and cells treated at low pH are shown in grey. For each cell line, 3,000 cells/well (96-well plate) were seeded, allowed to adhere overnight, treated for 2 hours, washed once with media and grown for 72 hour in complete medium at physiologic pH. Cell viability was assessed with the MTT assay. All measurements were normalized to the media control (0 μM, pH 7.4), as 100% cell viability. Results are shown as mean ± SEM (n=9–12). To assess statistical significance two-tailed Student's t test analyses at 95% confidence level were performed for the comparison of pH 7.4 and pH 5.0 treatments. For Figures (A) and (B), none of the differences between treatment at pH 7.4 and pH 5.0 were statistically different ($p > 0.05$).

Figure 5. PAR1 Expression correlates to inhibition of cell growth

Viability of cells after treatment of 10 μ M pHLIP-P1AP correlates to relative PAR1 expression. Cell viability (n=9–12) was determined as shown in Figure 3, and PAR1 expression (n=3) was analyzed by flow as shown in Figure 2. Results are shown as mean $\pm$ SEM ($R^2 = 0.9985$).

Figure 6. pHLIP-Scrambled P1AP is not cytotoxic

All cell lines were seeded at 3,000 cells/well (96 well plate) and allowed to adhere overnight. Cell media was removed and replaced with 10 μ M of pHLIP-Scrambled P1AP at pH 7.4 (black bars) or pH 5.0 (grey bars) for 2 hours, washed once with media and grown for 72 hours in complete medium at physiologic pH. Cell viability was assessed with the MTT assay, and all measurements were normalized to the media control (0 μ M, pH 7.4), as 100% cell viability. Results are shown as mean $\pm$ SEM (n=9–12). None of the differences between treatment at pH 7.4 and pH 5.0 were statistically different ($p > 0.05$) using the Student's t test.

Figure 7. PAR1-Mediated Calcium Mobilization

MDA-MB-231 cells were seeded at a density of 40,000 cells/well and allowed to adhere overnight. Cells were treated at (A) pH 7.4, (B) pH 5.0 or (C) with pHLIP-P1AP at both pH. After 10 min incubation at 37°C, the treatment media was removed, and the calcium indicator fura-2, NM was added. Cells were incubated for 30 min at 37 °C and then for 30 min at room temperature. 10 nM thrombin was then added (A, B; black lines and C; black

and dashed lines) or not (A, B; dotted lines), and fluorescence emission intensity at 530 nm (excitation at 485 nm) was immediately measured.

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