Teleocidin A2 inhibits human proteinase-activated receptor 2 signaling in tumor cells

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Abstract

Enhanced expression of the proteinase-activated receptor 2 (PAR2) is linked to cell proliferation and migration in many cancer cell types. The role of PAR2 in cancer progression strongly illustrates the need for PAR2-inhibiting compounds. However, to date, potent and selective PAR2 antagonists have not been reported. The natural product teleocidin A2 was characterized against PAR2-activating peptide SLIGKV-NH2, and trypsin-induced PAR2-dependent intracellular Ca2+ mobilization in tumor and in primary endothelial or epithelial cells. Further biochemical and cell-based studies were conducted to evaluate teleocidin specificity. The antagonizing effect of teleocidin A2 was confirmed in PAR2-dependent cell migration and rearrangement of actin cytoskeleton of human breast adenocarcinoma cell line (MDA-MB 231) breast cancer cells. Teleocidin A2 antagonizes PAR2-dependent intracellular Ca2+ mobilization induced by either SLIGKV-NH2 or trypsin with IC50 values from 15 to 25 nmol/L in MDA-MB 231, lung carcinoma cell line, and human umbilical vein endothelial cell. Half maximal inhibition of either PAR1 or P2Y receptor-dependent Ca2+ release is only achieved with 10- to 20-fold higher concentrations of teleocidin A2. In low nanomolar concentrations, teleocidin A2 reverses both SLIGKV-NH2 and trypsin-mediated PAR2-dependent migration of MDA-MB 231 cells, and has no effect itself on cell migration and no effect on cell viability. Teleocidin A2 further controls PAR2-induced actin cytoskeleton rearrangement of MDA-MB 231 cells. Thus, for the first time, the small molecule natural product teleocidin A2 exhibiting PAR2 antagonism in the low nanomolar range with potent antimigratory activity is described.

Abbreviations

A549, lung carcinoma cell line; ANOVA, analysis of variance; ATP, adenosine 5′ triphosphate; DMEM, Dulbecco’s modified Eagle’s medium; ERK1/2, signal-regulated kinase 1/2; FCS, fetal calf serum; GPCR, G protein-coupled receptor; HMEC, human mammary epithelial cells; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; MDA-MB 231, human breast adenocarcinoma cell line; mPASMC, murine pulmonary artery smooth muscle cells; NSCLC, non-small cell lung cancer; PAR1, proteinase-activated receptor 1; PAR2, proteinase-activated receptor 2; PASMC, pulmonary artery smooth muscle cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PyMt, polyoma middle T; Rac1, ras-related C3 botulinum toxin substrate 1; RhO, ras homolog family member A; ROCK1/2, rho-associated, coiled-coil containing protein kinase 1/2; TF, tissue factor; WT, wild type.
Introduction

Proteinase-activated receptors (PARs) are the members of the seven transmembrane G protein-coupled receptor (GPCR) superfamily. To date, four PARs (PAR 1–4; cloned and named in order of their discovery) have been described (Vu et al. 1991; Nystedt et al. 1994; Ishihara et al. 1997; Xu et al. 1998). PARs share a unique mechanism of receptor activation by proteolytic cleavage of the extracellular N-terminal domain by trypsin serine proteases. In contrast to thrombin-regulated PAR1, 3, and 4, proteinase-activated receptor 2 (PAR2) can be specifically activated by trypsin-like proteases, including trypsin, trypstatin, and coagulation proteases upstream of thrombin, complexes of tissue factor (TF) with FVIIa or FXa, respectively (Nystedt et al. 1994; Rothmeier and Ruf 2012). Short synthetic PAR2 peptide sequences representing the first six amino acids of the cleaved N-terminus can directly activate the receptor (Scarborough et al. 1992).

Upon activation, PAR2 couples to heterotrimeric G proteins Gq/11, G16, and G12/13 and additionally recruits β-arrestins subsequently initiating receptor internalization and degradation. Moreover, β-arrestins have been reported to induce G protein-independent signaling and regulation of cell migration (DeFea et al. 2000; Zoudilova et al. 2010). Despite the described role of PAR2 in the migration of various cell types (Rothmeier and Ruf 2012), the respective underlying signaling cascade still remains elusive, presumably based on cell-type specificity. For instance, Zhu et al. (2011) demonstrated a PAR2-ras homolog family member A (RhoA)-dependent migration of endothelial cells, whereas in colon cancer a pathway encompassing c-jun/AP-1 activation and upstream protein kinase C (PKC)ζ and ERK1/2 activation contributed to cell migration (Hu et al. 2013). In general, small Rho GTPases are important regulators of a dynamic actin cytoskeleton in migratory cells (Rafitopoulos and Hall 2004). In line, rhoA activation was demonstrated downstream to PAR2 inducing the formation of stress fibers and focal adhesions (Greenberg et al. 2003; Siritai et al. 2013; Suen et al. 2014). Furthermore, Su et al. (2009) described PAR2-induced activation of a c-src – ras-related C3 botulinum toxin substrate 1 (Rac1) – JNK 1/2 signaling axis leading to paxillin phosphorylation finally resulting in increased cell motility of breast cancer cells.

Beside a role in regulating various physiological functions ranging from vasoregulation to inflammation and nociception (reviewed in Rothmeier and Ruf 2012; Gieseler et al. 2013; Bao et al. 2014), there is growing evidence for a function of PAR2 in cancer progression (Elste and Petersen 2010). In this context, various in vitro studies including breast (Hjortoe et al. 2004; Su et al. 2009), colon (Hu et al. 2013), pancreatic (Shi et al. 2013; Xie et al. 2015), lung adenocarcinoma (Huang et al. 2013), and hepatocellular carcinoma (Nakanuma et al. 2010; Kaufmann et al. 2011) cells revealed a role of PAR2 signaling in cancer cell proliferation as well as in migration and invasion, presumably linked to increased metastatic potential. In an in vivo study of spontaneous development of mammary adenocarcinoma in polyoma middle T (PyMt) mice, PAR2, in contrast to PAR1, promoted the transition to invasive carcinoma (Versteeg et al. 2008). Furthermore, from a clinical perspective, elevated PAR2 expression in isolated tumor tissue could be associated with increased malignancy grades in breast and high-grade astrocytoma tumors, lung carcinoma, and gastric and esophageal cancer (D’Andrea et al. 2001; Rydén et al. 2010; Svensson et al. 2011; Li et al. 2014). Moreover, in patients suffering from breast cancer, elevated levels of PAR2 were linked to a bad prognosis (Rydén et al. 2010).

Lessons from studies using receptor-activating peptides and genetically receptor-deficient mice have predominantly contributed to elucidation of functions of PARs. However, the distinct role of PAR2 in multiple pathophysiological contexts illustrates the need for PAR2 targeting and inhibiting compounds to further investigate and understand PAR2 signaling. Primarily, the described tumor-promoting effects of PAR2 provide the basis for the development of a receptor antagonist as a novel therapeutic strategy in cancer treatment. To date, all PAR2 antagonists described are limited in potency, namely ENMD-1068 (IC₅₀ 2.5–5 mmol/L; Kelso et al. 2006), K14585 (IC₅₀ 5–10 μmol/L; Goh et al. 2009; Kanke et al. 2009), GB88 (IC₅₀ 2 μmol/L; Suen et al. 2012), and C391 (IC₅₀ 1.3 μmol/L; Boitano et al. 2015).

Here, we present for the first time the natural product teleocidin A2 (Fig. 1) as a potent inhibitor of PAR2 signaling. In previous decades, teleocidins were mainly categorized as tumor promoters via a PKC-activating mechanism, however, in concentrations ranging from 100 nmol/L up to 10 μmol/L (Fujiki et al. 1984; Arcoleo and Weinstein 1985; Imamoto et al. 1993). In this study, teleocidin A2 specifically inhibits PAR2-induced Ca²⁺ release in the investigated tumor cell lines human breast adenocarcinoma cell line (MDA-MB 231), lung carcinoma cell line (A549), as well as in human umbilical vein endothelial cells (HUVEC) at low nanomolar concentrations (15–25 nmol/L). Teleocidin A2 suppresses PAR2-induced Ca²⁺ mobilization rather than inhibiting Ca²⁺ mobilization initiated by other endogenously expressed Gq-coupled GPCRs. Finally, our studies reveal teleocidin A2 to be able to inhibit PAR2-induced, Rac1-dependent cell migration by controlling PAR2-initiated actin cytoskeletal changes.
Materials and Methods

Cell culture

To explore PAR2 signaling and to study the effect of teleocidin A2 on endogenous receptors, human breast adenocarcinoma MDA-MB 231, lung carcinoma A549, HUVEC, noncancerous human mammary epithelial cells (HMEC), and murine pulmonary artery smooth muscle cells isolated from pulmonary arteries of wild-type and PAR2-deficient mice (mPASMC WT, mPASMC PAR2<sup>−/−</sup>) (ten Freyhaus et al. 2015) were used. MDA-MB 231 cells were obtained from European Collection of Cell cultures (Salisbury, U.K.) and grown in Leibovitz’s L15 containing 15% fetal calf serum (FCS) and 2 mmol/L glutamine. A549 cells were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) along with 10% FCS. Murine PASMC WT and PAR2<sup>−/−</sup> were a kind gift from Prof. Dr. Rosenkranz (University Hospital Cologne). They were cultured in DMEM, 20% FCS. Media was supplemented with 100 units mL<sup>−1</sup> penicillin and 100 μg mL<sup>−1</sup> streptomycin. HUVECs and HMECs were cultured as indicated by Lonza (Basel, Switzerland). Cells were incubated in humidified atmosphere at 37°C containing 5% CO<sub>2</sub> or in case of MDA-MB 231 in the absence of CO<sub>2</sub>. For cell dissociation during passaging, trypsin-free cell dissociation buffer was applied.

Intracellular calcium mobilization

Kinetic measurements of intracellular calcium mobilization were performed as previously described by Suen et al. (2012) using Fluo-8 (4 μmol/L) as indicator dye. To examine inhibitory effects of PAR2 compounds, cells were incubated for 10 min with different compound concentrations before agonist stimulation. Stimulation with the agonists SLIGKV-NH<sub>2</sub>, SLIGRL-NH<sub>2</sub>, trypsin, TFLLR-NH<sub>2</sub>, thrombin, and adenosine 5′ triphosphate (ATP) was performed with the injector unit from Tecan Infinite M1000 Pro microplate reader Tecan (Maennedorf, Switzerland) after 16 sec of baseline measurement. Fluorescence was measured using excitation at λ = 490 nm and emission at λ = 525 nm. The maximum fluorescence signal was generated with the calcium ionophore A23187; H<sub>2</sub>O served as a control for background fluorescence.

Cell migration assay

PAR2-mediated cell migration was measured using Oris<sup>TM</sup> Cell Migration Assay (Platypus Technologies, Madison, USA) according to Gough et al. (2011). MDA-MB 231 cells were grown to 80% confluency and seeded overnight at 1.5 × 10<sup>5</sup> cells per well. Compounds were added as indicated in the results and the cells incubated for 48 h to allow migration. Cells were fluorescently labeled with Track It<sup>TM</sup> Green purchased from AAT Bioquest (Sunnyvale, CA). Images of single wells were taken using fluorescent microscope Zeiss AxioVert A1 (Zeiss, Jena, Germany) and AxioCam MRm, and migration into detection zones was evaluated by particle analysis in ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/, 1997–2014).

Cell viability assay

MDA-MB 231 cells were cultivated to 80% confluency and seeded overnight at 1.5 × 10<sup>5</sup> cells per well into white 96-well clear bottom plates. Compounds were added as indicated in results and in parallel to the cell migration assay and the cells incubated 48 h at 37°C to assess a possible cytotoxic or proliferative effect of different compounds tested in the migration assay. Staurosporin, Dimethyl sulfoxide (DMSO), and cell-free medium were applied as controls. The amount of viable cells was quantified using CellTiter-Glo® Luminescent Cell Viability Assay purchased from Promega (Madison, WI).

Immunofluorescent microscopy

MDA-MB 231 cells were grown to 80% confluency and seeded overnight at 7 × 10<sup>5</sup> cells per well into black 96-well clear bottom plates. Cells were stimulated with different compounds at defined time points as indicated in results. Fixed and permeabilized cells were stained with 100 nmol/L Alexa Fluor 546-conjugated phalloidin in Phosphate buffered saline (PBS) for 20 min and cell nuclei were visualized with 3 μmol/L a fluorescent DNA
of generated ATP was quantified using ADP-Glo glycerol and calcium. After 60-min reaction, the amount of generated ATP was measured via luminescence using a Tecan Infinite M1000 Pro microplate reader.

Radioligand binding assay
Teleocidin A2 binding was tested in a PAR2 (agonist radioligand) binding assay which was mandated to CEREP (ref. no. 2424, Celle l’Evescault, France) as described by Kanke et al. (2005). A competition assay was performed testing teleocidin A2 (0.5 μmol/L, 5 μmol/L) versus radiolabeled PAR2 peptide [3H]-2-f-LIGRL-NH2 using human PAR2 from recombinant Hela cells. Binding of the radiolabeled peptide was detected after incubation at room temperature for 7 h by scintillation counting. Results showing an inhibition of radiolabeled PAR2 peptide binding higher than 50% indicate a significant binding of test compounds.

Biochemical PKC activation assay
Measurement of PKC activation by teleocidin A2 was based on biochemical PKC and ADP-Glo™ kinase assay (Promega). PKC was incubated with phorbol 12-myristate 13-acetate (PMA) and teleocidin A2 in the presence of phosphatidylyserine, ATP, and the PKC substrate neurogranin. Incubation was performed in the absence of diacylglycerol and calcium. After 60-min reaction, the amount of generated ATP was quantified using ADP-Glo™ reagent and kinase detection reagent via luminescence using a Tecan Infinite M1000 Pro microplate reader.

Intracellular PKC activation
The PathScan® phospho-MARCKS (Ser152/156) sandwich ELISA (Cell Signaling Technology, Danvers, MA) was used to measure the level of cellular PKC activation in MDA-MB 231 cells according to the manufacturer’s information. Cells were seeded overnight at 1.8 × 10^6 cells in FCS-free Leibovitz’s L15 culture medium to 10 cm dishes (Greiner Bio-One, Kremsmünster, Austria) and treated with 10 μmol/L PMA for control, 25 μmol/L SLIGKV-NH2, 25 nmol/L teleocidin A2, or both. Cell lysates of controls and compound-treated cells were generated using lysis buffer supplemented with phenylmethanesulfonyl fluoride. Lysate protein concentrations were determined with Bradford protein assay. Phosphorylated myristolated alanine-rich protein kinase C substrate (MARCKS) protein was detected colorimetrically by biotinylated phospho-MARCKS antibody, horseradish peroxidase (HRP)-coupled streptavidin, and HRP substrate tetramethylbenzidine using Tecan Infinite M200 Pro microplate reader.

Materials
Cell culture media and reagents were purchased from Life Technologies (Carlsbad, CA) or Lonza (Basel, Switzerland). Fetal calf serum was obtained from Biochrom (Berlin, Germany), and Quest Fluo-8™ AM and probenecid from AAT Bioquest. SLIGKV-NH2 was ordered from Bachem (Bubendorf, Switzerland). SLIGRL-NH2, TFLLR-NH2, trypsin, thrombin, ATP, and A23187, as well as EHop-016, G6983, Y-27632, PMA, and phosphatidylyserine were purchased from Sigma-Aldrich (St. Louis, MO). Cell staining reagents were purchased from Life Technologies. Plates (96 and 384 well) were obtained from Greiner Bio-One. Teleocidin A2, a small molecule originally isolated from Streptomyces species, was a kind gift from IMD Natural Solutions, Dortmund, Germany. PAR1 inhibitor vorapaxar was purchased from Axon Medchem (Groningen, the Netherlands).

Group sizes
All data were obtained from a minimum of nine biological replicates of at least three independent experiments. The exact group size for each experiment and the number of independent experiments is provided in the respective figure legend of each dataset. All data subjected to statistical analysis had equal group sizes and were performed with a minimum of nine biological replicates of at least three independent experiments.

Randomization
All cell-based assay samples were completely randomized to control and treatment.

Normalization
Data obtained for parametric statistical analysis were not normalized so all control group values became 1. For calculation of EC_{50} (agonist–response) and IC_{50} (antagonist–response) values, the logarithmic concentration of the agonist or antagonist was plotted against maximum fluorescence change in % of the experiment internal agonist-induced Ca^{2+} release. In Ca^{2+} mobilization studies, data were either normalized to the value of the experiment internal agonist or to the A23187-induced maximum Ca^{2+} release. Normalization had no effect to the overall result of the experiment.

In phospho-MARCKS (Ser152/156) ELISA, the level of MARCKS phosphorylation in untreated cells was taken as the experiment internal control and set to 1.

Data obtained from cell migration studies were normalized against each internal 48-h basal migration control.
Normalization had no effect on the overall outcome of the experiment.

**Statistical comparison**

Statistical data analysis was performed with equal sample values of a minimum of nine biological values using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test in case groups were compared with a control or followed by Tukey’s post hoc test in case all groups were compared with each other. P < 0.05 was used to announce statistically significant difference.

**Data analysis**

All data were obtained from a minimum of nine biological replicates of three independent experiments and expressed as mean ± SEM. Concentration–response curves were established using nonlinear regression. For calculation of EC_{50} (agonist–response) and IC_{50} (antagonist–response) values, the logarithmic concentration of the agonist or antagonist was plotted against maximum fluorescence change in % of the agonist-induced Ca^{2+} release. Statistical data analysis was performed with equal sample values using one-way ANOVA followed by Dunnett’s post hoc test in case groups were compared with a control or followed by Tukey’s post hoc test in case all groups were compared with each other. P < 0.05 was used to announce statistically significant difference. Curve fitting and data analysis using ANOVA were performed in GraphPad Prism v. 5.04 (GraphPad Software, San Diego, CA).

**Ethics**

There is currently no clinical relevance of the study. No human subjects were involved in this study.

**Results**

**Teleocidin A2 antagonizes efficaciously PAR2-induced Ca^{2+} mobilization**

Intracellular Ca^{2+} mobilization induced by the PAR2-activating protease trypsin or the receptor-specific tethered ligand SLIGKV-NH_{2} was examined in human cell lines and primary cells. Invasive triple negative breast cancer cell line MDA-MB 231 has been characterized by enhanced PAR2 expression and PAR2-linked increased cell migration (Su et al. 2009). In A549 non–small cell lung cancer (NSCLC) cells, PAR2 has been shown to prevent apoptosis (Huang et al. 2013). Moreover, HUVEC showed PAR2 upregulation and activation under hypoxic conditions resulting in release of proangiogenic factors (Svensson et al. 2011).

EC_{50} values of SLIGKV-NH_{2} (EC_{50} 109 ± 9 μmol/L, 23.2 ± 0.8 μmol/L, and 7.46 ± 0.88 μmol/L for HUVEC, A549, and MDA-MB 231, respectively) or trypsin (EC_{50} 112 ± 15 nmol/L, 43.1 ± 8.3 nmol/L, and 4.49 ± 0.41 nmol/L for HUVEC, A549, and MDA-MB 231, respectively) slightly varied between different cell lines, presumably hinting at distinct receptor expression (Fig. 2A–C; Table 1). A constant potency shift to 500- to 1500-fold higher potency of endogenous PAR2 receptor protease trypsin in comparison to SLIGKV-NH_{2} to trigger Ca^{2+} release was observed. In contrast, in noncancerous mammary epithelial cells (HMEC) no significant calcium mobilization was detected upon stimulation with SLIGKV-NH_{2} up to a concentration of 10 μmol/L or with trypsin up to 10 nmol/L (Fig. 2D). These findings are consistent with data from qPCR analysis revealing significant upregulated PAR2 expression levels in MDA-MB 231 cells in comparison to HMEC (Table S1).

A library of preselected natural products was subjected to a cell-based screening aiming at identifying novel antagonists of PAR2. Interestingly, this screen previously revealed the natural product teleocidin A2 as a putative PAR2 inhibitor. To confirm its capacity of antagonizing PAR2-induced intracellular Ca^{2+} mobilization, teleocidin A2 was evaluated against two structurally different PAR2 agonists (SLIGKV-NH_{2} and trypsin).

Teleocidin A2 itself had no significant effect on Ca^{2+} release in MDA-MB 231 and in healthy epithelial breast cells (HMEC) up to 1 μmol/L, suggesting a lack of intrinsic agonistic properties (Fig. 2C and D).

Importantly, in contrast to all PAR2 inhibitors reported to date, teleocidin A2 blocked PAR2-induced Ca^{2+} signaling with efficacy in the low nanomolar range (IC_{50} 14.0 ± 4.1 nmol/L, 25.8 ± 1.7 nmol/L, 18.1 ± 1.7 nmol/L for HUVEC, A549, and MDA-MB 231, respectively) with SLIGKV-NH_{2} applied at its respective cell-specific EC_{50} values (Fig. 3A–D, Table 1). It is noteworthy that independent of the cell type, teleocidin A2 displayed similar potency by blocking PAR2-dependent Ca^{2+} release. The amount of teleocidin A2-induced Ca^{2+} inhibition remained stable during the investigated time period of maximal 75 min (Fig. 3E). To further explore the mechanism of teleocidin A2 antagonism, varying concentrations of teleocidin A2 were tested against increasing concentrations of the PAR2 agonist peptide SLIGKV-NH_{2}. Increasing concentrations of teleocidin A2 reduced the maximum response of SLIGKV-NH_{2}-induced intracellular Ca^{2+} mobilization. In the presence of teleocidin, full receptor activation by SLIGKV-NH_{2} could not be achieved at the highest concentrations applied of the agonist (up to 333 μmol/L; Fig. 3F).
To start assessing selectivity of teleocidin for PAR2, the ability to inhibit Ca\(^{2+}\) signaling was observed for the endogenously expressed G\(_{q}\)-coupled GPCRs P2Y and PAR1. PAR1-activating peptide TFLLR-NH\(_2\) and thrombin stimulated PAR1-related intracellular Ca\(^{2+}\) release (Fig. 3B and C, Table 1). Teleocidin A2 was tested in the presence of TFLLR-NH\(_2\) at its respective EC\(_{50}\) to determine IC\(_{50}\) values for PAR1 inhibition. Although teleocidin markedly reduced intracellular Ca\(^{2+}\) mobilization initiated by PAR1 in A549 (IC\(_{50}\) 200 ± 38 nmol/L) and MDA-MB 231 cells (IC\(_{50}\) 371 ± 58 nmol/L), it is noteworthy that the compound inhibited PAR1 signaling with a 20-fold potency loss compared to PAR2 (Fig. 3B and C). Furthermore, differences in potency of teleocidin A2-induced blockade of P2Y-initiated Ca\(^{2+}\) mobilization could be confirmed. Teleocidin inhibited ATP-induced, P2Y-dependent Ca\(^{2+}\) mobilization with an IC\(_{50}\) of 179 ± 37 nmol/L (Fig. 3C, Table 1).

In contrast to PAR1-activating peptide TFLLR-NH\(_2\), the comparison of inhibition of trypsin- versus thrombin-induced Ca\(^{2+}\) release by teleocidin revealed inhibitory effects with IC\(_{50}\) values of 54.6 ± 10.4 nmol/L for trypsin- and of 116 ± 30 nmol/L for thrombin-induced Ca\(^{2+}\) signaling, respectively (Fig. 3D, Table 1). Thus, although less pronounced than for the respective activating peptides, differences in potency of teleocidin A2-induced blockade of PAR2-initiated Ca\(^{2+}\) mobilization compared to PAR1-induced Ca\(^{2+}\) release by thrombin could be confirmed. The discrepancy in inhibition of thrombin versus TFLLR-NH\(_2\) by teleocidin is consistent with the previously reported unspecificity of thrombin. The generated tethered ligand of PAR1 by thrombin can bind and
activate PAR2, resulting in intermolecular PAR2 signaling (Ossovskaya and Bunnett 2004). In contrast, vorapaxar, an FDA-approved PAR1 receptor antagonist (Chackalamanil et al. 2008), failed to inhibit trypsin-induced Ca²⁺ release confirming drug specificity for PAR1 and protease specificity for PAR2 (Fig. S3).

**In contrast to teleocidin, PMA inhibits PAR1 and PAR2 comparably**

To further evaluate compound selectivity, teleocidin A2 was examined in a biochemical PKC kinase assay. The alkaloid markedly activated purified PKC isofrom mix up to 1.3-fold compared to DMSO control in the absence of diacylglycerol and calcium. The level of induced PKC activity was comparable to the tumor-promoting agent PMA (applied at 100 μmol/L) serving as a positive control in the presence of respective agonists. In contrast to the described pronounced PAR2 blocking effect for teleocidin, PMA did not demonstrate a discrepancy in efficacy of PAR2 and PAR1 inhibition. SLIGKV-NH₂ itself initiated MARCKS phosphorylation up to threefold, representing a significant PKC activation.

To explore cellular stimulation of PKC, phosphorylation of the specific endogenous PKC substrate MARCKS at its serine residues 152 and 156 was determined (Heemskerk et al. 1993). As expected, the positive control PMA (10 nmol/L) induced a strong phosphorylation up to threefold, representing a significant PKC activation. SLIGKV-NH₂ itself initiated MARCKS phosphorylation up to twofold in comparison to untreated control cells (Fig. 4C). Furthermore, MARCKs was phosphorylated up to 2.7-fold compared to the untreated control in the presence of 25 nmol/L teleocidin A2. In case teleocidin was incubated along with SLIGKV-NH₂, the level of MARCKs phosphorylation was comparable to the level induced by teleocidin alone (Fig. 4C).

As PMA has demonstrated in numerous studies to be a strong PKC activator, the inhibitory potential of PMA on PAR2 and PAR1 agonist peptide-induced intracellular Ca²⁺ mobilization was evaluated, respectively. Importantly, PMA inhibited both SLIGKV-NH₂ and TFFLR-NH₂-mediated Ca²⁺ releases similarly in the low nanomolar range (IC₅₀ 5.89 ± 2.78 and 7.47 ± 3.18 nmol/L) (Fig. 4D). Thus, in contrast to the described pronounced PAR2 blocking effect for teleocidin, PMA did not demonstrate a discrepancy in efficacy of PAR2 and PAR1 inhibition.

**Teleocidin A2 confirms preference for PAR2 blockade in PAR2⁻/⁻ murine PASMC**

To assess Ca²⁺ mobilization in murine pulmonary smooth muscle cells (WT and PAR2⁻/⁻), cells were stimulated with
the PAR2 agonists murine peptide SLIGRL-NH₂ and trypsin or the PAR1 agonists TFLLR-NH₂ and thrombin. A markedly reduced stimulation of intracellular Ca²⁺ release by SLIGRL-NH₂ or trypsin in mPASMC PAR2⁻/⁻ compared to WT was noted. However, in contrast to the tethered PAR1 ligand for thrombin, a significantly reduced signal could be detected in mPASMC PAR2⁻/⁻ (Fig. 5A), which is consistent with the reported findings for thrombin in MDA-MB 231 cells (Fig. 3D). As thrombin significantly differentiated between PAR2⁻/⁻ and WT cells,
subsequent studies to assess the effects of teleocidin A2 in mPASMC were performed using the PAR1 agonist peptide TFLLR-NH₂ with comparably EC₅₀ values in both WT and PAR2⁻/⁻/⁻ cells. Determined IC₅₀ values (IC₅₀ 24.6 ± 4.2 nmol/L and 86.2 ± 214 nmol/L for mPASMC WT and PAR2⁻/⁻/⁻, respectively) confirmed that in the presence of PAR2, teleocidin is significantly more efficacious in inhibiting Ca²⁺ mobilization. Thus, the reported preference of teleocidin A2 for PAR2 blockade in human tumor cell lines (Fig. 5B, Table 1) could be verified in murine PAR2⁻/⁻/⁻ cells.

**Teleocidin A2 does not displace SLIGKV-NH₂ binding to PAR2**

Based on the striking efficacious PAR2 antagonistic effect, it was obvious to further address whether teleocidin A2 acts mechanistically via direct competition with SLIGKV-NH₂ or with the formed N-terminal sequence upon protease cleavage. Thus, binding of teleocidin A2 to human PAR2 in recombinant Hela cells was evaluated in competition with radiolabeled [³H]2-f-LIGRL-NH₂. Inhibition of PAR2-induced intracellular Ca²⁺ mobilization by 0.5 and 5 nmol/L teleocidin A2 was below 4%, and thus considered to be nonsignificant (Table 2). In conclusion, teleocidin A2 does not compete with the binding of radiolabeled [³H]2-f-LIGRL-NH₂ PAR2.

**Teleocidin A2 antagonizes cell migration**

PAR2 has been associated with increased cellular motility in various cancer cell types. Migration of MDA-MB 231 in the presence and absence of teleocidin A2 and various stimuli was examined in a cell migratory exclusion assay.
to explore the effect upon PAR2-stimulated chemokinesis. Trypsin (5 nmol/L) and SLIGKV-NH₂ (25 nmol/L) significantly enhanced the migration of MDA-MB 231 by twofold to basal values after 48 h (Fig. 6A and B). To examine whether teleocidin A2 suppresses PAR2-induced cell migration, cells were preincubated with 25 nmol/L compound and then stimulated with either 25 nmol/L SLIGKV-NH₂ or 5 nmol/L trypsin. Teleocidin A2 reduced SLIGKV-NH₂- and trypsin-stimulated migration to the level of basal values. When incubated alone, the compound had no effect upon basal cellular motility (Fig. 6B). To confirm the effect demonstrated in cell migration to be solely based on increased cellular motility, a cell viability assay was conducted in parallel at the same experimental conditions. No significant change in cell viability occurred upon PAR2 stimulation (with either trypsin or SLIGKV-NH₂) or after inhibition via teleocidin A2, respectively (Fig. 6C). Moreover, the reported data show enhanced cell proliferation upon PAR2 stimulation by neither trypsin or SLIGKV-NH₂ nor teleocidin A2 alone.

**Rac1 controls PAR2-mediated migration**

PAR2-induced signaling cascade leading to cell migration in MDA-MB 231 cells was further examined by applying various inhibitors of PAR2 downstream effector proteins. PKC antagonist Gö6983 (Gschwendt et al. 1996) was incubated along with 25 nmol/L SLIGKV-NH₂. However, PKC inhibition failed to significantly block PAR2-induced cell migration (Fig. 7A). On the other hand, PKC inhibitor Gö6983, applied at 100 nmol/L, distinctly reduced the level of PAR2-induced phosphorylation of the specific endogenous PKC substrate MARCKS in MDA-MB 231 cells (Fig. 7B), suggesting that the PAR2–Ca²⁺–PKC signaling axis is not involved in PAR2-induced migration in the selected cell line.

Rho-associated coiled-coil forming protein kinase (ROCK) family inhibitor Y27632 (Ishizaki et al. 2000) potentiated the SLIGKV-NH₂-induced migration by twofold in comparison to SLIGKV-NH₂ and by fourfold in comparison to the basal migration, whereas the inhibitor alone had no significant effect on MDA-MB 231 basal cell migration (Fig. 7C). Small GTPase Rac1 inhibitor EH0-016 (Montalvo-Ortiz et al. 2012) (2 µmol/L) was incubated along with SLIGKV-NH₂ in the cell migratory exclusion assay (Fig. 7D). EH0-016 reduced SLIGKV-NH₂ (25 µmol/L)-stimulated migration to 100% basal migration, and therefore completely blocked the SLIGKV-NH₂-induced effect. Furthermore, the stimulatory effect

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**Table 2. Radioligand binding assay.**

| Compound   | Test concentration | % Inhibition of control specific binding |
|------------|--------------------|-----------------------------------------|
| Teleocidin A2 | 0.5 µmol/L          | -2.05 ± 9.82                            |
|            | 5 µmol/L            | 3.75 ± 3.18                             |

IC₅₀ value of reference compound SLIGRL-NH₂ for inhibition of control specific binding was 0.12 µmol/L. Inhibition levels of control specific (labeled PAR2 peptide) binding higher than 50% indicate significant binding of test compounds. PAR2, proteinase-activated receptor 2.
of trypsin (5 nmol/L) on MDA-MB 231 cell migration was significantly reduced to 100% basal migration in the presence of EHop-016, confirming the significant role of Rac1 in PAR2-induced cell migration. Ehop-016 alone, however, had no influence on basal tumor cell migration (Fig. 7D).

Influence of PAR2 and teleocidin A2 on actin cytoskeleton rearrangement

The migration of cells is based on a dynamic actin cytoskeleton undergoing constant and directed rearrangement. PAR2 activation has been associated with both increased migratory capacities and rearrangement of actin cytoskeleton. To further determine the role of PAR2 and the influence of teleocidin A2 on actin cytoskeletal rearrangement, MDA-MB 231 cells were treated with PAR2 agonist SLIGKV-NH2 in the presence and absence of teleocidin A2 or inhibitors of putative PAR2 downstream effectors. SLIGKV-NH2 induced reorganization of the actin cytoskeleton and markedly increased the number of stress fibers (Fig. 8). Preincubation of the cells with either 1 μmol/L ROCK1/2 inhibitor Y27632 or 2 μmol/L Rac1 inhibitor EHop-016 followed by stimulation with SLIGKV-NH2 initiated distinct reorganization of

Figure 6. Teleocidin A2 interferes with PAR2-mediated cell migration. (A) Teleocidin A2 reverses SLIGKV-NH2 or trypsin induced migration in a cell exclusion assay. Microscopy images show representative data of migration into detection zone after 48 h from three independent experiments. Cell culture medium was used as negative control. (B) Cell migration into detection zone shown in (A) was analyzed by particle analysis in ImageJ and basal migration after 48 h (control) was set to 100%. (C) Cell viability of MDA-MB 231 is influenced neither by teleocidin A2 nor by SLIGKV-NH2 or trypsin. MDA-MB 231 cells were treated at the same conditions as in the migration assay and the amount of viable cells was detected using CellTiter-Glo® assay reagent. After a treatment period of 48 h, testing conditions had no statistically significant impact on cell viability when compared to DMSO control. Data shown in bar charts are mean values from three independent experiments with n ≥ 3 (n, number of replicates); ns, not statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001. PAR, proteinase-activated receptor; MDA-MB 231, human breast adenocarcinoma cell line.
the actin cytoskeleton. Actin cytoskeletal organization of cells pretreated with Rac1 inhibitor EHop-016 was markedly changed in the majority of observed cells, supporting the prominent role of Rac1 in PAR2-mediated tumor cell migration. The inhibition of ROCK1/2 which is a downstream target of small GTPase RhoA led to a less frequent alteration of cell morphology, but reduced the number of visible stress fibers in comparison to PAR2 AP-treated control cells (Fig. 8).

With respect to the anti-PAR2 migratory effect of teleocidin A2 in the cell migration assay, the compound was examined for its potential to inhibit PAR2-induced actin rearrangement. PAR2-induced stress fiber formation was suppressed by 20 nmol/L teleocidin A2, however, teleocidin A2 treatment led to the development of distinct actin fiber accumulation at the cell membrane and membrane ruffles (Fig. 8). Although this effect was also observed after treatment with teleocidin A2 alone, suggesting a second, presumably PAR2-independent role of the compound in regulating cytoskeleton rearrangement, teleocidin A2 did not alter basal cellular motility of MDA-MB 231.

Discussion and Conclusions

Due to their importance in the control of many pathophysiological processes, GPCRs are the primary targets for over 30% of clinically used drugs (reviewed in Jacoby et al. 2006; Zhao et al. 2014). Most PAR-related drug discovery research to date has focused on PAR1. PAR1 receptor antagonist vorapaxar (SCH 530348, Merck) gained FDA approval in 2014 for antiplatelet therapy and is the first marketed antagonist of the PAR family (Chackalamannil et al. 2008). However, the development of effective PAR2 antagonists remains in the early stages. The distinct role of PAR2 in multiple pathophysiological contexts strongly illustrates the need for PAR2 inhibitors to further investigate PAR2 biology. All PAR2 antagonists described to date, however, are limited in potency and selectivity, combined with a deficiency to completely antagonize PAR2 signaling. For example, the small molecule antagonist ENMD-1068 achieved in vitro potency only in the low millimolar concentration range (Kelso et al. 2006). The peptide mimetic antagonist K14585 failed to inhibit protease-dependent PAR2 signaling (Goh...
et al. 2009; Kanke et al. 2009). The PAR2 antagonist GB88 achieved antagonistic activity at low micromolar concentrations in vitro and demonstrated efficacy in vivo. However, subsequent studies revealed biased agonist or antagonist activity (Barry et al. 2010; Lohman et al. 2012; Suen et al. 2014). The most recently published antagonist C391 was able to inhibit PAR2-dependent Ca$^{2+}$ release and mitogen-activated protein kinase (MAPK) signals (IC$_{50}$ 1.3 μmol/L), but showed partial agonistic activity already at concentrations of 10 μmol/L (Boitano et al. 2015).

In this study, we confirm the natural product teleocidin A2 as a potent inhibitor of PAR2 signaling. For the first time, our data reveal that teleocidin A2 inhibits PAR2-dependent intracellular Ca$^{2+}$ mobilization with remarkable efficacy (IC$_{50}$ values between 15 and 25 nmol/L) in MDA-MB 231 breast cancer, A549 lung carcinoma, and HUVEC. The high potency of teleocidin A2 was observed for both structurally divergent activating PAR2 agonists, the peptide tethered ligand-derived agonist SLIGKV-NH$_2$, as well as the endogenous activating protease trypsin. In contrast to MDA-MB 231 cells, noncancerous primary breast epithelial cells (HMEC) showed only insignificant Ca$^{2+}$ mobilization upon PAR2 stimulation. These data are consistent with previous findings (Su et al. 2009) which demonstrated upregulated PAR2 protein level in cancerous breast tissue in comparison to healthy normal breast tissues, supporting PAR2 as an attractive cancer target. In contrast to the PAR2 agonists described earlier, teleocidin A2 itself, however, did not induce significant agonistic effects on intracellular Ca$^{2+}$ mobilization up to concentrations of 1 μmol/L. Furthermore, the amount of teleocidin A2-induced Ca$^{2+}$ inhibition remained stable for at least 75 min.

To date, teleocidin derivatives are mainly categorized as tumor-promoting agents. Although studies suggest that activation of PKC might play a major role in the mechanism of teleocidin-induced tumor promotion, the decisive PKC-activating concentration of teleocidins is still unclear and mainly based on biochemical data. In radioactive kinase assays, PKC was activated by either 100 nmol/L of an undefined teleocidin (Arcoleo and Weinstein 1985) or by 1–10 μmol/L of teleocidin A2 (Imamoto et al. 1993). Indolactam V, representing a teleocidin scaffold, was shown to activate the PKC substrate MARCKS in a cell-based assay at 200 nmol/L (Meseguer et al. 2000).
Based on the reported significant variations in PKC activating potency, a biochemical PKC kinase assay has been established. Our assay data reveal that teleocidin A2 is able to activate a PKC isoform mixture (primarily \(\alpha, \beta\), and \(\gamma\) and lesser amounts of \(\delta\) and \(\zeta\) isoforms) at high micromolar concentrations, which is consistent with the reported findings from Imamoto et al. (1993). Thus, in comparison to the demonstrated remarkable IC\(_{50}\) values in the PAR2 \(Ca^{2+}\) mobilization assay, a significant potency loss for teleocidin A2-induced PKC activation in the biochemical kinase assay was noted. In contrast, in the established cellular readout monitoring PKC-dependent phosphorylation of MARCKs teleocidin was able to induce significant phosphorylation at a much lower concentration (25 nmol/L teleocidin), results of which are consistent with previously reported findings (Meseguer et al. 2000).

Moreover, in additional studies to evaluate compound specificity, teleocidin A2 inhibited \(Ca^{2+}\) mobilization induced by PAR1 or P2Y with a significant potency loss in comparison to PAR2 inhibition, respectively. In contrast, the PKC activator phorbol ester tumor promoter PMA inhibited both PAR2- and PAR1-mediated \(Ca^{2+}\) releases, similarly in the low nanomolar range. Thus, in contrast to the described pronounced PAR2 blocking effect for teleocidin, PMA did not demonstrate a preference for PAR2 inhibition. Former studies showed PMA to be able to reduce bradykinin-induced \(Ca^{2+}\) release, indicating that PMA might act through activation of PKC (Luo et al. 1995). Consistent with a hypothesis of a general PKC-mediated effect, \(Ca^{2+}\) mobilization of the \(G_{q}\)-coupled GPCRs PAR1 and P2Y are influenced by teleocidin A2. However, the PMA data clearly reveal that a solely PKC-mediated effect cannot explain the remarkable efficacy of teleocidin A2 on PAR2-mediated signaling. The cell-based kinase assay results are consistent with our findings that teleocidin-mediated blockade of PAR2 \(Ca^{2+}\) release was suppressed in the presence of a PKC inhibitor, although PAR2-induced \(Ca^{2+}\) release alone was not influenced by PKC inhibition. Despite the undisputable role of teleocidin as a potent PKC activator, it remains elusive how the ability to activate PKC mechanistically acts on PAR2-induced \(Ca^{2+}\) mobilization (Fig. 9).

Although increasing concentrations of teleocidin A2 reduced the maximum response of SLIGKV-NH\(_2\)-induced intracellular \(Ca^{2+}\) mobilization, in a radioligand binding assay, teleocidin A2 did not displace binding of the labeled PAR2 agonist peptide \([^{3}H]2\)-furoyl-LIGRL-NH\(_2\), presumably hinting at an unknown modulation site of teleocidin A2 to mediate its inhibitory potency. Identification of the modulation site of teleocidin A2 is in the focus of our current research activities.

Importantly, the reported results from murine pulmonary smooth muscle cells (WT and PAR2\(^{-/-}\)) confirmed that in the presence of PAR2, teleocidin is significantly more efficacious in inhibiting \(Ca^{2+}\) mobilization. However, studies in the PAR2\(^{-/-}\) cells further revealed a putative unspecificity for thrombin as a PAR1 agonist, indicating that the generated tethered ligand of PAR1 by thrombin can bind and activate PAR2, resulting in intermolecular signaling as described previously (Ossovskaya and Bunnett 2004). Moreover, a heterodimerization of PAR2 and PAR1 to form a functional signaling unit cannot be ruled out (Jaber et al.

**Figure 9.** Hypothesis of teleocidin A2-regulated blockade of PAR2 signaling pathways. Teleocidin A2 was able to potently inhibit PAR2-induced intracellular \(Ca^{2+}\) mobilization and antagonized PAR2-dependent cellular motility, presumably interfering with cancer cell migration by reorganization of the actin cytoskeleton. PAR2, proteinase-activated receptor 2; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; ROCK1/2, rho-associated, coiled-coil containing protein kinase 1/2.
and would require further experiments including knockout studies.

Our data show that stimulation by SLIGKV-NH₂ and trypsin significantly increased the number of migrated MDA-MB 231 cells, confirming the described role of PAR2 in promoting cell migration. Teleocidin A2 reduced PAR2-induced migration to the basal control level. The compound itself had no effect on cellular motility. In conclusion, additionally to the inhibition of intracellular Gq-coupled Ca²⁺ mobilization, teleocidin A2 is able to inhibit PAR2-induced cell motility at low nanomolar concentrations. Furthermore, teleocidin A2 does not enhance proliferation or migration of breast cancer cells and is not cytotoxic during the investigated incubation time.

As cell migration is based on a dynamic actin cytoskeleton rearrangement, the role of PAR2 and the distinct influence of teleocidin A2 on actin cytoskeletal rearrangement have been explored. The impact of PKC in PAR2 signaling contributing to cell migration is likely cell-type dependent (Ahamed and Ruf 2004; Wu et al. 2013). Our investigations suggest that PAR2 PKC inhibition failed to significantly block PAR2-induced cell migration, again indicating that teleocidin is able to induce cellular signaling independent of PKC. In contrast, the small GTPase Rac1 reveals to be one of the key regulators in promoting dynamic actin reorganization crucial for breast cancer cell migration. Small GTPases are well characterized to act on actin dynamics being important regulators for the balanced assembly and disassembly of actin bundles and focal adhesions (Raffopoulos and Hall 2004). Both RhoA and Rac1 were described to be involved in the PAR2 signaling cascade, either in case of RhoA leading to the formation of stress fibers and focal adhesions (Greenberg et al. 2003; Sriwai et al. 2013) or in case of Rac1 controlling cell migration via a c-src–Rac1–JNK 1/2 signaling pathway (Su et al. 2009). Rac1 inhibitor EHHop-016 inhibited PAR2-induced cell migration which is consistent with the findings of Su et al. (2009). Additionally, Rac1 inhibition blocked the PAR2 induced, uniquely defined actin bundles. Thus, upon Rac1 inhibition cell morphology resembled the diffuse actin staining of control cells.

ROCK is one downstream target activated by RhoA that was shown to be a key player in stress fiber formation and in focal adhesion organization (Ishizaki et al. 2000). Interestingly, inhibition of RhoA downstream target ROCK1/2 enhanced PAR2-induced migration, but not proliferation of MDA-MB 231 cells. RhoA and Rac1 were described to mediate opposing activities in cell migration. Moreover, a strong activation of RhoA can even be inhibitory to cell migration in certain cell types (McHardy et al. 2004; Brew et al. 2009). Our data confirm distinct stress fiber formation upon PAR2 stimulation. Furthermore, aggressive breast cancer cell line MDA-MB 231 has been characterized by constitutive activation and overexpression of RhoA (Pillé et al. 2005). Together, this may lead to significantly increased ROCK1/2 activation in MDA-MB 231 cells accompanied by rigid and less flexible network of actin bundles limiting cell migration, while ROCK1/2 inhibition results in enhanced cell migration.

Finally, the impact of teleocidin A2 on PAR2 characteristic actin rearrangement has been investigated. Teleocidin A2 abolished PAR2-dependent cytoskeletal effects. Thus, we propose that the inhibitory function of teleocidin A2 on PAR2-induced cell migration is dependent on reorganization of actin cytoskeleton. Teleocidin A2 led to prominent lamellipodia formation and actin enrichment at the cell membrane. These findings are consistent with results from Deng et al. (2010) who observed an increase in cell spreading upon stimulation with a teleocidin mixture in fibroblasts proposing an underlying signaling cascade involving PKD and Rac1 activation.

In summary, this study reveals teleocidin A2 as a potent inhibitor of PAR2-induced signaling cascades relevant for tumor progression in cancer cells (Fig. 9). Teleocidin A2 is able to inhibit Ca²⁺ mobilization induced by PAR2-specific agonists. Interestingly, in contrast to PAR1 or P2Y, PAR2-dependent signaling is inhibited by teleocidin A2 with remarkable potency, suggesting a preference of teleocidin for PAR2 blockade. Although we cannot rule out an involvement of PKC in the mechanism of action of teleocidin A2 to date, based on our knowledge from GPCR Ca²⁺ and PMA inhibition data, the blocking effect on PAR2 signaling might not be exclusively dependent on activation of PKC. Furthermore, teleocidin A2 inhibits PAR2-induced cellular motility at low nanomolar concentrations presumably independent from PKC activation. Moreover, teleocidin A2 might control cancer cell migration by modulating PAR2-induced actin cytoskeletal reorganization.

To further explore the divergent biological functions of teleocidin A2 independent from PKC in a cellular context, we intend to validate functional receptor knockout studies. Additionally, screening of a teleocidin library might hint at a structure activity relationship regarding PAR2 inhibition. Thus, a medicinal chemistry approach has been started to design novel derivatives depicting high efficacy in PAR2 antagonism, but no activation of cellular PKC to provide the basis for the development of a novel therapeutic anti-cancer strategy.

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Disclosures

The company IMD Natural Solutions GmbH, Dortmund, Germany, provided teleocidin A2 used in this study.

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**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. PAR2 gene expression analysis in MDA-MB 231 and HMEC cells. Total RNA was isolated from cells and transcribed (1 μg) to cDNA. The cDNA content was determined by qPCR amplification using gene-specific primers. PAR2 expression was normalized to the housekeeping genes GAPDH and HPRT1. The data represent ΔCq mean ± SEM from three independent experiments performed in triplicates with ΔCq = Cq (PAR2) – Cq (housekeeping gene).

**Figure S1.** Cell viability. PAR2-mediated cell migration and its inhibition by EHop-016 as shown in Figure 7 is not mediated by proliferative or toxic effects, respectively. MDA-MB 231 cells were treated at the same conditions as described in the migration assay. Amount of viable cells was detected using CellTiter-Glo® assay reagent. (A) Testing conditions had no statistically significant impact on cell viability compared to DMSO control after a treatment period of 48 h as determined with one-way ANOVA and Dunnett’s post hoc test relative to the respective mean value of 0.1% DMSO. (B) Single inhibitor controls had no statistically significant impact on cell viability as determined with one-way ANOVA and Dunnett’s post hoc test relative to the respective mean value of 0.1% DMSO. All data represent mean value ± SEM of five independent experiments performed in triplicate; ns, not statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure S2.** Stimulation of MDA-MB 231 cells with PAR4 AP AYPGKF-NH2. Kinetics of intracellular Ca2+ release in MDA-MB 231 upon stimulation with 10 μmol/L SLIGKV-NH2, 5 nmol/L trypsin, or 10 μmol/L/30 μmol/L AYPGKF-NH2. Maximum fluorescence was detected by 10 μmol/L of the ionophore A23187. Results represent data from at least three independent experiments performed in triplicates.

**Figure S3.** Inhibition of trypsin- and thrombin-induced Ca2+ release in MDA-MB 231 by vorapaxar. (A, B) Vorapaxar inhibited thrombin-induced PAR1 Ca2+ signaling in a dose-dependent manner, but not trypsin-induced PAR2-dependent Ca2+ signaling. Data shown in bar charts are mean values from three independent experiments with n ≥ 3 (n, number of replicates); ns, not statistically significant; *P < 0.05, **P < 0.01, ***P < 0.001.