Identification and characterization of novel small molecule Protease Activated Receptor 2 (PAR2) agonists

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Running Title

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14 pages of text

2 tables

9 figures

40 references

Abstract: 243 words

Introduction: 708 words

Discussion: 1137 words

Abbreviations

PAR: protease activated receptor

PAR2 AP: protease activated receptor 2 activating peptide

ABSTRACT

We report the first small molecule protease activated receptor 2 (PAR2) agonists, AC-55541 and AC-264613, each representing a distinct chemical series. AC-55541 and AC-264613 each activated PAR2 signaling in cellular proliferation assays, phosphatidyl inositol (PI) hydrolysis assays, and Ca^{2+} mobilization assays with potencies ranging from 200 to 1000 nM for AC-55541, and 30 to 100 nM for AC-264613. In comparison, the PAR2 activating peptide (PAR2 AP) 2-furoyl-LIGRLO-NH$_2$ had similar potency, whereas SLIGRL-NH$_2$ was 30-300 times less potent. Neither AC-55541 nor AC-264613 had activity at any of the other PAR receptor subtypes, nor did they have any significant affinity for over 30 other molecular targets involved in nociception. Visualization of EYFP-tagged PAR2 receptors showed that each compound stimulated internalization of PAR2 receptors. AC-55541 and AC-264613 were well absorbed when intraperitoneally administered to rats, each reaching micromolar peak plasma concentrations. AC-55541 and AC-264613 were each stable to metabolism by liver microsomes and maintained sustained exposure in rats, with elimination half lives of 6.1 and 2.5 hours, respectively. Intra-paw administration of AC-55541 or AC-264613 elicited robust and persistent thermal hyperalgesia and edema. Co-administration of either a tachykinin 1 (NK1) receptor antagonist or a transient receptor potential vanilloid 1 (TRPV1) antagonist completely blocked these effects. Systemic administration of either AC-55541 or AC-264613 produced a similar degree of hyperalgesia as was observed when the compounds were administered locally. These compounds represent novel small molecule PAR2 agonists that will be useful in probing the physiological functions of PAR2 receptors.

Introduction

Protease activated receptors (PARs) are a family of four G-protein coupled receptors (PAR1, PAR2, PAR3, and PAR4) that are self-activated by tethered peptide ligands exposed by proteolytic cleavage of the extracellular amino-terminus. PAR1, PAR3 and PAR4 are activated by thrombin, whereas PAR2 and to a lesser degree PAR4 are activated by trypsin. In addition, PAR2 can be proteolytically activated by a variety of other substances including tryptase, factor Xa/tissue factor/factor VIIa, and the dust mite allergens Der p3 and Der p9 among others. Exposure to soluble synthetic peptides matching or approximating the sequences of their cognate tethered ligands also activates PAR1, PAR2 and PAR4, thus mimicking the effects of activating proteases (see Ramachandran and Hollenberg, 2008 for review).

PAR2 receptors have been implicated in numerous physiological processes necessitating therapeutic intervention, especially pain and inflammation (Vergnolle et al, 1999; 2001; Steinhoff et al, 2000), and syndromes with a strong inflammatory component including colitis (Fiorucci et al, 2001; Nguyen et al., 2003; Hansen et al., 2005, Cenac et al, 2007), gastritis (Kawabata et al, 2005), pancreatitis (Kawabata et al, 2006), asthma and pulmonary disease (Cocks et al, 1999; Chow et al, 2000; Ricciardolo et al, 2000; Cicala et al, 2001; Schmidlin et al., 2002; Moffatt et al, 2002; Kawabata et al, 2004; Ebeling et al, 2005; Su et al, 2005; Morello et al, 2005; De Campo and Henry, 2005), and arthritis (Ferrell et al, 2003; Kelso et al, 2006). PAR2 receptors are widely distributed, with expression throughout the central nervous system, gastrointestinal system, pulmonary system, liver, kidney, exocrine glands (pancreas, salivary glands, tear ducts), heart, vascular tissue, reproductive organs, skin and inflammatory cells including eosinophils, mast cells, macrophages, and neutrophils (D'Andrea et al, 1998; also see Ramachandran and Hollenberg, 2008; and Henry, 2006; for reviews). PAR2 receptors are found on primary spinal afferent neurons co-expressed with substance P, calcitonin gene-related peptide (CGRP), and transient receptor potential vanilloid receptor 1 (TRPV1) (Steinhoff et al, 2000; Amadesi et al, 2004; Dai et al, 2004). Convincing evidence has been reported showing that PAR2 receptors mediate many of their actions through a neurogenic mechanism (Steinhoff et al, 2000; Ricciardolo et al, 2000; Vergnolle et al, 2001; Fiorucci et al, 2001; Nguyen et al., 2003; Amadesi et al, 2004; Su

et al, 2005; Kawabata et al, 2005; Cenac et al, 2007). Given these physiological roles for PAR2 receptors, PAR2 agonists would be expected to exacerbate most nociceptive and inflammatory processes, however PAR2 also exerts a protective effect in many settings (Cocks et al, 1999; Fiorucci et al, 2001; Cicala et al, 2001; Morello et al, 2005; Kawabata et al, 2004; 2006; De Campo and Henry, 2005; D'Agostino et al, 2007), especially in models of asthma and pulmonary inflammation, and thus there may be therapeutic roles for PAR2 agonists (Henry, 2006; De Campo and Henry, 2006). The therapeutic potential for PAR2 antagonists, which relieve symptoms in models of rheumatoid arthritis, appears clearer (Kelso et al, 2006; Cenac et al., 2007).

Little progress has been made in identifying non-peptidic, non-proteolytic agonists or antagonists of PAR2 receptors. Ideally, such compounds would be metabolically stable, small organic compounds. Mainly peptides, and peptide-mimetics have been identified as PAR2 ligands, including the PAR2 APs SLIGKV, SLIGRL, and their amidated derivatives, and trans-cinnamoyl-LIGRLO-NH$_2$ (Ramachandran and Hollenberg, 2008). In general, the low potency and susceptibility to proteolytic degradation of these compounds are major limitations, particularly for *in vivo* use. Recently peptide agonists of PAR2 such as 2-furoyl-LIGRLO-NH$_2$ with significantly enhanced potency and stability have been developed (Ferrell et al, 2003; Kawabata et al, 2004b; McGuire et al, 2004),. PAR2 peptide antagonists have been reported, however their mechanism of action is unclear as they do not block the actions of PAR2 APs, and they do not inactivate trypsin (Al-Ani et al, 2002). Recently a low potency small molecule antagonist for PAR2 has been described (Kelso et al, 2006).

Utilizing high-throughput functional screening, we have identified the first non-peptide agonists at the PAR2 receptor. We have characterized these compounds in a variety of *in vitro* and *in vivo* functional assays. These compounds are highly selective for PAR2 over the other PAR subtypes, are metabolically stable, and have persistent activity *in vivo*. These compounds will be useful in further exploring the physiological roles of PAR2 receptors.

Methods

Ligands – SLIGRL-NH$_2$, SFLLRN-NH$_2$, AYPGKF-NH$_2$ and 2-furoyl-LIGRLO-NH$_2$ were obtained from Peptides International (Louisville, KY). AC-55541 (N-[[1-(3-Bromo-phenyl)-eth-(E)-ylidene-hydrazinocarbonyl]-(4-oxo-3,4-dihydro-phthalazin-1-yl)-methyl]-benzamide) and AC-264613 (2-Oxo-4-phenyl-pyrrolidine-3-carboxylic acid [1-(3-bromo-phenyl)-(E/Z)-ethylidene]-hydrazide) were synthesized at ACADIA (see Seitzberg et al., in press). Compound structure was verified by NMR. Purity was greater than 99% measured by HPLC and gas chromatography.

Cell culture – NIH-3T3 cells (ATCC (Manassas, VA) CRL 1658) were incubated at 37 °C in a humidified atmosphere (5% CO2) in Dulbecco's modified Eagles medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U per ml penicillin G, 50 U per ml streptomycin (Invitrogen-Gibco, Carlsbad, CA) and 10% calf serum (Sigma, St Louis, MO) or 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD). HEK293T (ATCC (Manassas, VA) CRL-11268) cells were cultured similarly except 10% fetal calf serum was substituted for 10% calf serum.

Constructs. The human PAR1, PAR2, PAR3 and PAR4 receptors used in this study were cloned by polymerase chain reaction using oligonucleotides derived from the GenBank accession entries M62424, U34038, U92971, and AF055917, respectively, and subcloned into pSI (Promega Corp.) expression vectors. PCRs were performed using Pfu Turbo (Stratagene, La Jolla, CA). All mutations described were generated using the Quickchange mutagenesis protocol (Stratagene). PAR2-EYFP and PAR2-S37P-EYFP constructs in pSI expression vectors were produced by ligating PCR products of PAR2 or PAR2-S37P with the stop codons removed in frame with EYFP fused to the carboxy-terminus. All clones were sequence verified.

Functional assays - Receptor Selection and Amplification Technology (R-SAT^{TM}) assays were performed as described (Burstein et al, 2006) with the following modifications. Briefly: Cells were plated one day before transfection using 7 x 10^3 cells in 0.1 ml of media per well of a 96-well plate (Falcon). Cells were transiently transfected with 10 ng of receptor DNA and 30 ng pSI-β-galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (Qiagen, Valencia, CA) according to the

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manufacturer's instructions. One day after transfection medium was changed and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD) instead of calf serum to a final volume of 200 μl per well. After five days in culture β-galactosidase levels were measured essentially as described (Burstein et al, 2006). Cells were rinsed with phosphate buffered saline (PBS), pH = 7.4 before the addition of 200 μl PBS supplemented with 3.5 mM O-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, St Louis, MO). After incubation (2-4 h) the plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices).

Phosphatidyl inositol (PI) hydrolysis assays – Phosphatidyl inositol hydrolysis (PI) assays were performed as follows: For PAR2 WT, human embryonic kidney (HEK) 293T cells were seeded at 10,000 cells/well in DMEM (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in a 37°C humidified atmosphere containing 5% $CO2$. Eighteen hours later, the cells were transfected as described above with the indicated plasmid DNAs (30 ng/well of a 96-well plate). Approximately 20 to 24 h after transfection, the cells were washed and labeled overnight with DMEM culture medium containing 0.2 μCi [myo-2-3H]inositol (NET1114, 37 MBq/ml; PerkinElmer Life and Analytical Sciences, Boston, MA) per well (0.1 ml). The cells were washed and incubated with Hanks' balanced salt solution (Invitrogen) supplemented with 1 mM $CaCl2$, 1 mM $MgCl2$, 10 mM LiCl, and 0.2% bovine serum albumin for 45 min. The buffer was removed, and the cells were incubated for another 45 min at 37°C in the same buffer with the concentrations of freshly made ligands indicated in the figure legends. The reaction was stopped by exchange with ice-cold 20 mM formic acid, and the total [3H]inositol phosphate (IP1, IP2, and IP3) formation was determined by ion-exchange chromatography on 1-ml minicolumns loaded with 200 μl of a 50% suspension of AG 1-X8 resin (200-400 mesh, formate form; Bio-Rad, Hercules, CA). The columns were washed with 1 ml of 40 mM ammonium hydroxide, pH 9, after loading the cell extracts and then eluted with 0.4 ml 2 M ammonium formate in 0.1 M formic acid. The eluates (0.1 ml) were loaded on LumaPlate-96 plates (Yttrium silicate scintillator coated; PerkinElmer Life and Analytical Sciences), air-dried overnight, and counted on a Microplate Scintillation and

Luminescence Counter (TopCount NXT; PerkinElmer Life and Analytical Sciences). For PAR2 S37P, PAR1 S42P, and PAR R47A, HEK 293T cells were seeded at 4.2 x 10^6 cells per 10 cm dish as described above. Eighteen hours later, the cells were transfected using FuGene-HD (Roche, Mannheim, Germany) according to the manufacturer's instructions with the indicated plasmid DNAs (10 ug/plate). Approximately 20 to 24 h after transfection, the cells were lifted with PBS/EDTA and resuspended in inositol-free DMEM (Caisson, North Logan, Utah; cat# DME-1013) (100 ul total vol/well) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and 2 µCi/ml Myo-inositol ([myo-2-3H]inositol (NET1114, 37 MBq/ml; PerkinElmer Life and Analytical Sciences, Boston, MA) and seeded into poly-D-Lysine (Sigma, St. Louis, MO; cat# P7280) coated 96 well dishes at 80,000 cells/well in 100 µl. 24-hours later, medium was carefully removed, and the cells rinsed with 2x200 ul/well serum free medium containing 0.3% BSA (wash medium). 50 ul wash medium containing 10 mM LiCl was added per well, and incubated at 37°C for 15 min, followed by addition of another 50 ul wash medium containing 10 mM LiCl, and 2x concentrations of the indicated ligands, and incubation for 1 hour. The assay was terminated by carefully removing the medium, lysing the cells with 50 ul 0.1 M formic acid for 20 min at room temp. 80 ul of diluted SPA beads (Amersham, Piscataway, New Jersey; Cat # RPNQ0013), followed by 30 ul of each cell lysate was added per well of Perkin Elmer Pico plates, the plates sealed, and placed on a shaker for at least 1 hour. Radioactivity was determined using a Topcount (manufacturer, place).

Ca^{2+} mobilization assays - Intracellular changes in calcium concentrations due to activation of PAR2 receptors were detected using the calcium binding bioluminescence protein aequorin which was expressed as part of a tripartite chimeric protein: MT-GFP-AEQ as described (Burstein et al, 2006) except KNRK cells (CRL-1569 from ATCC) transiently transfected with human PAR2 receptors using the Geneporter II transfection kit (Genlantis, San Diego, CA) were used for these studies.

Data analysis – Concentration-response graphs for all functional assays were plotted and EC50 values were determined by nonlinear regression analysis using Prism software (GraphPad version 4.0, San Diego, CA, USA) according to the following equation:

$$Y = Bottom + (Top\text{-}Bottom)/(1+10\text{^}((LogEC50\text{-}X)))$$

where X is the logarithm of concentration. Y is the response; Y starts at Bottom and goes to Top with a sigmoid shape. Allowing the Hill coefficient to vary did not significantly change the fits of the curves and thus the Hill coefficient was constrained to unity.

Immunofluorescence Confocal Microscopy - Transiently-transfected HEK293T cells expressing PAR2-EYFP or PAR2-S37P-EYFP receptors were grown overnight on CC2 chamber slides (Nunc Inc., Napperville, IL). Treatment with 100 μM SLIGRL-NH$_2$, 1 μM 2-furoyl-LIGRLO-NH$_2$, 3 μM 55541 and 1 μM 264613 were carried out at 37 °C for 15, 30 or 60 min. Following treatment, cells were fixed for 10-15 min at room temperature with 3.7% paraformaldehyde in PBS then washed three times with PBS and once with water. Slides were mounted using Fluoromount G. Images were collected on a Delta Vision Optical Sectioning microscope consisting of an Olympus (Tokyo, Japan) IX-70 microscope and a photometrics CH 350 cooled CCD camera. An Olympus oil immersion 60X objective was used to collect the images.

Animals - Male Sprague Dawley rats (Harlan Sprague Dawley, San Diego, CA), 150–200 gm at the time of testing, were maintained in a climate-controlled room on a 12 hr light/dark cycle (lights on at 7:00 A.M.), and food and water were available *ad libitum.* Rats were housed in pairs for at least 2 days prior to use. All of the testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of the ACADIA Pharmaceuticals, Inc.

Paw edema assays – Drugs were administered to rats directly into the paw (i. paw) in a vehicle of 25 ul DMSO using a Hamilton syringe. The controls were given the same volume of vehicle as in test groups. Paw edema or extent of the edema thickness (foot-pad) was measured in conscious rats 4h after injections using a caliper, and normalized to the width of the contralateral paw.

Thermal hyperalgesia assays – The method of Hargreaves et al. (1988) was used to assess paw withdrawal latency to a thermal nociceptive stimulus. Rats were placed in a clear acrylic box on a glass platform and allowed to acclimatize. A constant-intensity radiant-heat source (Model #7371 Plantar Test, Ugo Basile, Varese, Italy) was applied

through the platform to the mid-plantar area of the hind paw. The response to the stimulus was recorded by a photocell detecting withdrawal of the paw and the latency expressed in seconds. To prevent tissue damage, stimulation to the paw was terminated if the rats failed to withdraw after 20 seconds. Drugs were dosed intraperitoneally (i.p.) or directly into the paw (i. paw) as indicated. A significant reduction in paw withdrawal latency from pre-treatment baseline or vehicle-treated control values was interpreted as thermal hperalgeisa.

Pharmacokinetics- AC-55541 and AC-264613 were prepared at 10 mg/mL in DMSO. Three rats per compound were dosed intraperitoneally at 10 mg/kg. Plasma samples were collected at 0, 0.083, 0.176, 0.25, 0.5, 1, 2, 4, 6 and 24 hr, and analyzed by LC/MS/MS. The LC/MS/MS analysis was performed using a 4000 QTRAP (Applied Bioscience, Foster City, CA) hybrid triple quadrupole linear ion-trap mass spectrometer equipped with electrospray ionization (ESI) and operated in multiple reaction monitoring (MRM) mode. AC-055541 and AC-264613 ion pairs were 518.2/ 105 and 401.2/213, respectively. The mass spectrometer was coupled to a HPLC system consisting of two Shimadzu (Columbia, MD) LC-20AD high performance pumps interfaced with a Shimadzu CBM-20A controller and a CTC HTC PAL(Carrboro, NC) autosampler. Separation was performed using a 4.6- x 50-mm Phenomenex Synergi Fusion (Torrance, CA) reversed-phase C18 column equipped with a guard column. LC solvent A was water and B was acetonitrile, each containing 1% formic acid. Data collection and processing were performed using Analyst software v 1.4.2. The pharmacokinetic parameters were calculated with WinNonlin 5.0 (Pharsight, CA).

Results

Using a cellular proliferation assay (RSAT®, see Burstein et al, 2006) we screened the human PAR2 receptor against a diverse chemical library derived from commercial and proprietary sources containing over 250,000 compounds. This compound library had a significant overlap in chemical space with the MDDR library of known drugs, and was heavily weighted to compounds within a molecular weight range suggested by standard drug-likeness rules (see Lipinski et al, 1997). Screening hits were chemically optimized

and two compounds called AC-55541 and AC-264613 (see **Figure 1**), each representing a distinct chemical series (see Seitzberg et al., in press), were characterized in detail. Both compounds obeyed the Lipinski rule-of-five (see Lipinski et al, 1997) with the exception that AC-55541 is slightly over Mol wt. 500 (AC-55541: Mol wt. 518.37, logD 4.65, H-bond donors: 3, H-bond acceptors: 8, AC-264613: Mol wt. 400.27, logD 2.20, H-bond donors: 2, H-bond acceptors: 5). Thus both compounds have drug-like properties.

Concentration-response experiments demonstrated that AC-55541 and AC-264613 each stimulated cellular proliferation in a PAR2-specific manner. To eliminate proteolysis as a contributing factor to receptor activation, the protease insensitive mutant PAR2 S37P was used (**Figure 2A**). The potencies of AC-55541 and AC-264613 in the cellular proliferation assay were approximately 200 nM and 50 nM, respectively, and were virtually the same at wild-type PAR2 receptors (**Table 1**). In comparison, the potencies of SLIGRL-NH$_2$ and 2-furoyl-LIGRLO-NH$_2$ were approximately 10 uM and 25 nM, respectively, comparable to previous reports (Kawabata et al, 2004b; McGuire et al, 2004). Both AC-55541 and AC-264613 displayed essentially full efficacy compared with SLIGRL-NH$_2$ in this assay. Neither the compounds, nor the PAR2 APs displayed activity in cells transfected with reporter alone (**Figure 2A**).

To assess their receptor selectivity, we tested AC-55541 and AC-264613 on cells transfected with the other PAR subtypes. We observed no activity of either compound at cells transfected with the protease insensitive mutants PAR1 S42P or PAR4 R47A (**Figure 2B, 2C**). PAR3 does not respond to soluble peptides derived from its tethered ligand; however it has been shown to interact with, and potentiate the responses to both PAR1 and PAR4 (Nakanishi-Matsui et al, 2000; McLaughlin et al, 2007). We observed that co-transfection of PAR3 receptors strongly potentiated the responses of PAR1 S42P and PAR4 R47A to SFLLRN-NH$_2$ and AYPGKF-NH$_2$, respectively, indicating PAR3 is functionally active in the cellular proliferation assay (**Figure 2B, 2C**). The potency of PAR1 was increased approximately 6-fold when PAR3 was co-expressed (pEC$_{50}$ 6.0$\pm$0.1 for PAR1 plus PAR3 *versus* 5.2$\pm$0.1 for PAR1 alone; p<0.01) while the maximum response of PAR4 was increased 2.3$\pm$0.3 fold (p<0.01) when PAR3 was co-expressed. However we observed no activity of either AC-55541 or AC-264613 at cells transfected with PAR1 S42P plus PAR3, or PAR4 R47A plus PAR3, or PAR3 alone (**Figure 2B,**

2C). Further, neither compound had significant affinity for over thirty other targets implicated in nociception and inflammation including A1, A2A, A2B and A3 adenosine receptors, B1 bradykinin receptors, CB1 and CB2 cannabinoid receptors, cholecystokinin B (CCKB) receptors, CGRP receptors, CXCR4 receptors, EP4 and FP prostanoid receptors, endothelin A (ETA) receptors, fMLP receptors, H1 and H4 histamine receptors, LTB4 and LTD4 leukotriene receptors, QNB-labeled muscarinic receptor binding sites and neuropeptide Y labeled binding sites in rat cerebral cortex, NK1 and NK3 tachykinin receptors, naloxone labeled binding sites in rat cerebral cortex, platelet activating factor (PAF) receptors, TRKA receptors, NMDA, K+V channel, Na+ channel (site 2), GABAA, Ca^{2+} channel (N), and P2X ion channels, and serotonin and norepinephrine transporters (data not shown).

PAR2 receptors stimulate production of inositol phosphates and release of intracellular Ca^{2+}. Therefore, to verify the results obtained using the cellular proliferation assay, AC-55541 and AC-264613 were tested for agonist activity at PAR2 in phosphatidyl inositol (PI) hydrolysis assays, and Ca^{2+} mobilization assays (**see Figure 3 and 4, Table 1**). The potencies of SLIGRL-NH$_2$ and 2-furoyl-LIGRLO-NH$_2$ in the PI hydrolysis, and Ca^{2+} mobilization assays were approximately 10 uM in both assays for SLIGRL-NH$_2$, and approximately 100 nM in both assays, for 2-furoyl-LIGRLO-NH$_2$. In the PI hydrolysis assays AC-55541 had potencies of approximately 1-3 uM and AC-264613 had potencies of approximately 100-300 nM, respectively, and in the Ca^{2+} mobilization assay, AC-55541 and AC-264613 had potencies of approximately 250 nM and 100 nM, respectively. To confirm the observation made above in the cellular proliferation assays that AC-55541 and AC-264613 specifically activate PAR2 receptors, we tested them in PI hydrolysis assays on cells transfected with control vector, PAR1 or PAR4 receptors (Figure 3A-3C). Robust responses to the PAR1 and PAR APs were observed, with pEC$_{50}$ values of 6.1$\pm$0.1 for SFLLRN-NH$_2$ on PAR1, and 4.5$\pm$0.2 for AYPGKF-NH$_2$ on PAR4 (Figure 3C). However little or no response to either AC-55541 (Figure 3A) or AC-264613 (Figure 3B) was observed except for a slight effect of AC-55541 at high (>10 uM) concentrations. This slight effect was observed in all conditions including cells transfected with empty vector, and therefore is probably not due to activation of either PAR1 or PAR4. Similarly, we observed no significant calcium signal

in untransfected KNRK cells (data not shown), in agreement with previous observations that KNRK cells do not express functional PAR2 receptors (Al-Ani et al., 1999). In general, all the compounds had full or nearly full efficacy compared to SLIGRL-NH$_2$ in all the functional assays. The rank order of potencies of all tested ligands was consistent across all functional assays, and was 2-furoyl-LIGRLO-NH$_2$ > AC-264613 > AC-55541 > SLIGRL-NH$_2$ (**Table 1**).

PAR2 receptors have been reported to rapidly internalize upon activation by trypsin or PAR2 APs (Dery et al, 1999). Using EYFP-tagged PAR2 S37P receptors transiently transfected into HEK293T cells, we examined the internalization behavior of PAR2 receptors exposed to either PAR2 APs or the small organic molecules described here. As shown in **Figure 5**, receptor internalization could be visualized as a redistribution of fluorescence from a uniform border located around the periphery of the cells into punctate vesicles located inside the cells. All four ligands induced receptor internalization and vesicle formation. However, SLIGRL-NH$_2$ appeared to induce this process slightly faster.

Poor bioavailability is a major limitation of PAR2 APs for *in vivo* experiments. Intraperitoneal administration of AC-55541 or AC-264613 resulted in peak plasma concentrations of 1035 and 1233 ng/ml, at 50 and 10 minutes, respectively in male Sprague-Dawley rats (**Figure 6 and Table 2**). The elimination half lives of AC-55541 and AC-264613 were 6.1 hrs and 2.5 hrs, respectively (**Figure 6 and Table 2**). *In vitro* testing revealed that both AC-55541 and AC-264613 are reasonably stable to metabolism in the presence of liver microsomes, with values of 6 and 19 ul/min/mg for AC-55541 and values of 9 and 37 ul/min/mg for AC-264613 in human and rat liver microsomes, respectively.

PAR2 receptors have been strongly implicated in nociceptive and inflammatory processes. Therefore we characterized AC-55541, AC-264614 and the PAR2 APs in hotplate and paw edema assays, commonly used models of acute thermal nociception, and acute inflammation. Administration of SLIGRL-NH$_2$ or trypsin into the paw elicited robust thermal hyperalgesia and edema in male Sprague-Dawley rats (**Figure 7A and 7B**), which persisted for a period of at least 4 hours, the longest time point examined. This finding is consistent with previous reports (Vergnolle et al, 1999; 2001; Steinhoff et

al, 2000). Similarly, intrapaw administration of AC-55541 (**Figure 7C and 7D**) or AC-264613 (**Figure 7E or 7F**) resulted in hind paw edema and thermal hyperalgesia at doses as low as 30 ng. The maximum effects of SLIGRL-NH$_2$, trypsin, AC-55541 and AC-264613 on thermal hyperalgesia and edema were similar to each other (see **Figure 7**), and to the maximal effects we have previously observed using carrageenan (data not shown).

PAR2 receptors have been reported to elicit pain and inflammation through a neurogenic mechanism of action, causing release of substance P, activation of NK1 receptors, and sensitization of TRPV1 receptors (Steinhoff et al, 2000; Vergnolle et al, 2001; Amadesi et al, 2004; Dai et al, 2004). Therefore we tested the effects of a L732,138, a selective NK1 receptor antagonist, and capsazepine, a TRPV1 receptor antagonist on the pronociceptive effects of AC-55541 and AC-264613. As shown in **Figure 8**, both L732,138 and capsazepine each completely blocked the pro-nociceptive actions of AC-55541 and AC-264613.

We next examined the hyperalgesic effects of AC-55541 and AC-264613 in male Sprague-Dawley rats following systemic administration. As shown in **Figure 9**, AC-55541 and AC-264613 each showed dose dependent pronociceptive effects, comparable to their effects when administered locally. The maximum effects of AC-55541 were delayed, and extended compared to the maximum effects of AC-264613, which is consistent with their pharmacokinetic profiles.

Discussion

We have described the discovery and characterization of the first small molecule PAR2 agonists AC-55541 and AC-264613. Both compounds displayed high potency and efficacy for PAR2 receptors in a variety of functional assays including cellular proliferation, calcium mobilization and PI hydrolysis assays, and both compounds stimulated internalization of PAR2 receptors (see Figure 2-5). To assess the selectivity of AC-55541 and AC-264613 we tested them at PAR1 and PAR4 receptors, each expressed alone or together with PAR3. PAR3 does not respond to soluble peptides derived from its tethered ligand; however in agreement with previous reports (Nakanishi-Matsui et al, 2000; McLaughlin et al, 2007) it significantly potentiated the responses to both PAR1 and

PAR4 (see Figure 2B and 2C). We observed no response to either AC-55541 or AC-264613 under any of these conditions. In terms of assay sensitivity, the potencies of the PAR1, PAR2 and PAR4 APs reported in our cellular proliferation assays and our PI hydrolysis assays are very similar to their potencies reported in other systems (Kawabata et al, 2004b; McGuire et al, 2004; Lerner et al., 1996; Quinton et al., 2004). Thus, our assessment is that these compounds are highly selective for PAR2, and would be expected to show similar selectivity in systems commonly employed in other laboratories. Ultimately it will be important to test them in native systems with each of the PAR subtypes deleted to fully characterize their selectivity *in vivo*. Both compounds were metabolically stable, were well absorbed systemically, and remained at effective plasma concentrations for several hours, with AC-264613 achieving higher initial exposure, but AC-55541 maintaining more sustained exposure (Table 2).

Injection of PAR2 APs into the rat paw has been previously shown to cause a strong acute inflammatory response characterized by marked edema and granulocyte infiltration (Vergnolle et al, 1999). Consistent with their profiles as PAR2 agonists, both AC-55541 and AC-264613 produced robust hyperalgesia and edema in rats, either administered locally (intra-paw) or systemically (intra-peritoneally) (see **Figure 7** and **9**). AC-55541 produced more robust effects *in vivo*, especially when given systemically (see **Figure 9**) despite the fact that it is less potent than AC-264613 *in vitro* (**Table 1**), possibly due to its more sustained exposure (**Figure 6**). However since both compounds offer advantages, both will be useful in further probing the physiological roles of PAR2.

Ample evidence indicates PAR2 receptors mediate pro-nociceptive and pro-inflammatory effects through a neurogenic mechanism of action (Steinhoff et al; 2000; Vergnolle et al, 2001; Fiorucci et al, 2001; Nguyen et al., 2003; Ricciardolo et al, 2000; Su et al, 2005; Kawabata et al, 2005). Treatment with PAR2 APs or trypsin can stimulate the release of calcitonin gene-related peptide (CGRP) and substance P (SP) from both central and peripheral terminals of primary afferent nociceptive neurons, which in turn may mediate states of pain and inflammation (Steinhoff et al; 2000). In addition, PAR2 receptors are co-expressed with TRPV1 receptors on primary afferent neurons where activation of PAR2 receptors leads to potentiation of capsaicin-induced currents, and antagonism or deletion of TRPV1 receptors blocks PAR2-induced thermal hyperalgesia

(Amadesi et al, 2004; Dai et al, 2004). Consistent with their actions as PAR2 agonists, we observed that thermal hyperalgesia induced by AC-55541 or AC-264613 was completely blocked by either the NK1 antagonist L732,138 or the TRPV1 antagonist capsazepine (see **Figure 8**).

The possible therapeutic utility of PAR2 agonists is controversial, though therapeutic roles for PAR2 agonists have been proposed (Henry, 2006; De Campo and Henry, 2006). PAR2 activation has been shown to cause airway smooth muscle relaxation (Cicala et al, 2001), primarily in an epithelium-derived, PGE2-dependent manner (Cocks et al, 1999; Kawabata et al, 2004; De Campo and Henry, 2005), as well as by CGRP and PGE2 (Morello et al, 2005), however other studies have demonstrated that PAR2 agonists may also cause bronchoconstriction (Schmidlin et al, 2002) through a neural mechanism (Su et al, 2005). One study showed PAR2 activation caused relaxation of trachea and main bronchi in an epithelium-derived, PGE2-dependent manner, but constriction of intrapulmonary bronchi in a tachykinin receptor-dependent manner (Ricciardolo et al, 2000). Interestingly, PAR2 agonists potentiate histamine-induced contractions of human isolated bronchial rings from non-smokers, but antagonize histamine-induced contraction in isolated bronchial rings from smokers (Risse et al, 2004). More recently it was shown that activation of PAR2 reduced airway inflammation and airway hyper-responsiveness in rabbits sensitized to pollen allergen (D'Agostino et al, 2007). These authors speculate that the use of pollen allergen instead of ovalbumin as the sensitizing agent may represent an asthma model more relevant to the human condition. Thus the net effect of PAR2 activation on lung airflow may depend on species differences, anatomical differences (trachea and main bronchi versus deeper bronchioles), differences in the inflammatory state of the lungs, differences in the sensitizing agent, and differences in route of administration, which could affect exposure of the ligand to neurons *versus* epithelium.

Similarly, single high doses of PAR2 agonists inhibit infiltration of inflammatory cells into the lungs and reduce airway resistance of lipopolysaccharide-challenged mice and ovalbumin sensitized mice (Moffatt et al, 2002; De Campo and Henry, 2005; Morello et al, 2005) whereas administration of multiple doses of SLIGRL-NH$_2$ or genetic overexpression of PAR2 increased airway hyperresponsiveness, infiltration of

eosinophils and mononuclear cells, and levels of pro-inflammatory cytokines (Schmidlin et al, 2002; Ebeling et al, 2005). Thus PAR2 receptors may play a dual role in the lung.

In both HCl-induced and indomethacin-induced models of gastric ulcer PAR2 agonists prevent/limit tissue damage to the stomach lining by inducing mucus secretion, possibly through a neurogenic mechanism (Kawabata et al., 2005). Likewise, caerulein-induced pancreatitis and associated abdominal hyperalgesia/allodynia was exacerbated in PAR2 KO mice, and suppressed by 2-furoyl-LIGRLO-NH$_2$ in WT but not PAR2-KO mice (Kawabata et al, 2006). PAR2 activation limits 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice (Fiorucci et al, 2001), however more recently PAR2 has been implicated as pro-inflammatory both in animal models of colitis, pancreatitis, and in the development and progression of inflammatory bowel disease (IBS) in humans (Nguyen et al, 2003; Hansen et al, 2005; Cenac et al, 2007, Hyan et al, 2008, Laukkarinen et al, 2008).

The identification of potent, selective, and metabolically stable small molecule PAR2 ligands offers a number of new avenues for further exploration, including use in chronic studies, the development of radiolabeled probes for use in medical diagnostics and for binding studies, and development of antagonist analogs. Besides AC-264613 and AC-55541, we have successfully identified a series of active structural analogs of both compounds (see Seitzberg et al., in press) demonstrating that these scaffolds are amenable to chemical modification. Given the controversial, but clearly crucial role PAR2 receptors play in a wide variety of conditions with a strong inflammatory component, and the substantial number of published studies indicating possible therapeutic roles for PAR2 agonists (discussed above), it will be important to further test the compounds described herein in those models.

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Footnotes

*These authors contributed equally to this work

Legends for Figures

Figure 1. Structures of AC-55541 and AC-264613.

Figure 2. Functional responses in cellular proliferation assays. The indicated compounds were tested for proliferative responses using transiently transfected NIH3T3 cells in the R-SAT® functional assay as described in the methods. To eliminate proteolysis as a contributing factor to receptor activation, the protease insensitive mutants PAR1 S42P, PAR2 S37P, and PAR4 R47A were used. Proliferative responses were quantified using a beta-galactosidase reporter system and normalized to the responses to the PAR activating peptides SLIGRL-NH$_2$ for **A**) PAR2 S37P (P2); SFLLRN-NH$_2$ for **B**) PAR1 S42P (P1), and AYPGKF-NH$_2$ for (**C**) PAR4 R47A (P4). 100% represents 5 to 10-fold responses over baseline in typical experiments with these receptors. PAR3, which does not itself respond to PAR3 APs, but which acts as a co-receptor for PAR1 and PAR4 (see Nakanishi-Matsui et al, 2000; McLaughlin et al, 2007), was co-transfected with PAR1 and PAR4 receptors where noted (P1 & P3, P4 & P3, respectively). Data points represent the means of duplicate determinations. The ND label in the abscissa represents no drug added. Vector represents control cells that were transfected and analyzed with reporter plasmid only.

Figure 3. Functional responses in cellular PI hydrolysis assays. The indicated compounds were tested for activity in phosphatidyl inositol hydrolysis assays using HEK293T cells transiently transfected with PAR1 S42P, PAR2 S37P, PAR4 R47A or empty vector as described in the methods. **A**) AC-55541 on cells transfected with the indicated receptors; **B**) AC-264613 on cells transfected with the indicated receptors; **C**) 2-f-LIGRLO-NH$_2$ on PAR2 S37P transfected cells, SFLLRN-NH$_2$ on PAR1 S42P transfected cells, AYPGKF-NH$_2$ on PAR4 R47A transfected cells. Data points represent the means of triplicate determinations. The ND label in the abscissa represents no drug added.

Figure 4. Functional responses in cellular Ca^{2+} mobilization assays. The indicated compounds were tested for activity in Ca^{2+} (aequorin) mobilization assays using KNRK cells transiently transfected with PAR2 as described in the methods. Responses were normalized to the response to the PAR2 activating peptide SLIGRL-NH$_2$ which was 20% of the maximal possible luminescence (MT-GFP-aequorin) response. Data points represent the means of triplicate determinations. The ND label in the abscissa represents no drug added.

Figure 5. Internalization of PAR2 receptors. HEK293T cells were transiently transfected with PAR2 S37P-EYFP and grown in chamber slides overnight. Cells were treated with ligands (100 uM SLIGRL-NH$_2$, 1 uM 2-furoyl-LIGRLO-NH$_2$, 3 uM 55541 and 1 uM 264613) for indicated times at 37°C. Images were collected as described in the methods.

Figure 6. Pharmacokinetics of AC-55541 and AC-264613. Compounds were dissolved in DMSO and administered intraperitoneally to male, Sprague-Dawley rats at time=0. Blood samples were obtained at the indicated time points, and the concentrations of AC-55541 and AC-264613 quantified using LC-MS. Data points represent the means of three independent experiments.

Figure 7. Pro-inflammatory and hyperalgesic effects of PAR2 agonists. Paw edema assays (**A, C**, and **E**) were used to assess acute inflammatory responses in male, Sprague-Dawley rats. The indicated amounts of drugs were dosed directly into the paw (i. paw) as described in the methods. The controls were given the same volume of sterile saline as in test groups. Foot-pad thickness was measured in conscious rats using a caliper, and normalized to the width of the contralateral paw (assigned a value of 100 %). Data shown were taken at 4 hours post-dose, and are the means +/- SEM of 6 animals per group. Thermal hyperalgesia (**B, D**, and **F**) was evaluated by recording the paw withdrawal latencies (in seconds) to a radiant thermal stimulus according to Hargreaves' method (plantar test), using a 7371 Plantar Test (Ugo Basile, Varese, Italy) (Hargreaves et al., 1988). Data shown were taken at 2 hours post-dose, and are the means +/- SEM of

6 animals per group. *denotes $p < 0.05$ compared to vehicle. **denotes $p < 0.01$ compared to vehicle.

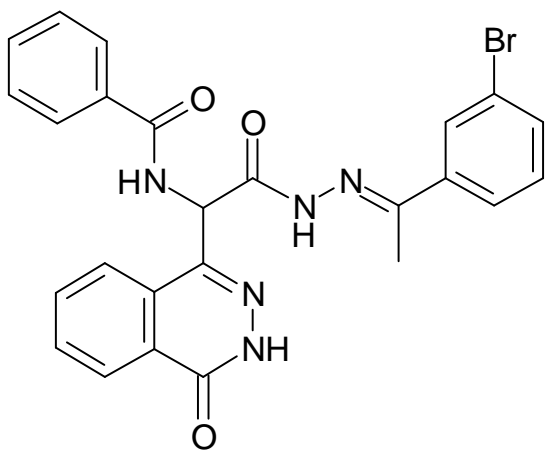**Figure 8. PAR2 agonists cause hyperalgesia through a neurogenic mechanism.** Thermal hyperalgesia was measured as described above. One hour prior to i.paw administration of 10 ug of either AC-55541 or AC-264613, male Sprague-Dawley rats were dosed intraperitoneally with 10 mg/kg of either the NK1 receptor antagonist L732,138, the TRPV1 receptor antagonist capsazepine, or saline where indicated. **A**) AC-55541 and L732,138; **B**) AC-264613 and L732,138; **C**) AC-55541 and capsazepine; **D**) AC-264613 and capsazepine. Data shown were taken at 2 hours post-dose of the antagonists (1 hour post-dose of AC-55541 or AC-264613), and are the means +/- SEM of 6 animals per group. **denotes $p < 0.01$ compared to vehicle.

Figure 9. Systemic actions of PAR2 agonists. Male Sprague-Dawley rats were dosed intraperitoneally with the indicated doses (mg/kg) of either AC-55541 or AC-264613, and thermal hyperalgesia measured as described above at the indicated time points. Data shown are the means +/- SEM of 6 to 12 animals per group. *denotes $p < 0.05$ compared to vehicle. **denotes $p < 0.01$ compared to vehicle.

Ligands	Receptor	Cellular Proliferation		PI hydrolysis		Ca^{2+} mobilization	
		pEC50	Eff (%)	pEC50	Eff (%)	pEC50	Eff (%)
AC-55541	WT	6.7 $\pm$ 0.1	81 $\pm$ 4	5.9 $\pm$ 0.1	96 $\pm$ 10	6.6 $\pm$ 0.1	82 $\pm$ 4
	S37P	6.7 $\pm$ 0.3	78 $\pm$ 6	5.4 $\pm$ 0.2	110 $\pm$ 7	n.d.	
AC-264613	WT	7.5 $\pm$ 0.1	93 $\pm$ 15	6.9 $\pm$ 0.1	88 $\pm$ 14	7.0 $\pm$ 0.2	110 $\pm$ 2
	S37P	7.3 $\pm$ 0.2	88 $\pm$ 23	6.5 $\pm$ 0.1	100 $\pm$ 8	n.d.	
2-f-LIGRLO-NH$_2$	WT	7.6 $\pm$ 0.1	89 $\pm$ 11	7.4 $\pm$ 0.1	82 $\pm$ 3	7.1 $\pm$ 0.1	103 $\pm$ 5
	S37P	7.5 $\pm$ 0.2	91 $\pm$ 21	6.9 $\pm$ 0.0	96 $\pm$ 5	n.d.	
SLIGRL-NH$_2$	WT	5.0 $\pm$ 0.3	100 $\pm$ 0	5.0 $\pm$ 0.1	100 $\pm$ 0	5.0 $\pm$ 0.1	100 $\pm$ 0
	S37P	5.0 $\pm$ 0.3	100 $\pm$ 0	5.3 $\pm$ 0.3	100 $\pm$ 0	n.d.	

Table 1. Pharmacological activity of PAR2 agonists. Functional assays were performed as described in Materials and Methods. Values represent the means $\pm$ S.E.M. of three to nine independent experiments. n.d. indicates not done. WT indicates human wild-type PAR2. S37P indicates the protease-insensitive mutant of PAR2.

Parameter	AC-55541			AC-264613		
Tmax (hr)	0.8	±	0.2	0.2	±	0.0
Cmax (ng/mL)	1035	±	140	1233	±	395
T1/2 (hr)	6.1	±	1.5	2.5	±	2.0
AUC$_{INF\ obs}$ (hr*ng/mL)	7936	±	997	1341	±	448
MRT$_{INF\ obs}$ (hr)	6.9	±	1.6	1.4	±	0.4

Table 2. Pharmacokinetic properties of PAR2 agonists. AC-55541 or AC-264613 were administered (10 mg/kg, intraperitoneally (i.p.)) to male, Sprague-Dawley rats, a series of blood samples were taken at various intervals over twenty-four hours, and analyzed for the presence of each compound. Parameters are the averages $\pm$ S.E.M. from three different animals. Tmax refers to the time of peak plasma concentration. Cmax refers to the peak plasma concentration. T1/2 refers to the half-life of the compound in plasma. AUC represents area under the curve. MRT represents mean residence time.

Figure 1.

AC-55541
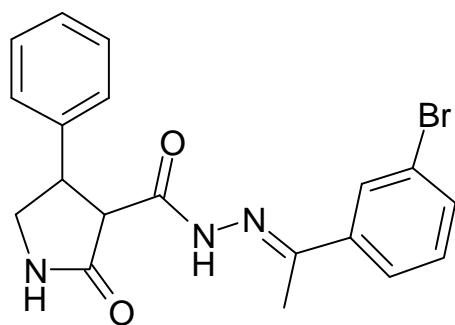
AC-264613

Figure 2A.
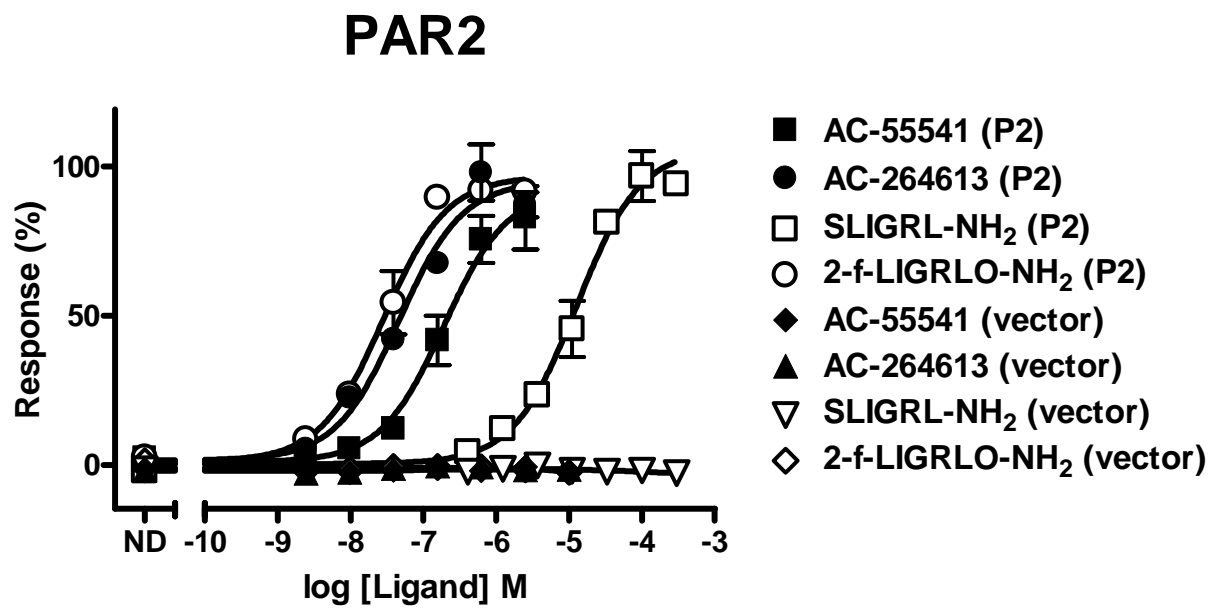
Figure 2B.

Figure 2C.

Figure 3A.

AC-55541

Figure 3B.

Figure 3C.

Reference ligands

- ■ 2-f-LIGRLO-NH$_2$ (PAR2)
- ▼ SFLLRN-NH$_2$ (PAR1)
- ▲ AYPGKF-NH$_2$ (PAR4)

Figure 4.

PAR2 - Ca^{2+} mobilization

- ■ AC-55541
- ● AC-264613
- □ SLIGRL-NH$_2$
- ○ 2-f-LIGRLO-NH$_2$

Figure 5.

Figure 6.

Pharmacokinetics of PAR2 agonists

Figure 7.

A

Trypsin and PAR2 Activating Peptide Produce Local Edema

B

Trypsin and PAR2 Activating Peptide Elicit Thermal Hyperalgesia

Figure 7.

C AC-55541 Produces Dose-dependent Paw Edema

D AC-55541 Produces Dose-dependent Thermal Hyperalgesia

Figure 7.

E

AC-264613 Produces Dose-dependent Paw Edema

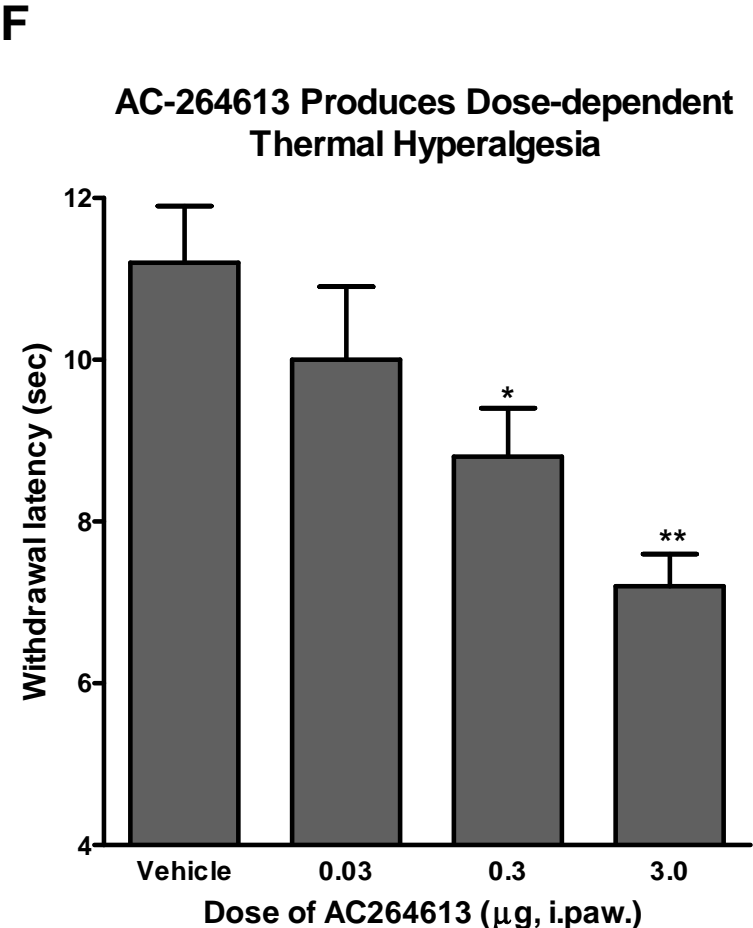**F**

AC-264613 Produces Dose-dependent Thermal Hyperalgesia

Figure 8.

A

**Thermal Hyperalgesia Produced by AC-55541
is Blocked by an NK1 Antagonist**

Legend:
☐ Vehicle
■ AC-55541 (10μg)

Y-axis: Withdrawal latency (sec) — 6, 7, 8, 9, 10, 11, 12, 13

X-axis: Treatment conditions — +Vehicle, +L732,138

B

**Thermal Hyperalgesia Produced by AC-264613
is Blocked by an NK1 Antagonist**

Legend:
☐ Vehicle
■ AC-264613 (10μg)

Y-axis: Withdrawal latency (sec) — 6, 7, 8, 9, 10, 11, 12, 13

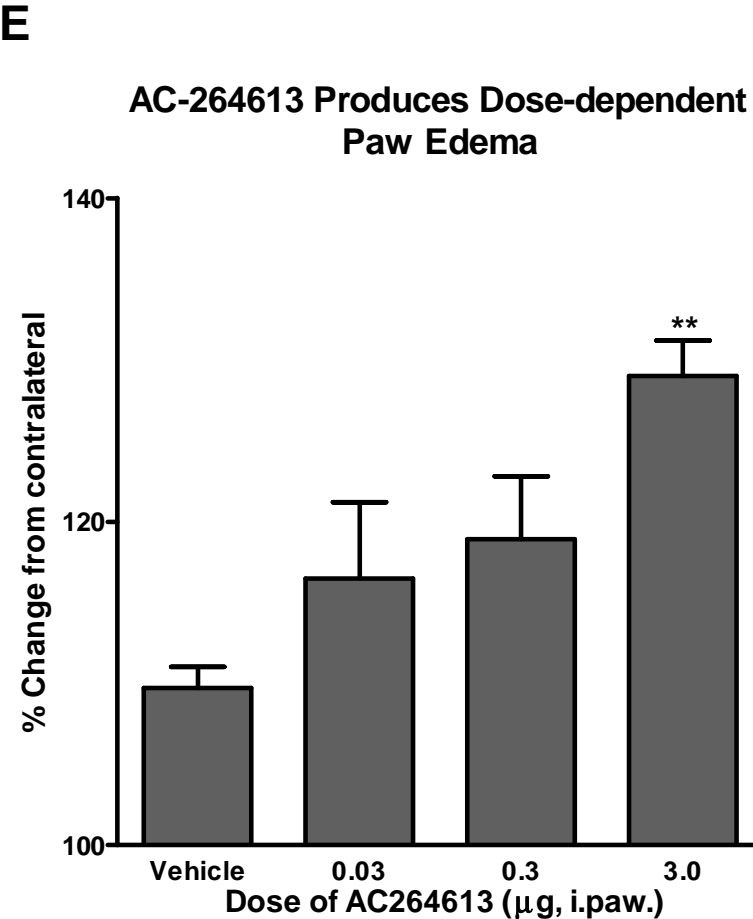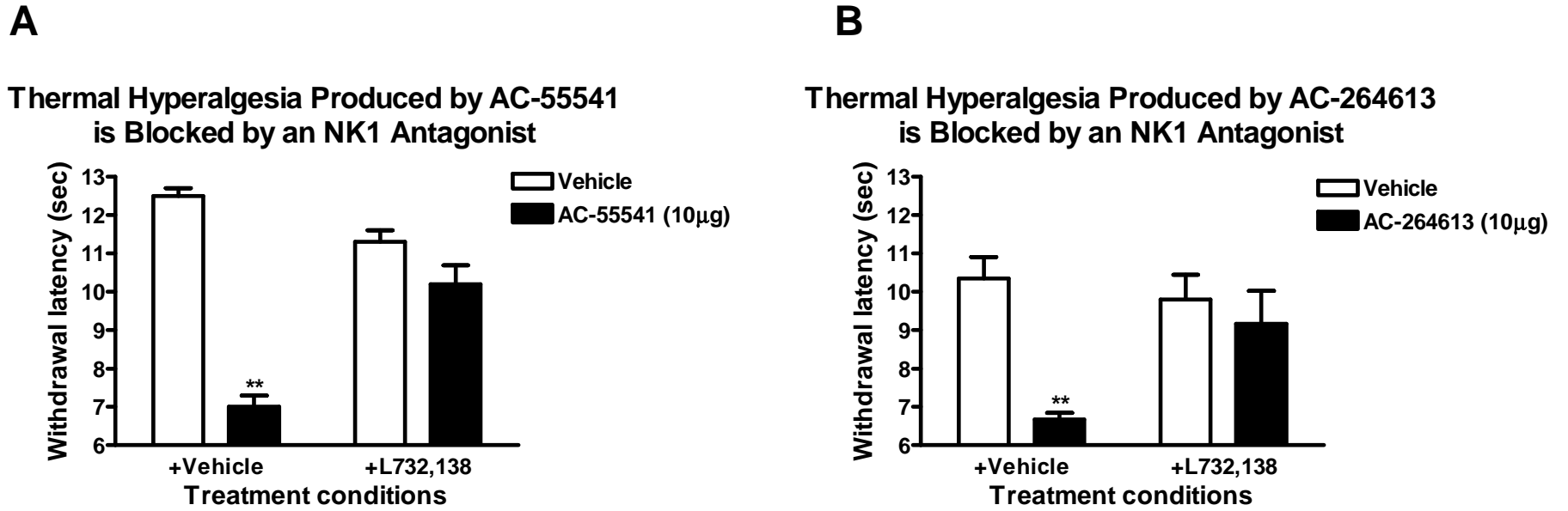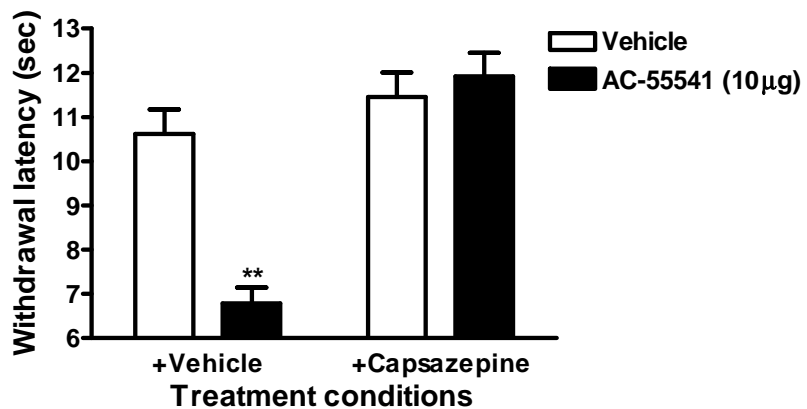X-axis: Treatment conditions — +Vehicle, +L732,138

Figure 8.

C

Thermal Hyperalgesia Produced by AC-55541
is Blocked by a TrpV1 Antagonist

D

Thermal Hyperalgesia Produced by AC-264613
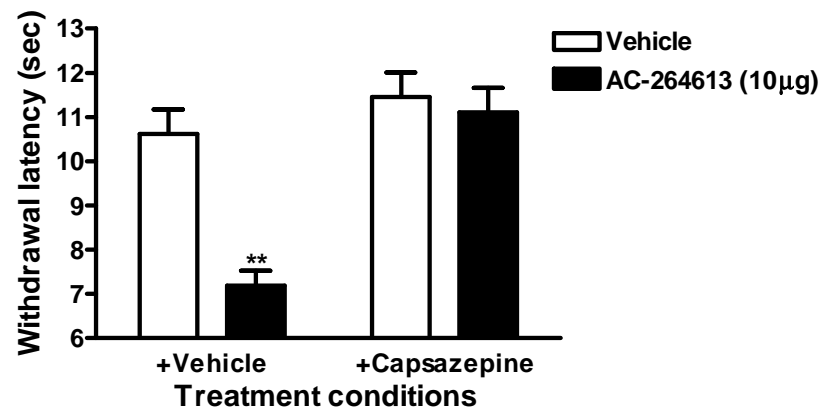is Blocked by a TrpV1 Antagonist

Figure 9.

A

Systemic Administration of AC-55541 Produces Dose-dependent Thermal Hyperalgesia

B

Systemic Administration of AC-264613 Produces Dose-dependent Thermal Hyperalgesia