

## RESEARCH PAPER

# Antagonism of the proinflammatory and pronociceptive actions of canonical and biased agonists of protease-activated receptor-2

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## BACKGROUND AND PURPOSE

Diverse proteases cleave protease-activated receptor-2 (PAR2) on primary sensory neurons and epithelial cells to evoke pain and inflammation. Trypsin and tryptase activate PAR2 by a canonical mechanism that entails cleavage within the extracellular *N*-terminus revealing a tethered ligand that activates the cleaved receptor. Cathepsin-S and elastase are biased agonists that cleave PAR2 at different sites to activate distinct signalling pathways. Although PAR2 is a therapeutic target for inflammatory and painful diseases, the divergent mechanisms of proteolytic activation complicate the development of therapeutically useful antagonists.

## EXPERIMENTAL APPROACH

We investigated whether the PAR2 antagonist GB88 inhibits protease-evoked activation of nociceptors and protease-stimulated oedema and hyperalgesia in rodents.

## KEY RESULTS

Intraplantar injection of trypsin, cathepsin-S or elastase stimulated mechanical and thermal hyperalgesia and oedema in mice. Oral GB88 or *par2* deletion inhibited the algescic and proinflammatory actions of all three proteases, but did not affect basal responses. GB88 also prevented pronociceptive and proinflammatory effects of the PAR2-selective agonists 2-furoyl-LIGRLO-NH<sub>2</sub> and AC264613. GB88 did not affect capsaicin-evoked hyperalgesia or inflammation. Trypsin, cathepsin-S and elastase increased [Ca<sup>2+</sup>]<sub>i</sub> in rat nociceptors, which expressed PAR2. GB88 inhibited this activation of nociceptors by all three proteases, but did not affect capsaicin-evoked activation of nociceptors or inhibit the catalytic activity of the three proteases.

## CONCLUSIONS AND IMPLICATIONS

GB88 inhibits the capacity of canonical and biased protease agonists of PAR2 to cause nociception and inflammation.

## Abbreviations

DRG, dorsal root ganglion; GB88, N-[(2S)-3-cyclohexyl-1-[[[(2S,3R)-3-methyl-1-oxo-1-spiro[indene-1,4'-piperidine]-1'-yl]pentan-2-yl]amino]-1-oxopropan-2-yl]-1,2-oxazole-5-carboxamide; PAR, protease-activated receptor; TRP, transient receptor potential

## Tables of Links

TARGETS	
<b>GPCRs<sup>a</sup></b>	<b>Enzymes<sup>c</sup></b>
PAR2	Cathepsin-S
<b>Voltage-gated ion channels<sup>b</sup></b>	Elastase
TRPV1	Trypsin
TRPV4	

LIGANDS
Capsaicin
GB88

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (<sup>a,b,c</sup>Alexander *et al.*, 2015a,b,c).

## Introduction

Serine, cysteine and metalloproteases can signal to cells by cleaving protease-activated receptors (PARs), a family of four G-protein coupled receptors (PAR1–4) (Ossovskaya and Bunnett, 2004; Hollenberg *et al.*, 2014; Zhao *et al.*, 2014b). PAR2 is expressed by epithelial, endothelial and smooth muscle cells, as well as by cells of the immune and nervous systems (Nystedt *et al.*, 1994, 1995; Bohm *et al.*, 1996). Proteases that activate PAR2 in primary sensory neurons stimulate the release of substance P and CGRP in peripheral tissues, leading to neurogenic inflammation (Steinhoff *et al.*, 2000). PAR2 can also sensitize and activate transient receptor potential (TRP) ion channels in primary sensory neurons, including TRP vanilloid 1 and 4 (TRPV1 and TRPV4) and ankyrin 1 (TRPA1) (Amadesi *et al.*, 2004; Dai *et al.*, 2007; Grant *et al.*, 2007), which results in central transmission, neuropeptide release in the spinal cord and nociceptive transmission (Vergnolle *et al.*, 2001). Proteases that activate PAR2 on epithelial cells can promote disassembly of tight junctions (Jacob *et al.*, 2005), induce cyclooxygenase 2 (Wang *et al.*, 2008) and stimulate release of proinflammatory cytokines (Wang *et al.*, 2010). PAR2 deletion ameliorates inflammatory and painful disorders of the airways, joints, colon and skin (Lindner *et al.*, 2000; Schmidlin *et al.*, 2002; Ferrell *et al.*, 2003; Shichijo *et al.*, 2006; Cottrell *et al.*, 2007; Cattaruzza *et al.*, 2011). These observations suggest that PAR2 is an important target for inflammatory and painful disorders. However, the development of therapeutically useful antagonists has been hampered by the unusual mechanism of PAR2 auto-activation.

The canonical mechanism by which trypsin and mast cell tryptase activate PAR2 involves hydrolysis of Arg<sup>36</sup>↓Ser<sup>37</sup> and exposure of the tethered ligand S<sup>37</sup>LIGKV- (human PAR2), which binds to and activates the cleaved receptor (Nystedt *et al.*, 1994; Bohm *et al.*, 1996). Synthetic peptides that mimic the tethered ligand can directly activate PAR2 and are useful tools to probe receptor function. Trypsin-activated PAR2 couples to G<sub>αq</sub> and phospholipase C<sub>β</sub>, leading to mobilization of intracellular calcium and activation of PKC and D (Amadesi *et al.*, 2006; Amadesi *et al.*, 2009). Trypsin-activated PAR2 also recruits G protein receptor kinase 2 and β-arrestins, which mediate PAR2 endocytosis and ERK1/2 signalling from endosomes (Dery *et al.*, 1999; DeFea *et al.*, 2000; Ayoub and

Pin, 2013; Jensen *et al.*, 2013). The development of PAR2 antagonists is complicated by this mechanism of intramolecular receptor activation by a proteolytically exposed tethered ligand. Another complication is the existence of divergent mechanisms of proteolytic activation (Hollenberg *et al.*, 2014; Zhao *et al.*, 2014b). Proteases that cleave PAR2 distal to the canonical cleavage site can disarm the receptor by removing the trypsin-activation site. For example, neutrophil/leukocyte elastase cleaves PAR2 at Ser<sup>67</sup>↓Val<sup>68</sup>, which removes the trypsin cleavage site and thereby blocks the capacity of trypsin to activate the receptor (Dulon *et al.*, 2003). However, proteases that cleave PAR2 at different sites within the N-terminal domain can create different tethered ligands or stabilize unique receptor conformations and thereby can act as biased agonists that promote PAR2 coupling to divergent signalling pathways. Cathepsin-S, a cysteine protease secreted by antigen-presenting cells, cleaves PAR2 at Glu<sup>56</sup>↓Thr<sup>57</sup>, to reveal a different tethered ligand that promotes PAR2 coupling to G<sub>αs</sub>, adenylyl cyclase, cAMP and PKA, but not to G<sub>αq</sub> and β-arrestins (Zhao *et al.*, 2014a). Cathepsin-S can also cleave PAR2 at Gly<sup>41</sup>↓Lys<sup>42</sup> (Elmariah *et al.*, 2014). Elastase is also a biased agonist that promotes PAR2 coupling to G<sub>αs</sub>, adenylyl cyclase, cAMP and PKA, but not to G<sub>αq</sub> and β-arrestins, although elastase does not activate PAR2 by a tethered ligand mechanism (Ramachandran *et al.*, 2011; Zhao *et al.*, 2015). Despite these divergent mechanisms of PAR2 activation, both canonical and biased protease agonists cause PAR2- and TRPV4-dependent inflammation and pain (Grant *et al.*, 2007; Poole *et al.*, 2013; Zhao *et al.*, 2014a, 2015). Thus, therapeutically useful antagonists may need to disrupt the capacity of diverse proteases to activate PAR2 at different sites by canonical and biased mechanisms.

Although antibodies that target the canonical PAR2 cleavage site have efficacy in preclinical models of inflammatory disease (Kelso *et al.*, 2006; Yau *et al.*, 2013), it is uncertain whether they can block activation of the receptor by biased proteases that cleave at distant sites. The small molecule PAR2 antagonist ENMD-1068 and peptidomimetic antagonists based on the canonical tethered ligand domain, including K-14585 and C391, can also inhibit PAR2-mediated inflammation and pain, but their ability to suppress biased mechanisms of PAR2 activation has not been reported (Kelso *et al.*, 2006; Goh *et al.*, 2009; Yau *et al.*, 2013; Boitano *et al.*, 2015). GB83 and GB88 are small molecules that can inhibit

PAR2 activation by trypsin, tryptase and tethered ligand-derived agonists and are efficacious in preclinical models of inflammatory diseases (Barry *et al.*, 2010; Suen *et al.*, 2012; Lohman *et al.*, 2012a,b). However, it has not yet been reported whether GB88 can antagonize the actions of canonical and biased agonists of PAR2 on nociceptor activity and nociception. We examined the effects of GB88 on the capacity of canonical and biased proteases to activate rodent nociceptors and induce pain and inflammation.

## Methods

### Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). The Animal Ethics Committee of Monash University approved procedures using animals. Male C57BL/6, *par2*<sup>-/-</sup> and *par2*<sup>+/+</sup> mice littermates (Lindner *et al.*, 2000) (8–12 weeks-old), and male Sprague Dawley rats (7–8 weeks-old) were studied. Animals were maintained under temperature (22 ± 4°C) and light-controlled (12 h light/dark cycle) conditions with free access to food and water.

### Mechanical hyperalgesia and oedema

Mice were placed in individual cylinders on a mesh stand. They were acclimatized to the experimental room, restraint apparatus and investigator for 2 h periods on two successive days before experiments. To assess mechanical pain, paw withdrawal in response to stimulation of the plantar surface of the hindpaw with graded von Frey filaments (0.078, 0.196, 0.392, 0.686, 1.569, 3.922, 5.882, 9.804, 13.725 and 19.608 mN) was determined using the 'up-and-down' paradigm (Chaplan *et al.*, 1994). In this analysis, an increase in the filament stiffness required to induce paw withdrawal indicates mechanical analgesia, whereas a decrease in the filament stiffness required to induce withdrawal indicates mechanical hyperalgesia. On the day before the study, von Frey scores were measured in triplicate to establish a baseline for each animal. To assess inflammatory oedema of the paw, hind paw thickness was measured using digital callipers before and after treatments (Zhao *et al.*, 2014a, 2015).

### Thermal hyperalgesia

For studies of thermal hyperalgesia, paw withdrawal latencies to thermal stimulation of the plantar surface of the hind paw were measured in unrestrained mice using Hargreaves' apparatus (Amadesi *et al.*, 2006). Mice were placed in plastic chambers on a glass surface (25°C) and acclimatized for 1 h before baseline readings were collected. A radiant heat source was applied to the hind paw, and latency of the paw withdrawal was taken as the average of three trials per animal. A cut-off latency was set at 20 s to avoid tissue damage. In this analysis, an increase in latency indicates thermal analgesia, whereas a decrease in latency indicates thermal hyperalgesia.

### PAR2 antagonist and agonists

Investigators were blinded to the experimental treatments. GB88 (10 mg·kg<sup>-1</sup> in olive oil) or vehicle (control and olive

oil) was administered by gavage (150 µL) 2 h before intraplantar injections. For intraplantar injections, mice were sedated with 5% isoflurane. Trypsin (140 nM, 0.04 U·µL<sup>-1</sup>), elastase (1.18 µM, 0.03 U·µL<sup>-1</sup>), cathepsin-S (2.5 µM, 0.06 U·µL<sup>-1</sup>), 2-furoyl-LIGRLO-NH<sub>2</sub> (64 µM, 50 ng·µL<sup>-1</sup>), AC264613 (250 µM, 100 ng·µL<sup>-1</sup>), capsaicin (1.6 µM, 0.5 ng·µL<sup>-1</sup>) or vehicle (0.9% NaCl) was injected s.c. into the plantar surface of the left hind paw (10 µL). Mechanical hyperalgesia, paw thickness and thermal hyperalgesia were measured hourly for 4 h.

### In situ hybridization

cDNAs for mouse and rat PAR2 were amplified by using RNA from mouse or rat colon. The following forward and reverse primers were used: mouse PAR2, CACCGGGACGCAACAACAGTAAAG (mPar2\_F199) and GAATTCTAATACGACTCACTATAGGGAGATATGCAGCTGTTGAGGGTTCGACAG (mPar2\_R1136\_T7); rat PAR2, GAATGCACCGGGACCCAACAGTAA (rPAR2\_F165) and GAATTCTAATACGACTCACTATAGGGAGATGGAGGTGAGCGATATCTGCATGC (rPAR2\_R1216\_T7). The design of the reverse primers included the T7 promoter sequence (underlined), which allowed the PCR products to be used directly for the generation of digoxigenin (DIG)-labelled antisense cRNA probes by *in vitro* transcription with T7 RNA polymerase (Roche Products, Dee Why, NSW, Australia). Sections (12 µm) of mouse and rat dorsal root ganglia (DRG, lumbar) or trigeminal ganglia were processed for combined *in situ* hybridization and immunofluorescence as described previously (Bron *et al.*, 2014; Lieu *et al.*, 2014). The following primary antibodies were used: rabbit anti-CGRP (Sigma #C8198; 1:2000), mouse anti-heavy chain neurofilament (NF200, Sigma; #N0142; 1000). Biotinylated isolectin B4 (IB4) was from Sigma (#L2140). Secondary antibodies used were donkey anti-mouse-Alexa-488 (1:500), donkey anti-rabbit-Alexa568 (1:1000) and streptavidin-Alexa647 (1:500) (ThermoFisher Scientific, Carlsbad, CA, USA). Sections were imaged using 10× or 20× objective magnification on a Zeiss AxioScope.Z1 fluorescence microscope (Zeiss, Oberkochen, Germany). Images were processed using the Zeiss Zen software and exported as TIFF files to Adobe Photoshop for figure preparation.

### Dissociation of DRG neurons

DRG were collected from Sprague Dawley rats. Neurons were dispersed as described previously with modifications (Zhao *et al.*, 2014a; 2015). Briefly, DRG from all levels were incubated with collagenase IV (2 mg·mL<sup>-1</sup>), dispase II (2 mg·mL<sup>-1</sup>) and DNase I (100 µg·mL<sup>-1</sup>) for 40 min at 37°C. Cells were centrifuged (500 g, 5 min), re-suspended in HBSS and filtered through a 40 µm nylon mesh. Filtered cells were centrifuged, re-suspended in 1 mL of HBSS and layered onto a 20% Percoll gradient in Leibovitz's L-15 medium. The gradient was centrifuged (800 g, 9 min). The supernatant was removed, and the cell pellet was washed with L-15 medium. Neurons were placed in 96 well plates coated with laminin (0.004 mg·mL<sup>-1</sup>) and poly-L-lysine (0.1 mg·mL<sup>-1</sup>). Neurons were cultured in L-15 medium containing 10% FCS, with penicillin and streptomycin for 16 h at 37°C.

### Measurement of $[Ca^{2+}]_i$ in DRG neurons

Neurons were loaded with Fura2-AM (2  $\mu$ M, 1.5 h, 37°C). Neurons were incubated in calcium buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM  $CaCl_2$ , 1.18 mM  $MgCl_2$ , 10 mM D-glucose, 10 mM HEPES, pH 7.4) at 37°C on the stage of a Leica DMI6000B microscope equipped with a PL APO  $\times$  20 NA 0.75 objective (Leica Microsystems, North Ryde, NSW, Australia). Fluorescence was measured at 340 and 380 nm excitation with 530 nm emission using an Andor iXon 887 camera (Andor Technology, Belfast, UK) and METAFLUOR version 7.8.0 software (Molecular Devices, Sunnyvale, CA, USA) (Zhao *et al.*, 2014a; Zhao *et al.*, 2015). Neurons were challenged sequentially with trypsin (10 nM, 2.85  $mU \cdot \mu L^{-1}$ ), elastase (100 nM, 2.54  $mU \cdot \mu L^{-1}$ ) or cathepsin-S (100 nM, 2.4  $mU \cdot \mu L^{-1}$ ), followed by capsaicin (1  $\mu$ M) and KCl (50 mM). In some experiments, neurons were incubated with GB88 (10  $\mu$ M) or vehicle (control) (30 min pre-incubation and inclusion throughout). Images were analysed using a custom journal in METAMORPH software version 7.8.2 (Molecular Devices, Sunnyvale, CA, USA). A maximum intensity image was generated and projected through time to generate an image of all cells. Cells were segmented and binarized from this image using the Multi Wavelength Cell Scoring module on the basis of size and fluorescence intensity. Neurons of interest ( $<25 \mu$ m diameter) were selected. Results are expressed as the proportion of capsaicin- and KCl-responsive neurons that also responded to proteases.

### Fluorogenic protease assays

GB88 (10  $\mu$ M) was pre-incubated with the appropriate fluorogenic substrate (50  $\mu$ M): trypsin, H-D-Ala-Leu-Lys-AMC; elastase, MeOSuc-Ala-Ala-Pro-Val-AMC, cathepsin-S: Boc-Val-Leu-Lys-AMC. Proteases were added to give final concentrations of 10 nM trypsin, 100 nM elastase or 100 nM cathepsin-S. Substrate cleavage was assessed by measuring fluorescence during the initial 60–120 s (ex/em 360/440 nm). The slope was determined in the linear range and presented as % of the control.

### Covalent activity-based probe protease assays

Recombinant proteases (100 ng) were diluted in 20  $\mu$ L PBS containing GB88 (0, 1 or 10  $\mu$ M) and DMSO (1%) and were incubated for 30 min at 37°C. The appropriate activity-based probes were added: trypsin, PK-DPP (1  $\mu$ M); elastase, V-DPP (1  $\mu$ M), cathepsin-S, BMV109 (100 nM) (Pan *et al.*, 2006; Gilmore *et al.*, 2009; Verdoes *et al.*, 2013). Proteases were incubated with activity-based probes for 5 min at 37°C, solubilized with sample buffer, boiled and separated on a 15% SDS-PAGE gel. Probe labelling was detected by scanning gels for Cy5 fluorescence using a Typhoon FLA 7000 Scanner (GE Healthcare, Parramatta, NSW, Australia).

### Statistical Analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Results are expressed as mean  $\pm$  SEM. Data were analysed in GRAPHPAD PRISM 6.0 (GraphPad Software, La Jolla, CA, USA) using Student's *t*-test or ANOVA followed by Dunnett's *post hoc* test. *Post hoc* tests were used only if *F* achieved *P* < 0.05 and there was no

significant variance in homogeneity. Differences between means with a *P*-value < 0.05 were considered significant at the 95% confidence level.

### Materials

GB88 (N-[(2S)-3-cyclohexyl-1-[[[(2S,3R)-3-methyl-1-oxo-1-spiro[indene-1,4'-piperidine]-1'-yl]pentan-2-yl]amino]-1-oxopropan-2-yl]-1,2-oxazole-5-carboxamide) was prepared as described previously (Barry *et al.*, 2010; Suen *et al.*, 2012). The PAR2 agonists 2-furoyl-LIGRLO-NH<sub>2</sub> was from American Peptide Company Inc. (Sunnyvale, CA, USA) and AC264613 was from Tocris Biosciences (Bristol, UK). Human pancreatic trypsin (100 000 U·mL<sup>-1</sup>) was from Sigma-Aldrich (St. Louis, MO, USA). Human cathepsin-S (0.4 U·mL<sup>-1</sup>) was a gift from Medivir AB (Huddinge, Sweden) and has been described previously (Zhao *et al.*, 2014a). Human sputum elastase (864 U·mg<sup>-1</sup>) was from Elastin Products Company (Owensville, MO, USA). Fluorogenic protease substrates were from Bachem AG (Budendorf, Switzerland): trypsin, H-D-Ala-Leu-Lys-AMC; elastase, MeOSuc-Ala-Ala-Pro-Val-AMC; cathepsin-S, Boc-Val-Leu-Lys-AMC. The activity-based protease probes Cy5-ProLys-diphenyl phosphonate (PK-DPP), Cy5-Val-diphenyl phosphonate (V-DPP) and BMV109 were synthesized as described previously (Pan *et al.*, 2006; Gilmore *et al.*, 2009; Verdoes *et al.*, 2013). Unless otherwise indicated, other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

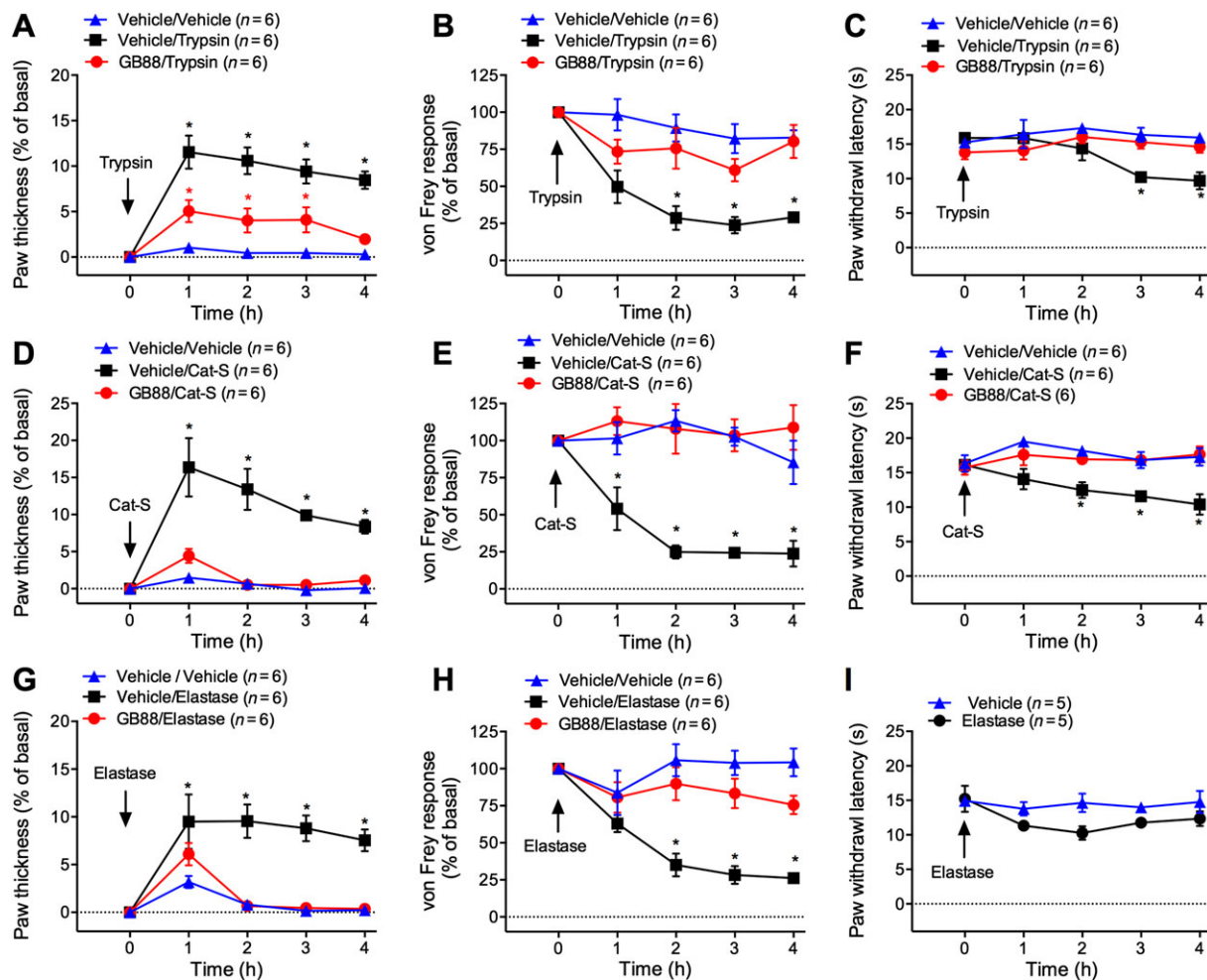
## Results

### GB88 antagonized the proinflammatory and pronociceptive actions of canonical and biased protease agonists of PAR2

Proteases that cleave PAR2 at different sites within the extracellular N-terminal domain can activate canonical or biased signalling pathways (Hollenberg *et al.*, 2014; Zhao *et al.*, 2014b). Although PAR2 deletion attenuates the pronociceptive and proinflammatory actions of trypsin, tryptase, elastase and cathepsin-S (Vergnolle *et al.*, 2001; Zhao *et al.*, 2014a; Zhao *et al.*, 2015), a pharmacological inhibitor of pain and inflammation induced by biased protease agonists of PAR2 has not been identified. We evaluated whether GB88 inhibits trypsin-, elastase- and cathepsin-S-evoked inflammation and nociception in mice.

Intraplantar injection of trypsin stimulated a 12% increase in paw thickness within 1 h that was sustained for 4 h, indicative of oedema (Figure 1A). Trypsin caused a significant reduction in von Frey responses between 2–4 h, consistent with mechanical hyperalgesia (Figure 1B), and decreased the latency of paw withdrawal to heat from 3 to 4 h, indicating thermal hyperalgesia (Figure 1C). P.o. administration of GB88 (10 mg·kg<sup>-1</sup>) 2 h before injection of trypsin reduced the effects of trypsin on paw thickness by ~50% and prevented trypsin-induced mechanical and thermal hyperalgesia (Figure 1A–C).

Intraplantar injection of cathepsin-S caused a 16% increase in paw thickness within 1 h, which was sustained for 4 h (Figure 1D). Cathepsin-S reduced the von Frey response from 1 to 4 h (Figure 1E) and decreased latency time to paw withdrawal from heat at 2–4 h (Figure 1F). GB88 abolished cathepsin-S-induced oedema and attenuated cathepsin-S-



**Figure 1**

Effects of GB88 on protease-evoked inflammation and nociception. Mice were treated with GB88 (10 mg·kg<sup>-1</sup> p.o.) or vehicle 2 h before intraplantar injections of trypsin (A–C; 30 ng), cathepsin-S (Cat-S, D–F; 14 µg), elastase (G–I; 290 ng) or vehicle. Paw thickness (A, D and G), paw withdrawal to mechanical stimulation (B, E and H) and paw withdrawal to thermal stimulation (C, F and I) were measured. \**P* < 0.05 compared with vehicle/vehicle control. Variable *n* indicates mouse number.

stimulated mechanical and thermal hyperalgesia (Figure 1 D–F). Intraplantar injection of elastase caused a 9% increase in paw thickness at 1 h that was sustained for 4 h (Figure 1G). Elastase reduced the von Frey response from 2 to 3 h, consistent with mechanical hyperalgesia (Figure 1H). In contrast to trypsin and cathepsin-S, elastase did not cause significant thermal hyperalgesia (Figure 1I). GB88 attenuated elastase-induced oedema and mechanical hyperalgesia (Figure 1G–I). Intraplantar injection of vehicle did not induce oedema or mechanical hypersensitivity, and GB88 did not affect baseline paw thickness (Figure 2A) or mechanical sensitivity (Figure 2B).

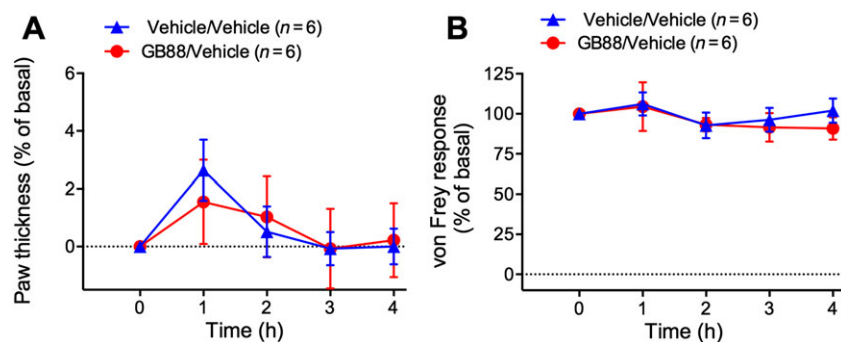
Thus, GB88 inhibits the proinflammatory and pronociceptive actions of proteases that activate PAR2 by canonical and biased mechanisms.

### *GB88 antagonized the proinflammatory and pronociceptive actions of synthetic PAR<sub>2</sub> agonists*

Synthetic peptides that mimic the trypsin-exposed tethered ligand can directly activate PAR2. Like trypsin, these

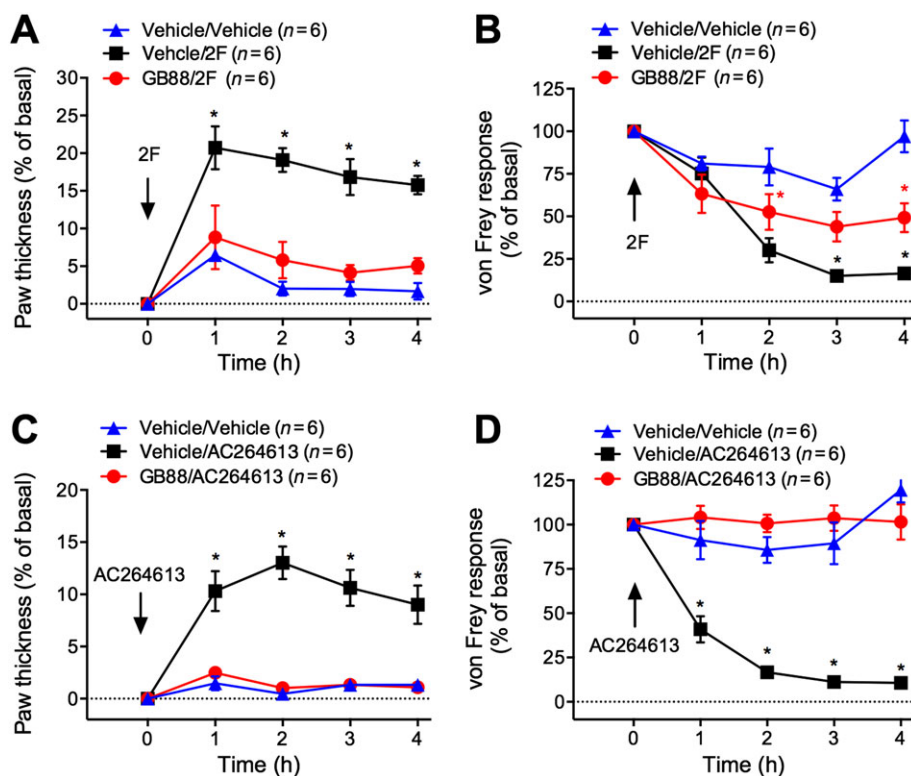
activating peptides induce PAR2 coupling to G<sub>αq</sub> and β-arrestins, sensitize TRP channels, and cause inflammation and pain (Amadesi *et al.*, 2004; Dai *et al.*, 2004; Grant *et al.*, 2007). We investigated whether GB88 inhibited the proinflammatory and pronociceptive actions of 2-furoyl-LIGRLO-NH<sub>2</sub>, an analogue of the tethered ligand domain (Kanke *et al.*, 2005), and AC264613, a small molecule agonist of PAR2 that elicits thermal hyperalgesia and oedema (Gardell *et al.*, 2008).

Intraplantar injection of 2-furoyl-LIGRLO-NH<sub>2</sub> caused a 21% increase in paw thickness at 1 h, which was sustained for 4 h (Figure 3A). 2-furoyl-LIGRLO-NH<sub>2</sub> reduced the von Frey withdrawal response from 2 to 4 h, indicative of mechanical hyperalgesia (Figure 3B). GB88 inhibited oedema induced by 2-furoyl-LIGRLO-NH<sub>2</sub> and reduced mechanical hyperalgesia by 30% (Figure 3A, B). Intraplantar injection of AC264613 induced a 10% increase in paw thickness at 1 h that persisted for 4 h (Figure 3C). AC264613 also evoked a robust mechanical hyperalgesia from 1 to 4 h (Figure 3D). GB88 prevented the proinflammatory and pronociceptive effects of



**Figure 2**

Effects of GB88 on basal inflammation and nociception. Mice were treated with GB88 (10 mg·kg<sup>-1</sup> p.o.) or vehicle 2 h before intraplantar injection of vehicle. Paw thickness (A) and paw withdrawal to mechanical stimulation (B) were measured hourly for 4 h. Variable *n* indicates mouse number.



**Figure 3**

Effects of GB88 on PAR2 agonist-evoked inflammation and nociception. Mice were treated with GB88 (10 mg·kg<sup>-1</sup> p.o.) or vehicle 2 h before intraplantar injections of 2-furoyl-LIGRLO-NH<sub>2</sub> (2F) (A and B; 500 ng) or AC264613 (C and D; 1 μg). Paw thickness (A and C) and paw withdrawal to mechanical stimulation (B and D) were measured hourly for 4 h. \**P* < 0.05 compared with vehicle/vehicle control. Variable *n* indicates mouse number.

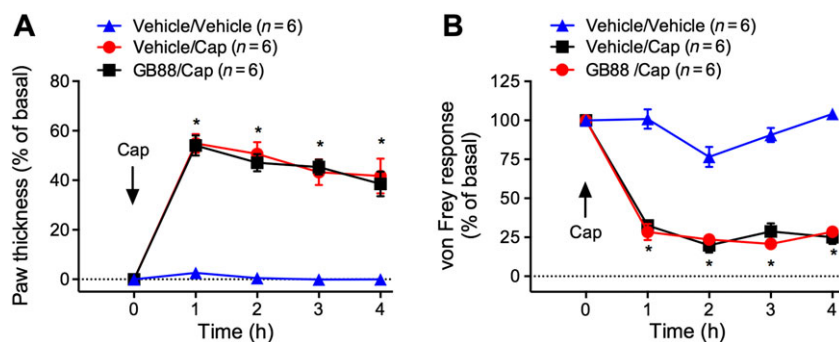
AC264613 (Figure 3C, D). Thus, GB88 inhibits mouse paw inflammation and nociception caused by small molecule synthetic agonists of PAR2.

### *GB88 did not affect the proinflammatory and pronociceptive actions of capsaicin*

The capacity of GB88 to inhibit protease- and PAR2-evoked inflammation and nociception could be due to antagonism of PAR2 or downstream mediators, such as TRP channels.

TRPV1 is a downstream target of PAR2 that contributes to the effects of proteases on inflammation and nociception (Amadesi *et al.*, 2004). Capsaicin directly activates TRPV1 on primary sensory neurons to cause neurogenic inflammation and nociception (Caterina *et al.*, 1997). We examined whether GB88 affected capsaicin-induced inflammation and nociception.

Intraplantar injection of capsaicin evoked a 55% increase in paw thickness within 1 h that persisted for 4 h (Figure 4A).



**Figure 4**

Effects of GB88 on capsaicin-evoked inflammation and nociception. Mice were treated with GB88 ( $10 \text{ mg}\cdot\text{kg}^{-1}$  p.o.) or vehicle 2 h before intraplantar injection of capsaicin (Cap,  $5 \mu\text{g}$ ). Paw thickness (A) and paw withdrawal to mechanical stimulation (B) were measured hourly for 4 h.  $*P < 0.05$  compared with vehicle/Cat-S control. Variable  $n$  indicates mouse number.

Capsaicin also induced a robust mechanical hyperalgesia at 1 h that was sustained for 4 h (Figure 4B). GB88 had no effect on capsaicin-stimulated oedema and mechanical hyperalgesia (Figure 4A, B). Thus, the anti-inflammatory and analgesic actions of GB88 are not due to antagonism of TRPV1, because the proinflammatory and nociceptive effects of capsaicin were unaffected.

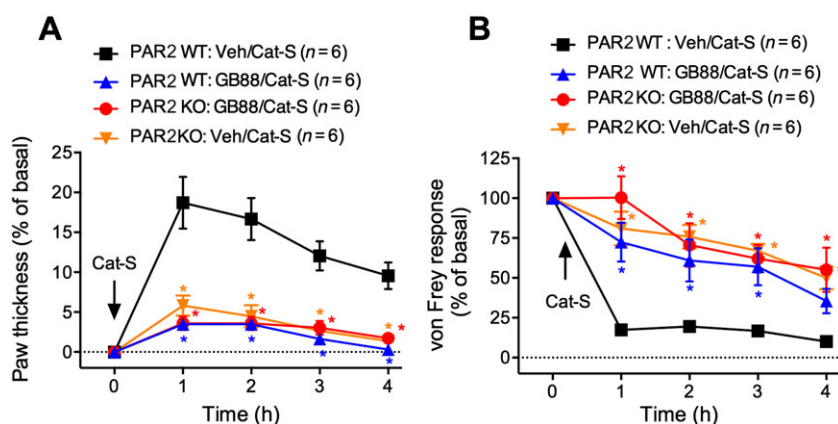
### The effects of GB88 on inflammation and nociception required expression of PAR2

Deletion of *par2* attenuates the capacity of trypsin, cathepsin-S and elastase to induce oedema and hyperalgesia (Vergnolle *et al.*, 2001; Zhao *et al.*, 2014a; Zhao *et al.*, 2015). Because *par2* deletion does not completely inhibit cathepsin-S-induced inflammation and nociception (Zhao *et al.*, 2014a), we examined whether GB88 had residual actions in *par2*<sup>-/-</sup> mice, consistent with PAR2-independent effects. In wild-type mice, cathepsin-S evoked a 19% increase in paw thickness (Figure 5A) and a sustained mechanical hyperalgesia

(Figure 5B). GB88 inhibited cathepsin-S-induced oedema and hyperalgesia. *Par2* deletion also inhibited cathepsin-S-induced oedema and hyperalgesia, and GB88 had no additional inhibitory actions in *par2*<sup>-/-</sup> mice. The inability of GB88 to exert additional anti-inflammatory and antinociceptive effects in *par2*<sup>-/-</sup> mice suggests that the actions of GB88 are mediated by antagonism of PAR2.

### PAR2 was highly expressed by rat nociceptors

Proteases can induce neurogenic inflammation and nociception directly by activating PAR2 on primary sensory neurons (Steinhoff *et al.*, 2000) or indirectly by releasing stimulants from keratinocytes, which express high levels of PAR2 (Steinhoff *et al.*, 1999). We used *in situ* hybridization to examine the expression of PAR2 mRNA by primary sensory neurons in dorsal root and trigeminal ganglia of rat and mouse. In mice, PAR2 was detected at low levels in DRG neurons (data not shown), but was more prominently expressed in trigeminal neurons



**Figure 5**

Effects of GB88 on inflammation and nociception in PAR2 deficient mice. *Par2*<sup>+/+</sup> (wild-type, WT) or *par2*<sup>-/-</sup> (knockout, KO) mice were treated with GB88 ( $10 \text{ mg}\cdot\text{kg}^{-1}$  p.o.) or vehicle 2 h before intraplantar injection of Cat-S ( $14 \mu\text{g}$ ). Paw thickness (A) and paw withdrawal to mechanical stimulation (B) were measured hourly for 4 h.  $*P < 0.05$  compared with vehicle/vehicle control. Variable  $n$  indicates mouse number.

(Figure 6A). In rats, PAR2 mRNA was readily detected in DRG neurons (Figure 6B, C). PAR2-positive neurons were of small diameter and included peptidergic neurons expressing immunoreactive CGRP and non-peptidergic neurons that bound IB4 (Figure 6C–G). PAR2-positive neurons did not express NF200, a marker for large diameter neurons. Thus, PAR2 mRNA is abundantly expressed in rat nociceptors.

### GB88 antagonized activation of nociceptors by canonical and biased protease agonists of PAR2

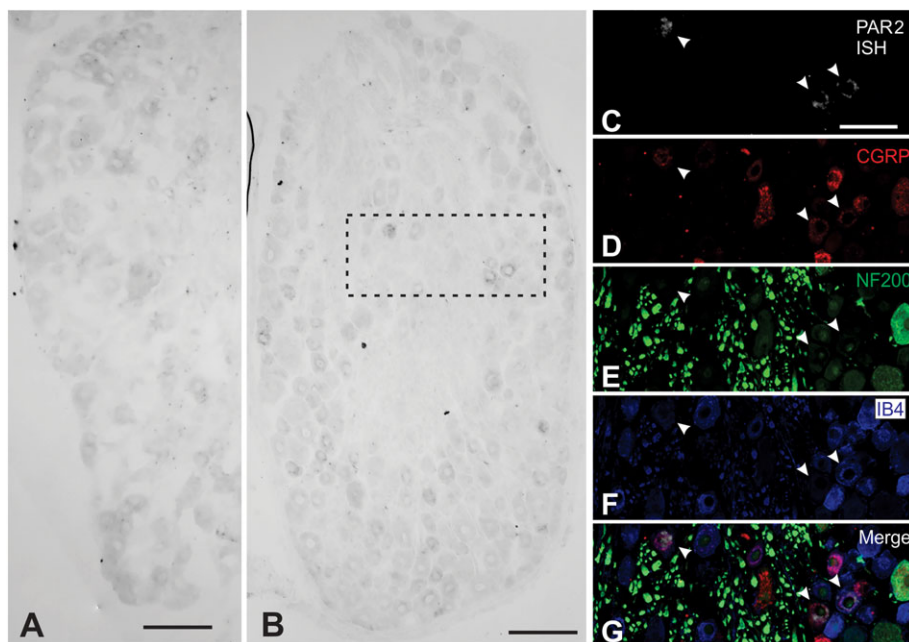
To determine whether GB88 can attenuate nociceptor activation by proteases that are canonical and biased agonists of PAR2, we examined protease-induced  $\text{Ca}^{2+}$  signalling in DRG neurons. We studied neurons from rats, rather than mice, due to the higher expression of PAR2 mRNA in rat nociceptors (Figure 6) and because PAR2 agonists generated larger signals in a higher proportion of DRG neurons from rats than mice (not shown). We have previously reported that canonical (trypsin, tryptase) and biased (cathepsin-S, elastase) proteolytic activators can induce PAR2-dependent  $\text{Ca}^{2+}$  signals in DRG neurons (Steinhoff *et al.*, 2000; Zhao *et al.*, 2014a; 2015). Whereas canonical proteases evoke PAR2 coupling to  $\text{G}\alpha_q$ , and mobilization of intracellular  $\text{Ca}^{2+}$ , cathepsin-S- and elastase-activated PAR2 does not couple to  $\text{G}\alpha_q$ , and instead causes  $\text{G}\alpha_s$ -, adenylyl cyclase- and PKA-mediated activation of TRPV4, which permits influx of  $\text{Ca}^{2+}$  ions from the extracellular fluid (Zhao *et al.*, 2014a, 2015).

Trypsin induced a rapid but transient increase in  $[\text{Ca}^{2+}]_i$  that was maximal at 2 min and returned to baseline after 5 min, consistent with mobilization of  $\text{Ca}^{2+}$  ions from

intracellular stores (Figure 7A). Cathepsin-S and elastase caused a gradual and sustained increase in  $[\text{Ca}^{2+}]_i$  that was maintained for at least 5 min, consistent with activation of TRPV4 and influx of extracellular  $\text{Ca}^{2+}$  ions (Figure 7C, F). GB88 markedly inhibited the magnitude of responses to trypsin, cathepsin-S and elastase. Of all the capsaicin- and KCl-responsive neurons,  $52 \pm 5\%$  responded to trypsin,  $49 \pm 7\%$  responded to cathepsin-S and  $57 \pm 10\%$  responded to elastase. GB88 reduced the proportion of responsive neurons by  $>60\%$  (Figure 7G). In contrast, GB88 neither affected the magnitude of the  $\text{Ca}^{2+}$  response to capsaicin nor the proportion of capsaicin-responsive neurons, consistent with its inability to inhibit capsaicin-evoked inflammation and nociception. The results suggest that GB88 inhibits proteolytic activation of nociceptive neurons, which has been shown to depend in a large part on PAR2 (Zhao *et al.*, 2014a, 2015).

### GB88 did not inhibit protease activity

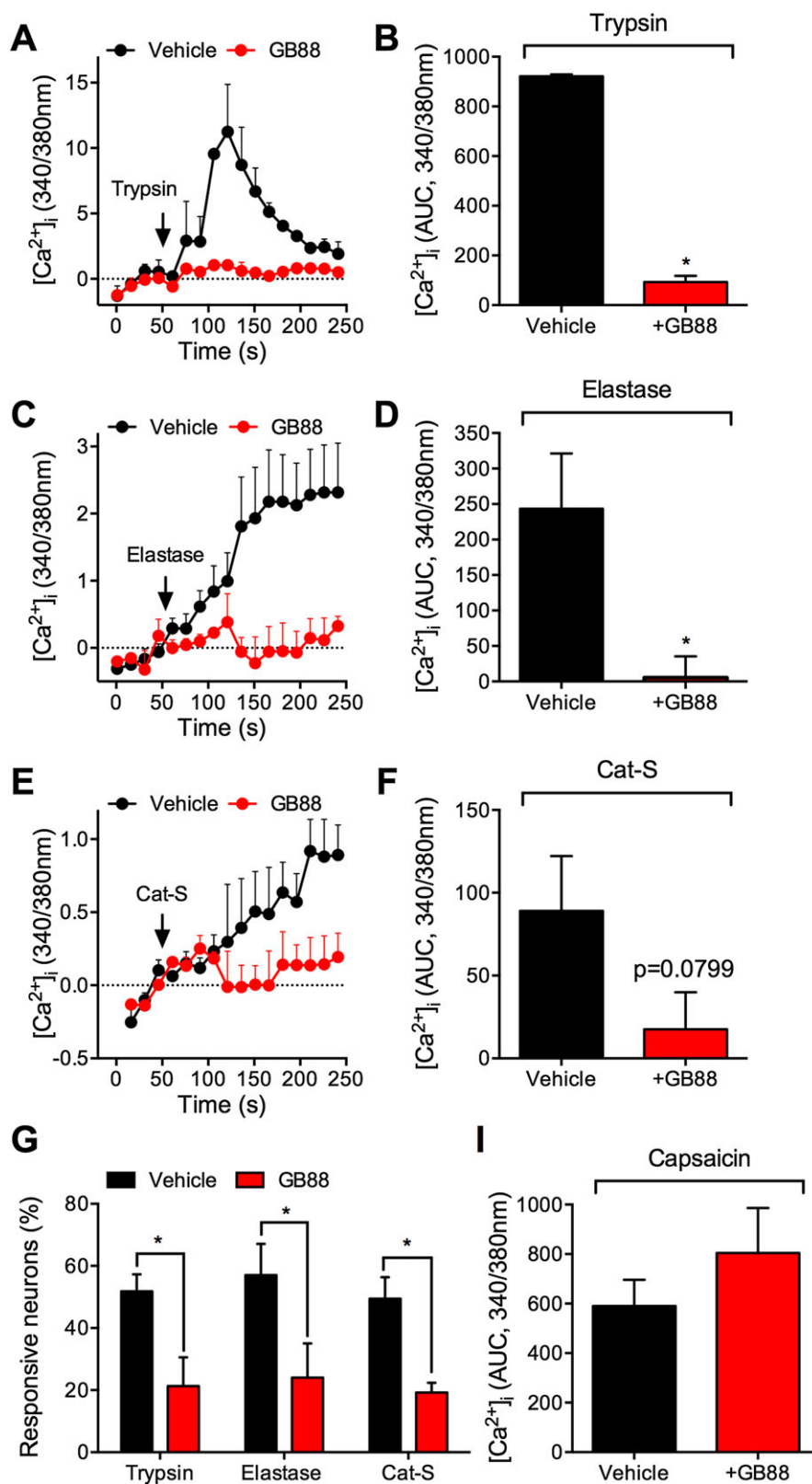
To eliminate the possibility that the anti-inflammatory and antinociceptive effects of GB88 were mediated by protease inhibition rather than PAR2 antagonism, we studied the ability of GB88 to prevent proteolytic activity. Using fluorogenic substrates, we monitored the activity of recombinant proteases upon initial interaction with GB88, mimicking the conditions that were used in the studies of DRG neurons. GB88 (1 or 10  $\mu\text{M}$ ) did not affect the activity of trypsin, elastase or cathepsin-S (Figure 8A). We also tested the ability of GB88 to inhibit the covalent binding of proteases to activity-based probes. In this assay, GB88 was incubated with the enzyme for 30 min. Trypsin activity was not affected at any concentration of GB88 tested (1, 10  $\mu\text{M}$ ) (Figure 8B, C). Cathepsin-S



**Figure 6**

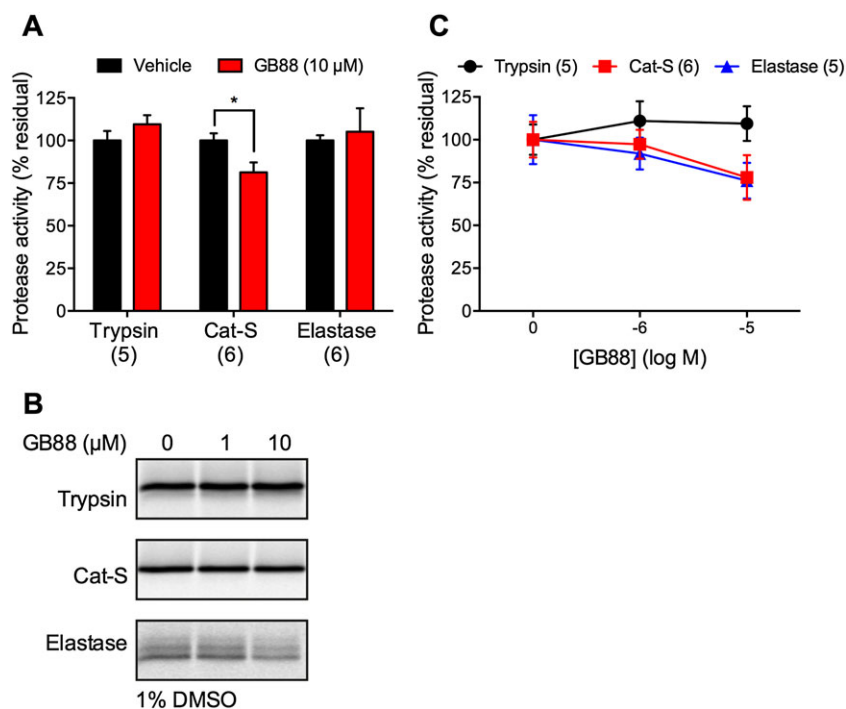
Localization of PAR2 mRNA in DRG. *In situ* hybridization on sections of mouse trigeminal ganglia (A) or rat DRG (B). (C–G) The inset shows an inverted image of PAR2 *in situ* hybridization (ISH; C), immunoreactive CGRP (D), immunoreactive neurofilament 200 (NF200; E), IB4 (F) and a merged image (G). Arrowheads show expression of PAR2 in small diameter neurons that expressed CGRP or bound IB4. Scale is 20  $\mu\text{m}$ .





**Figure 7**

Effects of GB88 on protease-induced  $Ca^{2+}$  signalling in DRG neurons. Rat DRG neurons were challenged with trypsin (A and B; 10 nM), elastase (C and D; 100 nM) or Cat-S (E and F; 100 nM) in the presence of GB88 (10  $\mu$ M) or vehicle (control). (A, C and E) Representative traces of kinetics of  $Ca^{2+}$  responses. (B, D and F) AUC from 50 to 250 s. (G) Effects of GB88 on the proportion of protease-responsive neurons that also responded to capsaicin. \* $P < 0.05$ ;  $n = 4-6$  rats, >100 neurons analysed from each rat.



**Figure 8**

Effects of GB88 on protease activity. (A) Effects of GB88 on protease cleavage of fluorogenic substrates. GB88 (10  $\mu$ M) was mixed with substrates (50  $\mu$ M). Proteases were added (final concentrations: trypsin, 10 nM; Cat-S, 100 nM; elastase, 100 nM), and fluorescence was monitored. The slope of the reaction was measured during the initial 60–120 s (in the linear range). (B and C) Effects of GB88 on protease labelling by fluorescent activity-based probes. Recombinant proteases were pretreated with GB88 (1, 10  $\mu$ M) in 1% DMSO. Residual activity was determined by labelling with activity-based probes and analysis by fluorescent SDS-PAGE. (B) A representative gel; (C) shows quantified signals. \* $P < 0.05$ ,  $n = 5$  or 6 separate experiments.

and elastase activities were modestly affected at 10  $\mu$ M (<25% inhibition). Hence, GB88 can directly inhibit proteases activity, but only at high concentrations that are unlikely to be achieved *in vivo*. Thus, the effects of GB88 on nociceptor activation, inflammation and nociception are unlikely to be due to direct effects on protease activity, but rather through antagonism of PAR2.

## Discussion and conclusions

### *GB88 antagonism of inflammation and nociception*

GB88 inhibited the proinflammatory and pronociceptive actions of proteases that are either canonical or biased agonists of PAR2. Trypsin, cathepsin-S and elastase exert proinflammatory and pronociceptive effects that are attenuated by *par2* deletion (Vergnolle *et al.*, 2001; Zhao *et al.*, 2014a; Zhao *et al.*, 2015). However, these proteases all cleave PAR2 at different sites and induce receptor coupling to distinct G proteins and signalling pathways. Trypsin causes PKC- and PKA-dependent sensitization of TRP channels and nociceptors, whereas cathepsin-S and elastase activate TRP channels and nociceptors solely *via* PKA (Amadesi *et al.*, 2006; Zhao *et al.*, 2014a; Zhao *et al.*, 2015). Despite these differences, we found that GB88 inhibited the proinflammatory and pronociceptive actions of trypsin, cathepsin-S and

elastase, consistent with the observation that *Par2* deletion inhibits trypsin-, cathepsin-S- and elastase-induced inflammation and nociception (Vergnolle *et al.*, 2001; Zhao *et al.*, 2014a; Zhao *et al.*, 2015). GB88 did not affect capsaicin-evoked and TRPV1-mediated inflammation and nociception and had no additional anti-inflammatory or antinociceptive actions in *par2*-deficient mice, consistent with PAR2 being the primary target of GB88 *in vivo*.

GB88 inhibited the proinflammatory and pronociceptive actions of the synthetic PAR2 agonists 2-furoyl-LIGRLO-NH<sub>2</sub> and AC264613. 2-furoyl-LIGRLO-NH<sub>2</sub> and AC264613 are selective for PAR2 over other PARs and induce oedema and hyperalgesia after intraplantar injection to mice (Kanke *et al.*, 2005; Gardell *et al.*, 2008; Suen *et al.*, 2012). These results are consistent with GB88 inhibiting protease-induced inflammation and pain and support the view that GB88 exerts anti-inflammatory and analgesic actions by antagonism of PAR2.

Trypsin, cathepsin-S and elastase each caused a sustained paw oedema and mechanical hyperalgesia in mice. Trypsin and cathepsin-S, but not elastase, also caused thermal hyperalgesia. The reason for the differences in the tendency of proteases to cause thermal hyperalgesia is unknown, but may relate to the activation of different signalling processes that differentially sensitize thermo-sensitive TRP channels. Although trypsin, cathepsin-S and elastase induced PAR2-dependent activation of TRPV4 (Zhao *et al.*, 2014a; Zhao *et al.*, 2015), trypsin can also sensitize TRPV1 and TRPA1

(Amadesi *et al.*, 2004; Dai *et al.*, 2007). Further studies are required to ascertain whether cathepsin-S and elastase can sensitize TRPV1 and TRPA1.

### GB88 antagonism of nociceptors

GB88 blocked the capacity of trypsin, cathepsin-S and elastase to activate nociceptors. DRG neurons expressing PAR2 mRNA were of small diameter and included peptidergic and non-peptidergic neurons with the characteristics of nociceptors. Our findings support other reports of prominent expression of PARs by nociceptors (Steinhoff *et al.*, 2000; Vellani *et al.*, 2010). Consistent with these findings, trypsin, cathepsin-S and elastase induced robust increases in  $[Ca^{2+}]_i$  in a substantial proportion of small diameter, capsaicin-sensitive rat DRG neurons. Whereas trypsin stimulated a rapid and transient increase in  $[Ca^{2+}]_i$ , consistent with mobilization of intracellular calcium stores, cathepsin-S and elastase induced a gradual and sustained increase in  $[Ca^{2+}]_i$ , which suggests activation of a plasma membrane channel and influx of extracellular  $Ca^{2+}$  ions. Regardless of the mechanism, GB88 inhibited the magnitude of protease-evoked signals and the proportion of neurons with detectable responses. Thus, PAR2 is a prominent mediator of protease signalling to nociceptive neurons. Residual responses in GB88-treated neurons may be attributed to activation of other receptors or channels. Elastase can also activate PAR<sub>1</sub>, and cathepsin-S activates MrgprC11, which are expressed in nociceptors (Vellani *et al.*, 2010; Mihara *et al.*, 2013; Reddy *et al.*, 2015).

### GB88 mechanism and selectivity

GB88 can antagonize mouse, rat and human PAR2, consistent with the high degree of PAR2 homology in these species (Ossovskaia and Bunnett, 2004). We observed that GB88 antagonizes mouse and rat PAR2, in agreement with reports that GB88 inhibits protease- and PAR2-mediated inflammation in rats (Barry *et al.*, 2010; Suen *et al.*, 2012; Lohman *et al.*, 2012a, b). GB88 also inhibits the effects of trypsin and synthetic PAR2 agonists on  $Ca^{2+}$  signalling in multiple human cell lines (Suen *et al.*, 2012; Lohman *et al.*, 2012a).

The molecular mechanism by which GB88 antagonizes PAR2 is not fully understood. GB88 is a competitive antagonist of SLIGRL-NH<sub>2</sub> and 2fLIGRLO-NH<sub>2</sub>, surmountable and PAR2-selective (Suen *et al.*, 2012), displaying antagonism of G<sub>α<sub>q</sub></sub> signalling but activation of other G-protein coupled signalling pathways (Suen *et al.*, 2014). However, it has not been reported yet whether GB88 binds at the same (orthosteric) site as the tethered ligand. We found that GB88 antagonizes the activation of PAR2 by multiple proteases and synthetic agonists. Trypsin and cathepsin-S cleave PAR2 at different sites to reveal distinctly different tethered ligands, whereas elastase activates PAR2 by a non-tethered ligand mechanism (Zhao *et al.*, 2014a, 2015). Although the trypsin-exposed tethered ligand interacts with the second extracellular loop of PAR2 (Ossovskaia and Bunnett, 2004), the binding site of the cathepsin-S-revealed tethered ligand is unknown. Thus, GB88 likely antagonizes PAR2 by stabilizing an inactive conformation because it does not inhibit cleavage nor binding of a specific tethered ligand, rather it inhibits activation by multiple proteases, multiple peptide agonists and also nonpeptide agonists. Further studies are required to define

the mechanisms by which GB88 specifically inhibits cathepsin-S and elastase activation of PAR2.

TRP channels are downstream targets of PAR2. PAR2 can sensitize TRPV1, and TRPV1 deletion or antagonism inhibits PAR2-dependent hyperalgesia (Amadesi *et al.*, 2004). We found that GB88 did not affect capsaicin-evoked calcium signals in nociceptors, consistent with its inability to inhibit the proinflammatory and hyperalgesic actions of capsaicin. These findings support the conclusion that GB88 prevents protease activation of nociceptors, inflammation and pain by antagonism of PAR2 rather than TRPV1.

To confirm that the effects of GB88 were not due to protease inhibition, we examined whether GB88 inhibits protease activity. By using a fluorogenic assay to mimic conditions of protease signalling to nociceptors in culture, we found that GB88 (10 μM) did not affect trypsin or elastase activity and had a modest effect on cathepsin-S activity. When pre-incubated with activity-based probes, GB88 did not affect trypsin, cathepsin-S or elastase binding. Thus, the effects of GB88 on inflammation and pain are more likely due to antagonism of PAR2 rather than inhibition of protease activity.

Multiple proteases become activated during injury and inflammation. The activity of these proteases is regulated by a large number of endogenous protease inhibitors. Thus, the balance between protease activation and inhibition is likely to be of crucial importance for the control of inflammatory and neuropathic pain. For example, cathepsin-S is activated in macrophages and spinal microglial cells during colitis and in neuropathic pain states (Clark *et al.*, 2007; Cattaruzza *et al.*, 2011), and mast cell tryptase is elevated in patients with visceral pain (Barbara *et al.*, 2004). Elastase released from leukocytes within sensory ganglia can contribute to neuropathic pain, which is exacerbated by deficiency in the elastase inhibitor serpinA3N (Vicuna *et al.*, 2015). Thus, the finding that GB88 inhibits the pronociceptive actions of diverse proteases suggests a potential to suppress different forms of inflammatory and neuropathic pain that are associated with the differential activation of proteases. The findings reported here support the usefulness of GB88 and potentially other PAR2 antagonists to inhibit inflammatory and painful conditions.

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## Author contributions

T.M.L. and E.S. analysed nociception and inflammation. P.Z. and D.P.P. studied nociceptor activation. R.B. localized receptors by *in situ* hybridization. L.E.M. and N.B. analysed the enzymatic activity. P.M., R.L. and D.F. provided GB88 and conceived the studies of nociception. N.W.B. designed the experiments and wrote the manuscript.

## Conflict of interest

The authors declare no conflicts of interest.

## Declaration of transparency and scientific rigour

This [Declaration](#) acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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