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RESEARCH PAPER

The anti-migraine component of butterbur extracts, isopetasin, desensitizes peptidergic nociceptors by acting on TRPA1 cation channel

Correspondence Serena Materazzi, PhD, Department of Health Sciences, Section of Clinical Pharmacology and Headache Center, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. E-mail: serena.materazzi@unifi.it

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Silvia Benemei[1,*], Francesco De Logu[1,*], Simone Li Puma[1], Ilaria Maddalena Marone[1], Elisabetta Coppi[1], Filippo Ugolini[1], Wolfgang Liedtke[2], Federica Pollastro[3], Giovanni Appendino[3], Pierangelo Geppetti[1], Serena Materazzi[1] and Romina Nassini[1]

[1]Department of Health Sciences, Section of Clinical Pharmacology and Headache Center, University of Florence, Florence, Italy, [2]Departments of Neurology, Anesthesiology and Neurobiology, Clinics for Headache, Head-Pain and Trigeminal Sensory Disorders, Duke University, Durham, NC, USA, and [3]Department of Pharmaceutical Sciences, University of Eastern Piedmont, Novara, Italy

*Equally contributing authors.

BACKGROUND AND PURPOSE

The mechanism of the anti-migraine action of extracts of butterbur [*Petasites hybridus* (L.) Gaertn.] is unknown. Here, we investigated the ability of isopetasin, a major constituent of these extracts, to specifically target TRPA1 channel and to affect functional responses relevant to migraine.

EXPERIMENTAL APPROACH

Single-cell calcium imaging and patch-clamp recordings in human and rodent TRPA1-expressing cells, neurogenic motor responses in rodent isolated urinary bladder, release of CGRP from mouse spinal cord *in vitro* and facial rubbing in mice and meningeal blood flow in rats were examined.

KEY RESULTS

Isopetasin induced (i) calcium responses and currents in rat/mouse trigeminal ganglion (TG) neurons and in cells expressing the human TRPA1, (ii) substance P-mediated contractions of rat isolated urinary bladders and (iii) CGRP release from mouse dorsal spinal cord, responses that were selectively abolished by genetic deletion or pharmacological antagonism of TRPA1 channels. Pre-exposure to isopetasin produced marked desensitization of allyl isothiocyanate (AITC, TRPA1 channel agonist)- or capsaicin (TRPV1 channel agonist)-evoked currents in rat TG neurons, contractions of rat or mouse bladder and CGRP release from mouse central terminals of primary sensory neurons. Repeated intragastric administration of isopetasin attenuated mouse facial rubbing, evoked by local AITC or capsaicin, and dilation of rat meningeal arteries by acrolein or ethanol (TRPA1 and TRPV1 channel agonists respectively).

CONCLUSION AND IMPLICATIONS

Activation of TRPA1 channels by isopetasin results in excitation of neuropeptide-containing nociceptors, followed by marked heterologous neuronal desensitization. Such atten uation in pain and neurogenic inflammation may account for the anti-migraine action of butterbur.

Abbreviations

AITC, allyl isothiocyanate; IMR90, human fetal lung fibroblasts; PAR2, proteinase activated receptor 2; SP, substance P; TG, trigeminal ganglion

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Introduction

For hundreds of years, butterburs (*Petasites*), herbaceous perennial plants belonging to the *Asteraceae*, which includes also *Tanacetum parthenium* L. (Materazzi *et al.*, 2013), have been used in folk medicine of northern Eurasia and America for therapeutic purposes, including treatment of fever, respiratory diseases, spasms and pain (Sutherland and Sweet, 2010). Among the many compounds contained in common butterbur [*Petasites hybridus* (L.) Gaertn.] (Aydin *et al.*, 2013), two major constituents, petasin and isopetasin (Avula *et al.*, 2012), are considered responsible for the anti-migraine effects of the herbal extract (Danesch and Rittinghausen, 2003). Clinical evidence (Grossmann and Schmidramsl, 2000; Lipton *et al.*, 2004; Pothmann and Danesch, 2005) of beneficial action in migraine prevention has been obtained with a preparation that contains standardized amounts (minimum 15%, corresponding to 7.5 mg) of petasin/isopetasin (Avula *et al.*, 2012; Danesch and Rittinghausen, 2003). The collective standardization is due to the instability of petasin, which spontaneously converts to isopetasin. Due to the stability issue, the specific role of each sesquiterpenoid for bioactivity has not been clearly identified.

Several hypotheses have been advanced to explain the anti-migraine action of petasin/isopetasin, including inhibition of leukotriene synthesis in leukocytes (Thomet *et al.*, 2001) and of voltage-sensitive calcium channels in arterial smooth muscle cells (Wang *et al.*, 2001; Wang *et al.*, 2002) or anti-muscarinic activity (Ko *et al.*, 2001). However, none of these actions seems to be relevant for migraine pathophysiology. Despite this mechanistic uncertainty, butterbur extract is currently recommended at high levels of strength for migraine prophylaxis (Holland *et al.*, 2012). Petasin and its cross-conjugated isomer, isopetasin, are eremophilane sesquiterpene esters of petasol and angelic acid (Supporting Information Figure S1A). Both compounds contain electrophilic double bonds and can potentially interact with bionucleophiles. Nevertheless, given the disubstitution at the β-carbon, they do not react with thiols, while giving a negative cysteamine assay (Avonto *et al.*, 2011).

The cation channel **TRPA1** belongs to the larger family of the TRP channels and, together with the **TRPV1** and **TRPV4** channels, is expressed by a sub-population of primary sensory neurons also characterized by their dual function of signalling pain and releasing the neuropeptides, substance P (**SP**) and CGRP, which mediate neurogenic inflammation (Nassini *et al.*, 2012). TRPA1 channels are selectively activated by a series of natural products, including **allyl isothiocyanate** (AITC, a constituent of horseradish) and cinnamaldehyde (a constituent of cinnamon). There is now robust evidence that TRPA1 channels transduce pain signals (Andersson *et al.*, 2008; Fusi *et al.*, 2014; Nassini *et al.*, 2015; Nassini *et al.*, 2014; Trevisan *et al.*, 2016) generated by an unprecedented series of reactive oxygen, nitrogen and carbonyl species that target the channel *via* nucleophilic attack of specific cysteine residues of the channels (Macpherson *et al.*, 2007).

A possible role of TRPA1 channels in migraine (Benemei *et al.*, 2014) is supported by its co-expression in the same nociceptor subpopulation (Bhattacharya *et al.*, 2008) with CGRP, the neuropeptide that markedly contributes to migraine pain (Edvinsson, 2015b; Ho *et al.*, 2008; Miller *et al.*, 2016; Olesen *et al.*, 2004; Sun *et al.*, 2016). Furthermore, agents known as potent headache and specific migraine triggers, such as **acrolein** (Kunkler *et al.*, 2011) or umbellulone (Benemei *et al.*, 2009), have been identified as agonists at TRPA1 channels (Kunkler *et al.*, 2011; Nassini *et al.*, 2012). It is relevant to the present study that another sesquiterpene, the lactone, parthenolide, a major constituent of feverfew (*Tanacetum parthenium*) (used for centuries for pain and headache treatment; Holland *et al.*, 2012), induces desensitization of TRPA1 channels and of nociceptors (Materazzi *et al.*, 2013). Other herbal medicines used for the treatment of pain and headaches, such as ligustilide, have been proposed to act by targeting the TRPA1 channel (Zhong *et al.*, 2011). Finally, the pyrazolone derivatives, dipyrone and propyphenazone (old analgesics with a well-established anti-migraine effect) (Bigal *et al.*, 2002) are poor cyclooxygenase inhibitors, while at clinically relevant doses, they selectively antagonize the TRPA1 channel (Nassini *et al.*, 2015).

Given the role of electrophilic compounds for the paradoxical induction and prevention of headache *via* modulation of the activity of the TRPA1 channel (Nassini *et al.*, 2012; Nilius and Szallasi, 2014), we wondered if the butterbur sesquiterpenoids could target the channel. Our findings indicated that the petasin family of compounds selectively targeted TRPA1 channel, leading to an initial neuronal excitation followed by marked desensitization of the afferent and efferent function of peptidergic nociceptors expressing TRPA1 channel. Similar to data reported for parthenolide, the present results suggest that the anti-migraine action of butterbur extracts may derive from the ability of isopetasin to evoke TRPA1-dependent desensitization of nociceptors that mediate neurogenic inflammation.

Methods

Animals

All animal care and experimental procedures were carried out according to the European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care procedures and were approved under University of Florence research permits #204/2012-B and #194/2015-PR. In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatments used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

C57BL/6 mice (male, 20–25 g, 5 weeks; Envigo, Milan, Italy; N = 208), littermate wild-type (*Trpa1*$^{+/+}$, N = 35) and TRPA1-deficient (*Trpa1*$^{-/-}$; N = 50) mice (25–30 g, 5–8 weeks), generated by heterozygotes on a C57BL/6 background (B6.129P–*Trpa1*$^{tm1Kykw/J}$; Jackson Laboratories, Bar Harbor, ME, USA), wild-type (*Trpv4*$^{+/+}$; N = 15) and TRPV4-deficient (*Trpv4*$^{-/-}$; N = 15) mice (25–30 g, 5–8 weeks), generated by heterozygotes on a C57BL/6 background (Liedtke and Friedman, 2003) and TRPV1-deficient mice (*Trpv1*$^{-/-}$; B6.129X1-*Trpv1*$^{tm1Jul/J}$; N = 15) backcrossed with C57BL/6 mice (*Trpv1*$^{+/+}$; N = 15) for at least 10 generations (Jackson Laboratories, Bar Harbor, ME, USA; 25–30 g,

5–8 weeks) or Sprague–Dawley rats (male, 75–100 g, Envigo, Milan, Italy; $N = 73$) were used.

Animals were housed in a controlled-temperature environment, 10 per cage (mice) or five per cage (rats), with wood shaving bedding and nesting material, maintained at 22 ± 1° C. Animals were housed with a 12 h light/dark cycle (lights on at 07:00 h) and fed with rodent chow (Global Diet 2018, Harlan, Lombardy, Italy) and tap water *ad libitum*. Animals were allowed to acclimatize to their housing environment for at least 7 days prior to experimentation and to the experimental room for 1 h before experiments. Behavioural experiments were done in a quiet, temperature-controlled (20 to 22°C) room between 09:00 and 17:00 h and were performed by an operator blinded to genotype and drug treatment. Animals were killed with inhaled CO_2 plus 10–50% O_2 under a valid institutional animal protocol.

Cell culture and isolation of primary sensory neurons

Naïve untransfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573™) were cultured according to the manufacturer's instructions. HEK293 cells were transiently transfected with the cDNAs (1 μg) coding for wild-type (wt-hTRPA1) or mutant 3C/K-Q human TRPA1 (C619S, C639S, C663S, K708Q; 3C/K-Q hTRPA1-HEK293) (both donated by D. Julius, University of California, San Francisco, CA, USA) (Hinman *et al.*, 2006) using the jetPRIME transfection reagent (Polyplus-transfection® SA, Strasburg, France) according to the manufacturer's protocol. HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293, donated by A.H. Morice, University of Hull, Hull, UK), or with cDNA for human TRPV1 (hTRPV1-HEK293, donated by M. J. Gunthorpe, GlaxoSmithKline, Harlow, UK), or with cDNA for human TRPV4 (hTRPV4-HEK293, donated by N.W. Bunnett, Monash Institute of Pharmaceutical Sciences, Parkville, Australia) were cultured as previously described (Nassini *et al.*, 2015). Human fetal lung fibroblasts (IMR90; American Type Culture Collection, Manassas, VA, USA; ATCC® CCL-186™), which express the native TRPA1 channel, were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Cells were plated on glass coated (poly-L-lysine, 8.3 μM) coverslips and cultured for 2–3 days before being used for recordings. For all cell lines, the cells were used as received without further authentication.

Primary trigeminal ganglion (TG) neurons were isolated from adult Sprague–Dawley rats and C57BL/6 or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice and cultured as previously described (Materazzi *et al.*, 2013). Briefly, TG were bilaterally excised under a dissection microscope and transferred to HBSS containing 1 $mg \cdot mL^{-1}$ of trypsin plus 2 $mg \cdot mL^{-1}$ of collagenase type 1A or papain, for rat or mouse ganglia, respectively, for enzymatic digestion (30 min, 37°C). Ganglia were then transferred to warmed DMEM containing: 10% FBS, 10% horse serum, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin and 100 $mg \cdot mL^{-1}$ streptomycin and dissociated into single cells by several passages through a series of syringe needles (23–25 G). Medium and ganglia cells were filtered to remove debris and centrifuged. The pellet was suspended in DMEM with added 100 $ng \cdot mL^{-1}$ mouse-NGF and 2.5 mM cytosine-D-arabinofuranoside free base. Neurons were then plated on glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM).

Cellular recordings

Mobilization of intracellular calcium ($[Ca^{2+}]_i$) was measured in untransfected or transfected HEK293 cells, in IMR90 and in primary sensory neurons, as previously reported (Materazzi *et al.*, 2013). Plated cells were loaded with 5 μM Fura-2 AM-ester (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37°C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After loading (40 min, 37°C), cells were washed and transferred to a chamber on the stage of an Olympus IX81 microscope for recording. Cells were excited alternately at 340 and 380 nm to indicate relative intracellular calcium changes by the $Ratio_{340/380}$ ($R_{340/380}$) and recorded with a dynamic image analysis system (XCellence Imaging software; Olympus srl, Milan, Italy). Results are expressed as the percentage of increase of $R_{340/380}$ over the baseline normalized to the maximum effect induced by ionomycin (5 μM) (% Change in $R_{340/380}$).

Whole-cell patch-clamp recordings were performed in untransfected and transfected HEK293 cells and in primary sensory neurons plated on coated coverslips as previously reported (Materazzi *et al.*, 2013). Briefly, coverslips were transferred to a recording chamber (1 mL volume), mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 2 $mL \cdot min^{-1}$ with a standard extracellular solution containing (in mM): 10 HEPES, 10 D-glucose, 147 NaCl, 4 KCl, 1 $MgCl_2$ and 2 $CaCl_2$ (pH adjusted to 7.4 with NaOH). Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 4–7 MΩ. Pipette solution used for HEK293 cells contained (in mM): 134 K-gluconate, 10 KCl, 11 EGTA, 10 HEPES (pH adjusted to 7.4 with KOH). When recordings were performed on rat neurons, 5 mM CaCl2 was present in the extracellular solution and pipette solution contained (in mM): 120 CsCl, 3 Mg_2ATP, 10 BAPTA, 10 HEPES-Na (pH adjusted to 7.4 with CsOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analysed with a pClamp 9.2 software (Axon Instruments, CA, USA). All the experiments were carried out at room temperature (20–22°C). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a ±10 mV voltage pulse. Currents were detected as inward currents activated on cell superfusion with the various stimuli in the voltage-clamp mode at a holding potential of −60 mV. Peak currents activated in each experimental condition were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Signals were sampled at 10 kHz and low-pass filtered at 10 kHz.

Cells and neurons were challenged with isopetasin (0.1 μM–3 mM), isopetasol (0.1 μM–3 mM) and angelic acid (Santa Cruz Biotechnology Inc.; TX, USA) (0.1 μM–3 mM) to assess their ability to promote a cellular response. To induce TRPA1 channel-dependent responses, cells and neurons were

challenged with the selective agonist, **AITC** (1–10 μM). **GSK1016790A** (0.05–0.1 μM) was used to induce a selective response from TRPV4 channel. Buffer solution containing DMSO 0.5% was used as vehicle. **Capsaicin** (0.1–1 μM) was used to induce a selective response of TRPV1 channel and to identify capsaicin-sensitive neurons. The activating peptide for human **proteinase-activated receptor 2** (hPAR2-AP; 100 μM) or KCl (50 mM) were used to elicit a TRP-independent cellular response. Some experiments were performed in the presence of selective antagonists for TRPA1, **HC-030031** (50 μM), for TRPV1, **capsazepine** (10 μM), for TRPV4 channels, **HC-067047** (30 μM) or their respective vehicles (0.5, 0.1 and 0.3% DMSO respectively).

Organ bath assays

Contractile response studies were performed on rat or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice urinary bladder strips. Briefly, urinary bladder was excised from rat or mouse and longitudinal strips were suspended at a resting tension of 1 g for rat and 0.5 g for mouse tissues in 10 mL organ bath bathed in aerated (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl and 11 D-glucose, maintained at 37°C. After 45 min of equilibration, tissues were contracted twice with carbachol (l μM), with a 45 min washing out period between the first and second administration. Tissues were challenged with isopetasin (10–300 μM), AITC (100 μM), GSK1016790A (10 μM) and capsaicin (0.3 μM) or their vehicles. In some experiments, tissues were pretreated with the TRPA1 channel antagonist, HC-030031 (50 μM), the TRPV1 channel antagonist, capsazepine (10 μM), the TRPV4 channel antagonist, HC-067047 (30 μM) or a combination of NK_1 and NK_2 receptor antagonists, L-733060 and **SR48968** respectively, (both 1 μM). Some tissue preparations were desensitized by exposure to capsaicin (10 μM for 20 min, twice). Similarly, some preparations were exposed to isopetasin (100–300 μM for 20 min, twice) before the challenge with various stimuli. Motor activity of tissue preparation was recorded isometrically on a force transducer (Harvard Apparatus, Ltd, Kent, UK). Responses were expressed as percentage (%) of the maximum contraction induced by carbachol (1 μM).

CGRP-like immunoreactivity (CGRP-LI) assay

For CGRP-LI outflow experiments, 0.4 mm slices of mouse spinal cord (combined cervical, thoracic and lumbosacral segments) were superfused with an aerated (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing 0.1% bovine serum albumin plus the angiotensin-converting enzyme inhibitor, captopril (1 μM), and the neutral endopeptidase inhibitor, phosphoramidon (1 μM), to minimize peptide degradation. Tissues were stimulated with isopetasin (10–100 μM) or its vehicle (1% DMSO) dissolved in modified Krebs–Henseleit solution and superfused for 10 min. Some tissues were pre-exposed to capsaicin (10 μM, 30 min) or superfused with a calcium-free buffer containing EDTA (1 mM). Other tissues were pre-exposed to a high concentration of isopetasin (300 μM, 30 min) and then, after a prolonged washing (40 min), stimulated with (100 μM), capsaicin (0.3 μM), GSK1016790A (10 μM) or KCl (40 mM). Fractions (4 mL) of superfusate were collected at 10 min intervals before, during and after administration of the stimulus and then freeze-dried, reconstituted with assay buffer and analysed for CGRP-LI by using a commercial enzyme-linked immunosorbent assay kit (Bertin Pharma, Montigny le Bretonneux, France). None of the used substances showed any cross reactivity with the CGRP antiserum. CGRP-LI was calculated by subtracting the mean prestimulus value from those obtained during or after stimulation. Detection limits of the assays were 5 pg·mL^{-1}. Results were expressed as femtomoles of peptide *per* gram of tissue. Stimuli did not cross-react with CGRP antiserum.

Behavioural experiments (face rubbing)

For behavioural experiments, after habituation, C57BL/6, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$, *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice were randomized to treatment groups, consistent with experimental design. Each experiment was repeated two to three times (using two or three animals in each experimental session). For the *in vivo* experiments, the first outcome assessed was acute spontaneous nociception evoked by the s.c. injection (10 μL), into the right mouse whisker pad (perinasal area), of AITC (10–100 nmol), capsaicin (0.5–3 nmol), GSK1016790A (1–5 nmol), isopetasin (5–50 nmol) or their vehicle (2.5% DMSO). Nociception was assessed by measuring the time (seconds) that the animal spent rubbing the injected area of the face with its paws (Luccarini *et al.*, 2006). These observations were made without knowledge of the treatments given (blinded). The nociceptive effects of s.c. injection (10 μL) of isopetasin (50 nmol) and AITC (50 nmol) or capsaicin (3 nmol) or GSK1016790A (2.5 nmol) were also tested in Trpa1$^{+/+}$ and Trpa1$^{-/-}$, Trpv1$^{+/+}$ or Trpv1$^{-/-}$, Trpv4$^{+/+}$ or Trpv4$^{-/-}$ mice respectively. To assess the pharmacological target of isopetasin, C57BL/6 mice received isopetasin (50 nmol in 10 μL, s.c.) 60 min after i.p. treatment with the selective TRPA1 channel antagonist, HC-030031 (100 mg·kg^{-1}) or 30 min after the selective TRPV1 channel antagonist, capsazepine (4 mg·kg^{-1}) or 60 min after the selective TRPV4 channel antagonist, HC-067047 (100 mg·kg^{-1}) or their vehicle (4% DMSO plus 4% tween 80 in isotonic saline).

Then, the preventive effect of isopetasin on nociceptive responses evoked by known TRPA1, TRPV1 and TRPV4 channel agonists was measured. Isopetasin (5 mg·kg^{-1}) was administered i.g. *quod diem* (q.d.) for 5 days in C57BL/6 or *Trpa1*$^{-/-}$ mice and each day, 60 min later, AITC (50 nmol) or capsaicin (3 nmol) or GSK1016790A (2.5 nmol) or their vehicle were injected (10 μL, s.c.) and behavioural tests performed, as described above. The dose of isopetasin for preventive purposes was calculated according to the National Institute of Health conversion formula in order to administer to animals a dose similar to that has been proved efficacious for migraine prevention in humans (Reagan-Shaw *et al.*, 2008).

Dural blood flow

Rats were anesthetized (urethane, 1.4 g in 10 mL·kg^{-1}, i.p.), and the head fixed in a stereotaxic frame. A cranial window (4 × 6 mm) was opened into the parietal bone to expose the *dura mater*. Changes in rat middle meningeal artery blood flow were recorded with a Laser Doppler Flowmeter (Perimed Instruments, Milan, Italy). The probe (needle type, tip diameter 0.8 mm) was fixed near a branch of the middle

meningeal artery (1 mm from the dural outer layer). The window was filled with synthetic interstitial fluid. In a first set of experiments, dural blood flow was monitored for 30 min after administration of isopetasin (5 mg in 10 mL·kg^{-1}, i.p.) or its vehicle. In a second set of experiments, dural blood flow was measured after the administration of acrolein (50 nmol, intranasal), ethanol (140 μL·kg^{-1}, i.v.), sodium nitroprusside (1 mM, 100 μL, topical to the exposed dura mater) or their vehicles (isotonic saline) in rats treated for 5 days with isopetasin (5 mg·kg^{-1}, i.g.) or its vehicle 60 min after the treatment. Baseline flow was calculated by the mean flow value measured during a 5 min period prior to stimulus. The increase in blood flow was calculated as change (%) over the baseline.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are reported as mean ± SEM. Statistical analysis was performed by the unpaired two-tailed Student's *t*-test for comparisons between two groups and the ANOVA, followed by the Bonferroni *post hoc* test, for comparisons between multiple groups (GraphPadPrism version 5.00, San Diego, CA, USA). Agonist potency was expressed as the EC_{50}. $P < 0.05$ was considered statistically significant.

Materials

^{1}H (500 and 400 MHz) NMR spectra were measured on Varian INOVA NMR spectrometers (Palo Alto, CA, USA). Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δH = 7.26). Silica gel 60 (70–230 mesh) used for gravity column chromatography was purchased from Macherey-Nagel (Düren, Germany). Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates (Kenilworth, NJ, USA), visualized by staining with 5% H_2SO_4 in methanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Chemical reagents and solvents were from Sigma-Aldrich (Milan, Italy) and were used without any further purification unless stated otherwise. All natural compounds were finally purified with Jasco-HPLC, Hichrom column 21.2 × 250 mm (Tokyo, Japan), normal phase with PU-2080 binary pump and UV-2075 plus detector to guarantee a purification degree for biological assays.

For petasin isolation, a sample (500 g) of common butterbur [*P. hybridus* (L.) Gaertn.] roots collected in the Swiss Alps was powdered using a kitchen blender. The root powder was extracted twice with 5 L of acetone (ratio plant:solvent 1:10), affording, after filtration and evaporation, 14.36 g (2.9%) of a dark brown oily extract. The latter was purified by gravity column chromatography on silica gel using petroleum ether-EtOAc (from 9:1 to 7:3) as eluant to afford 9 g (1.8%) of petasin as yellow oil, identified by comparison with spectroscopic data. For isomerization of petasin to isopetasin, 500 mg of NaH 95% (36.53 mmol) was added to a solution of petasin (390 mg, 1.23 mmol) in toluene (10 mL). After stirring for 20 h at room temperature, the reaction was quenched by dilution with water; 2N H_2SO_4 was added, and the reaction was extracted with ethyl acetate. The organic phase was dried, filtered and evaporated. The residue, a brown oil, was purified by gravity column chromatography on silica gel using petroleum ether-EtOAc (9:1) as eluant and further purified with HPLC to yield 256 mg (65.6%) of isopetasin (1b) as yellow oil. The hydrolysis of petasin in isopetasol has been obtained by adding petasin (350 mg, 1.10 mmol) to a 5% KOH methanol (10 mL) solution. After stirring for 20 h at room temperature, the reaction was diluted with 2N H_2SO_4 and extracted with EtOAc. The organic phase was dried, filtered and evaporated. The residue, a brown oil, was purified by gravity CC on silica gel using petroleum-ether (5:5) as eluant and further purified with HPLC to yield 187.7 mg (72.8%) of isopetasol as white crystals.

For *in vitro* experiments, isopetasin and isopetasol were dissolved in 100% DMSO at 10 mM concentration and then diluted in aqueous solution. For systemic *in vivo* treatment, isopetasin (5 mg in 10 mL·kg^{-1}) was dissolved in 0.5% carboxymethylcellulose solution (CMC) for intragastric (i.g.) administration or in 4% DMSO plus 4% tween 80 in isotonic saline for i.p. administration. **HC-030031** was synthesized as previously described (Andre *et al.*, 2008). If not otherwise indicated, all other reagents were from Sigma-Aldrich (Milan, Italy) and dissolved in appropriate vehicle solutions.

Nomenclature of targets and ligands

Key protein targets and ligands in this article 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 (Alexander *et al.*, 2015a,b).

Results

Isopetasin targets the human TRPA1 channel

In hTRPA1-HEK293 cells, isopetasin and isopetasol, but not angelic acid, produced concentration-dependent increases in $[Ca^{2+}]_i$ (EC_{50s} 10 and 100 μM respectively), responses that were attenuated by the selective TRPA1 channel antagonist, HC-030031 (Figure 1A and Supporting Information Figure S1C). hTRPV1-HEK293 and hTRPV4-HEK293 cells that were activated by capsaicin or GSK1016790A (TRPV1 and TRPV4 channel agonists respectively) did not respond to isopetasin (Figure 1C, D). In whole-cell patch-clamp recordings, isopetasin elicited inward currents in hTRPA1-HEK293 cells that were abolished by HC-030031 (Figure 1B). Isopetasin did not evoke any current in hTRPV1-HEK293 and hTRPV4-HEK293 cells (Figure 1C, D). Isopetasin and isopetasol did not induce any cellular response in naïve HEK293 cells (Supporting Information Figure S1B, D). 3C/K-Q hTRPA1-HEK293 cells, which express a TRPA1 which lacks three cysteine and one lysine residues, responded to the non-electrophilic agonist, menthol, as wild-type hTRPA1-HEK293, but failed to respond to isopetasin (50 μM) (Figure 1E). In IMR90, which constitutively express the TRPA1 channel (Jaquemar *et al.*, 1999), isopetasin concentration-dependently increased $[Ca^{2+}]_i$, (EC_{50} 8 μM), a response that was abolished by HC-030031 (Figure 1F and Supporting Information Figure S1E). Calcium responses

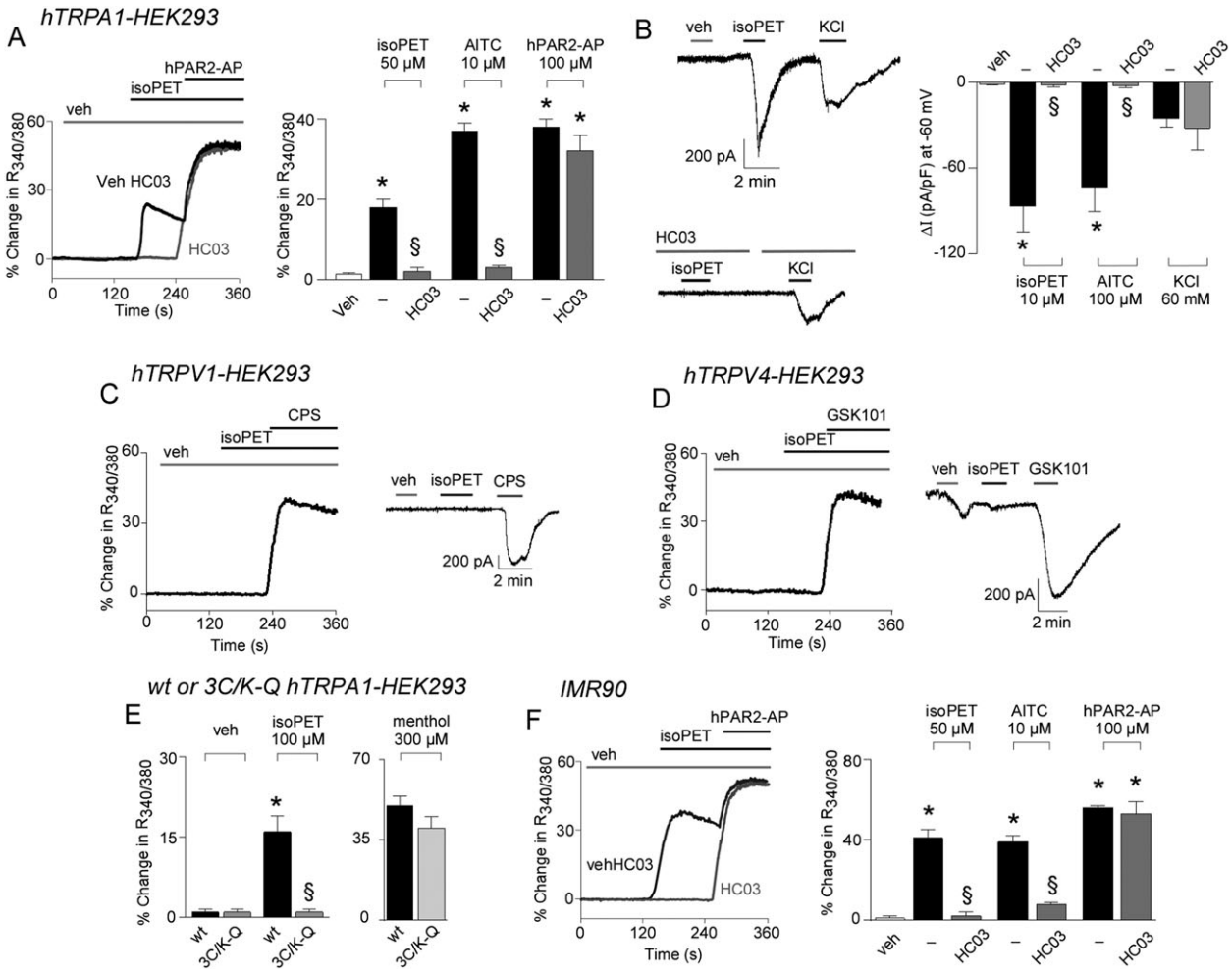

Figure 1

Isopetasin (isoPET) selectively activates the human TRPA1 channels. (A) Representative traces and pooled data of the $[Ca^{2+}]_i$ response evoked by isoPET (50 μM) in HEK293 cells transfected with the cDNA of human TRPA1 (hTRPA1-HEK293). $[Ca^{2+}]_i$ response evoked by isoPET (50 μM) and the selective TRPA1 channel agonist, AITC (10 μM) are abolished by the selective TRPA1 channel antagonist, HC-030031 (HC03; 30 μM), which does not affect the response evoked by the activating peptide for hPAR2 (hPAR2-AP (100 μM). (B) Representative traces and pooled data of the whole-cell patch-clamp inward currents evoked by isoPET (10 μM), AITC (100 μM) and KCl (60 mM) in hTRPA1-HEK293. HC03 abates the response to both isoPET and AITC without affecting the KCl response. (C, D) Representative traces of $[Ca^{2+}]_i$ responses and whole-cell patch-clamp inward currents in HEK293 cells transfected with the cDNA of human TRPV1 (hTRPV1-HEK293) (C) or with the cDNA of human TRPV4 (hTRPV4-HEK293) (D) show that isoPET (50 μM in $[Ca^{2+}]_i$ imaging or 10 μM in electrophysiological experiments) does not activate either TRPV1 or TRPV4 channels, which are activated by their respective agonist, capsaicin (CPS; 1 μM) and GSK1016790A (GSK101; 100 nM) respectively. (E) Pooled data of $[Ca^{2+}]_i$ responses evoked by isoPET (100 μM) and menthol (300 μM) in wild-type (*wt*) and mutant (3C/K-Q) hTRPA1-HEK293 transfected cells. (F) Representative traces and pooled-data of $[Ca^{2+}]_i$ response induced by isoPET in IMR90. HC03 inhibits the $[Ca^{2+}]_i$ response activated by isoPET (50 μM) but not that evoked by hPAR2-AP (100 μM). Veh is the vehicle of isoPET. Each column represents the mean ± SEM of $n > 25$ cells from three to six independent experiments for $[Ca^{2+}]_i$ recording or $n > 5$ cells from four to eight independent experiments for electrophysiological recording. Dash indicates combined vehicles of treatments. $^*P < 0.05$, significantly different from veh, $^{\S}P < 0.05$, significantly different from isoPET; ANOVA followed by Bonferroni test.

elicited by hPAR2-AP and ion currents evoked by KCl were not affected by HC-030031 indicating selectivity (Figure 1A, B, F and Supporting Information Figure S1B–D).

Isopetasin selectively activates the rodent and human TRPA1 channel

Exposure of cultures of rat TG neurons to isopetasin evoked a concentration-dependent (EC_{50} 10 μM) $[Ca^{2+}]_i$ increase in neurons that responded to capsaicin (Figure 2A and Supporting Information Figure S1F). Responses to isopetasin were abolished by HC-030031, but not by selective TRPV1 (capsazepine) or TRPV4 channel (HC-067047) antagonists (Figure 2A). Notably, while isopetasin, AITC and capsaicin activated TG neurons from *Trpa1*$^{+/+}$ mice, neurons from *Trpa1*$^{-/-}$ mice responded only to capsaicin (Figure 2C). In capsaicin-sensitive rat TG

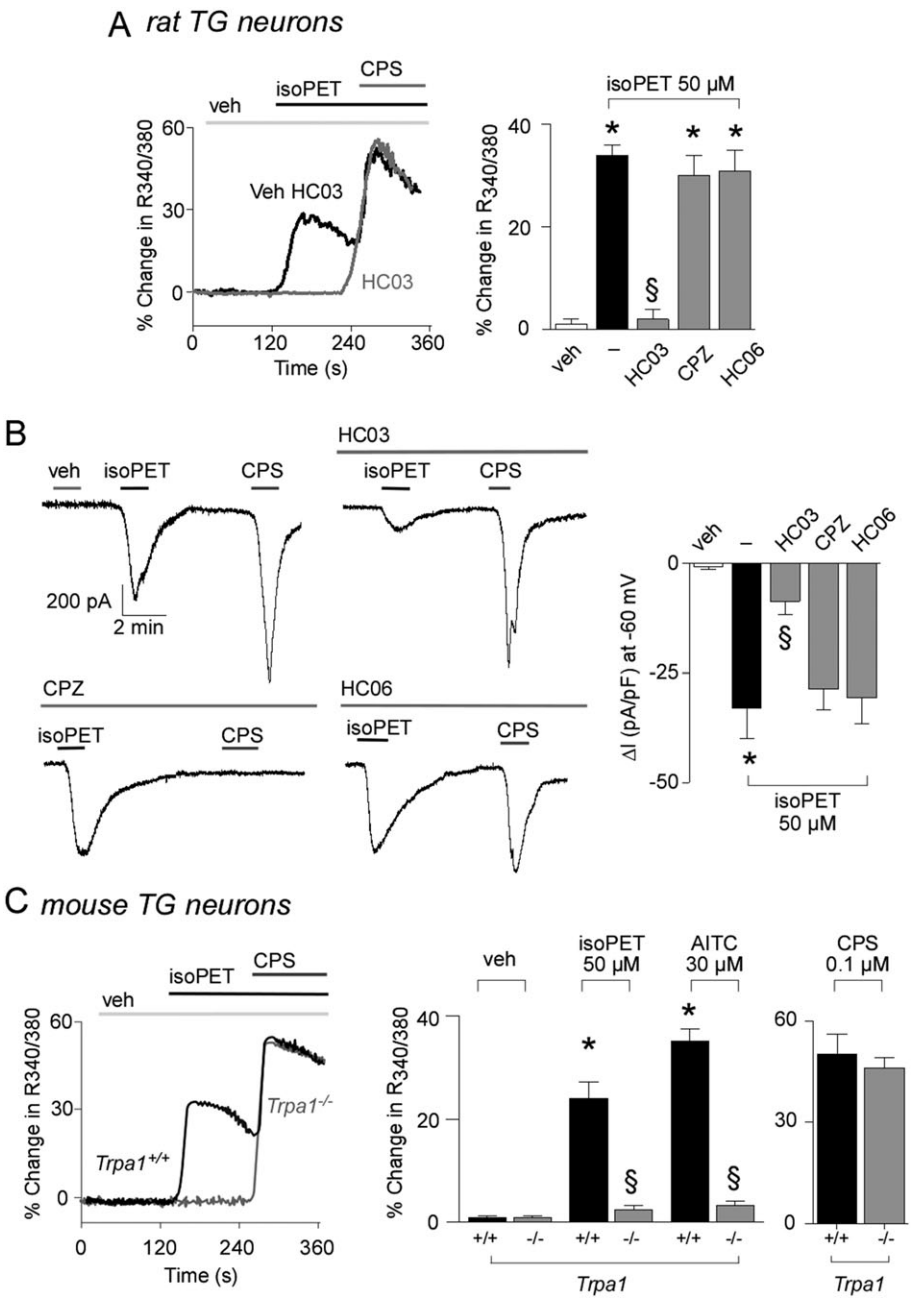

Figure 2

Isopetasin (isoPET) selectively activates TRPA1 channel in cultures of rodent TG neurons. (A, B) Representative traces and pooled data of $[Ca^{2+}]_i$ responses (A) and whole-cell patch-clamp inward currents (B) evoked by isoPET (50 μM) in rat TG neurons responding to capsaicin [CPS; 0.1 μM (A) and 1 μM (B)]. Both $[Ca^{2+}]_i$ responses and ion currents elicited by isoPET are abolished in the presence of the selective TRPA1 channel antagonist, HC-030031 [HC03; 30 μM (A) and 50 μM (B)], and unaffected in the presence of the selective antagonist for TRPV1 channels, capsazepine (CPZ; 10 μM), or TRPV4 channels, HC-067047 (HC06; 30 μM). (C) Representative traces and pooled data of the $[Ca^{2+}]_i$ response evoked by isoPET (50 μM) or AITC (30 μM) in cultured TG neurons from *Trpa1*$^{+/+}$ mice; both responses are absent in neurons from *Trpa1*$^{-/-}$ mice. Responses to capsaicin (CPS; 0.1 μM) are unchanged. Veh is the vehicle of isoPET. Each column represents the mean ± SEM of $n > 20$ neurons from three to six independent experiments for $[Ca^{2+}]_i$ recordings or of at least $n > 5$ cells from four to eight independent experiments for electrophysiological recordings. Dash indicates combined vehicles of treatments. *$P < 0.05$, significantly different from veh, §$P < 0.05$, significantly different from isoPET; ANOVA followed by Bonferroni test.

neurons, isopetasin evoked inward currents that were selectively blocked by HC-030031 but unaffected by capsazepine and HC-067047 (Figure 2B). Thus, calcium and electrophysiology data indicated that TRPA1 channel are necessary and sufficient for rodent nociceptors and human cells to respond to isopetasin.

Isopetasin excites and desensitizes rodent peptidergic nociceptors

In cultured rat TG neurons, currents evoked by AITC or capsaicin were unaffected by pre-exposure to AITC but were markedly desensitized after pre-exposure to isopetasin at high (50 μM), but not at low concentration (5 μM) (Figure 3A). Isopetasin-evoked CGRP release from mouse dorsal spinal cord slices was abolished in tissues desensitized to capsaicin or in the absence of extracellular Ca^{2+} (Figure 3D), indicating that isopetasin evokes a neurosecretory process from neurons expressing TRPV1 channel. The role of TRPA1 channel in neuronal excitation and the ensuing neurosecretion by isopetasin was demonstrated by its failure to evoke any release in spinal cord slices from *Trpa1*$^{-/-}$ mice (Figure 3E). Notably, exposure to an elevated concentration of isopetasin attenuated the release of CGRP produced by AITC, capsaicin and the non-specific depolarizing agent KCl (Figure 3F), indicating heterologous neuronal desensitization.

Terminals of peptidergic primary sensory neurons are widely expressed in most tissues and organs. The rodent urinary bladder contains a dense network of SP-containing nerve fibres, whose stimulation results in SP release from intramural sensory nerve endings that *via* **NK_1/NK_2** receptor stimulation contracts the bladder smooth muscle (Steinhoff *et al.*, 2014).

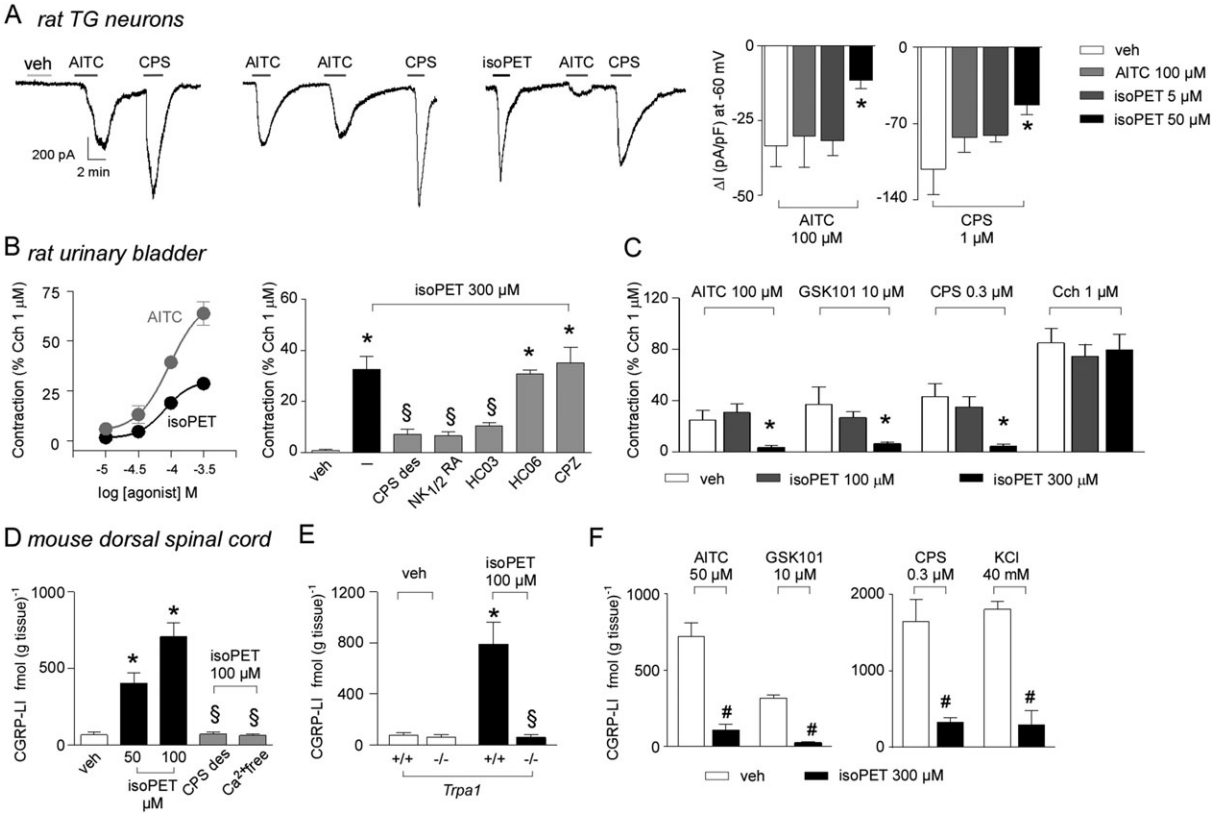

Figure 3

Isopetasin (isoPET) elicits characteristic desensitization of TRPA1 channel. (A) Representative traces and pooled data of whole-cell patch-clamp inward currents through rat TG neurons showing that the initial challenge with isoPET (50 μM), instead of AITC (100 μM), or its vehicle (veh), produces neuronal desensitization with a reduced response to the subsequent exposure to AITC (100 μM) and capsaicin (CPS; 1 μM). Each column represents the mean ± SEM of $n > 6$ independent experiments. (B) Concentration–response curve of the contractile response evoked by AITC and isoPET in rat urinary bladder strips. At the highest concentration used, isoPET (300 μM) evokes a contractile response that is selectively inhibited by the selective TRPA1 channel antagonist HC-030031 (HC03; 50 μM) and unaffected by the selective TRPV1 channel antagonist capsazepine (CPZ; 10 μM) or the selective TRPV4 channel antagonist, HC-067047 (HC06; 30 μM). The isoPET contraction is also abolished by a combination of NK_1 and NK_2 receptor antagonists (NK1/2 RA; L-733060 and SR48968, both 1 μM) and after exposure (20 min, twice) to a high concentration of capsaicin (CPS; 10 μM) that induces neuronal desensitization. (C) Exposure (20 min, twice) to the highest concentration of isoPET (300 μM) markedly reduces the contractile response evoked by a selective TRPA1, TRPV4 or TRPV1 channel agonist, AITC (100 μM), GSK1016790A (GSK101; 10 μM) or CPS (0.3 μM), but does not affect the response to carbachol (Cch; 1 μM). (D) isoPET increases the CGRP-LI outflow from mouse dorsal spinal cord slices in a concentration-dependent manner. Effect of isoPET (100 μM) is abolished by calcium removal (Ca^{2+}-free), capsaicin desensitization (CPS-des). (E) isoPET (100 μM) increases CGRP-LI outflow from spinal cord slices from *Trpa1*$^{+/+}$ mice, but not from *Trpa1*$^{-/-}$ mice. (F) Exposure to a high concentration of isoPET (300 μM, 30 min) blocks CGRP-LI release evoked by AITC (50 μM), GSK101 (10 μM), CPS (0.3 μM) or KCl (40 mM). Each column represents the mean ± SEM of $n = 4$ independent experiments. Dash indicates combined vehicles of the treatments. *$P < 0.05$, significantly different from veh; $^{§}P < 0.05$, significantly different from isoPET; ANOVA followed by Bonferroni test. $^{\#}P < 0.05$, significantly different from isoPET, Student's *t*-test.

Isopetasin caused a concentration-dependent contraction of isolated strips of the rat urinary bladder, a response attenuated by desensitization to capsaicin, NK_1 and NK_2 receptor (L-733060 and SR48968 respectively) antagonism and HC-030031, but not by capsazepine or HC-067047 (Figure 3B). In addition, isopetasin induced contractile responses in isolated strips of urinary bladders obtained from *Trpa1*$^{+/+}$ mice, but not in those obtained from *Trpa1*$^{-/-}$ mice (Supporting Information Figure S2A). Thus, isopetasin-evoked contractions were the result of SP release from peptidergic sensory nerve terminals, through activation of TRPA1 channels. Exposure of urinary bladder strips to a high concentration of isopetasin markedly attenuated contractions evoked by AITC and capsaicin, but not by carbachol, suggesting neuronal desensitization that, however, preserved the contractility of the bladder smooth muscle (Figure 3C). Furthermore, exposure to a high isopetasin concentration attenuated the contractile response to capsaicin in isolated strips of urinary bladders obtained from *Trpa1*$^{+/+}$ mice, but not in those obtained from *Trpa1*$^{-/-}$ mice (Supporting Information Figure S2B), suggesting that isopetasin induces neuronal desensitization by selective targeting of TRPA1 channels.

Isopetasin inhibits nociception and neurogenic dural vasodilatation via TRPA1 channel

Subcutaneous injection of irritant agents into the upper whisker pad of mice produces a typical nocifensor behaviour described as facial rubbing (Luccarini *et al.*, 2006). Injection (s.c.) of AITC into the upper whisker pad evoked a transient (<15 min) and dose-dependent facial rubbing that was abolished by HC-030031 (Figure 4A). Similarly, facial rubbing produced by capsaicin was attenuated by capsazepine (Figure 4B), while facial rubbing evoked by GSK1016790A was inhibited by the TRPV4 channel antagonist, HC-067047 (Figure 4C). Isopetasin produced facial rubbing that reproduced the features of the response to AITC, being abolished by HC-030031 and unaffected by capsazepine or HC-067047 (Figure 4D). Moreover, similar to AITC, isopetasin failed to provoke any facial rubbing in *Trpa1*$^{-/-}$ mice (Figure 4E). Isopetasin-evoked facial rubbing in *Trpv1*$^{-/-}$ and *Trpv4*$^{-/-}$ mice was similar to that observed in their respective wild-type littermates (Figure 4F).

A single dose of isopetasin (given i.g.) did not affect the ability of local AITC, capsaicin or GSK1016790A to evoke facial rubbing (Figure 5A). However, after giving a single dose of isopetasin for three consecutive days, rubbing responses to AITC and GSK1016790A were reduced, whereas that to capsaicin was unaffected (Figure 5A). Following isopetasin administration for five consecutive days, responses to AITC, GSK1016790A and capsaicin were further attenuated (Figure 5A). This indicates that longer-term repeated isopetasin i.g. administration leads to a desensitization of nocifensive behaviour specifically evoked by activators of TRPV1, TRPV4 and TRPA1 channels. Moreover, chronic administration of isopetasin for five consecutive days in *Trpa1*$^{-/-}$ mice did not affect facial rubbing evoked by TRPV1 or TRPV4 channel agonists (Supporting Information Figure S2C). These *in vivo* results support the hypothesis, originated by *in vitro* findings, that desensitization to isopetasin requires selective targeting of the TRPA1 channel. With more direct relevance for headaches and migraine, we next intended to test whether this would also affect CGRP-mediated dilation of meningeal arteries, which we subsequently assessed in rats. Firstly, i.p. administration of isopetasin did not produce any detectable change in meningeal perfusion (Supporting Information Figure S2D). Secondly, after isopetasin administrations for five consecutive days, increases in blood flow produced by the TRPA1 channel agonist, acrolein (Bautista *et al.*, 2006; Kunkler *et al.*, 2011), and the TRPV1 channel agonist, ethanol (Nicoletti *et al.*, 2008; Trevisani *et al.*, 2002), were reduced (Figure 5B). In contrast, the response to the direct vasodilator sodium nitroprusside remained unchanged (Figure 5B).

Discussion

Pharmacological and genetic data presented here show that isopetasin activates the universal irritant receptor ion channel, TRPA1. Mouse, rat and human (native and recombinant) TRPA1 channels are gated with similar efficacy and potency by isopetasin, suggesting that responses evoked by the drug *in vivo* in rodents could be reproduced in humans. Furthermore, failure to activate TRPV1 and TRPV4 channels indicates that nociceptor activation by isopetasin is caused by selective targeting of TRPA1 channels. As isopetasin failed to evoke calcium responses in the mutant transfected cells (3C/K-Q hTRPA1-HEK293), the molecular mechanism responsible for channel activation by this compound must be similar to that proposed for electrophilic and reactive agonists that gate TRPA1 channel *via* the involvement of specific cysteine/lysine residues (Hinman *et al.*, 2006; Trevisani *et al.*, 2007). Moreover, isopetasol, but not angelic acid, induced a calcium response in cells expressing TRPA1 channel. Thus, the hemiterpene moiety of angelic acid is not critical for activity, while the eremophilane sesquiterpenoid framework of isopetasol is essential for channel gating.

Peripheral sensory neurons expressing TRPA1 channel exert a dual function in as much as they both signal nociceptive stimuli to the brain and, by releasing the neuropeptides SP and CGRP, mediate neurogenic inflammation responses in the vasculature and other organs (Benemei *et al.*, 2014; Nassini *et al.*, 2014). In the rat urinary bladder, AITC, by targeting TRPA1 channel on intramural nerve terminals, elicits neurogenic SP-mediated smooth muscle contractions (Andrade *et al.*, 2006). Isopetasin produced an effect similar to that of AITC in terms of mechanism of action. However, the efficacy of isopetasin was much lower than that of AITC, indicating that the herbal compound may act as a partial agonist of the channel. Isopetasin, similarly to parthenolide (Materazzi *et al.*, 2013), caused desensitization of peptidergic primary sensory neurons either limited to TRPA1 channel, or involving other TRP channel stimulants and non-TRP depolarizing agents. Transition from homologous to heterologous desensitization seems to depend on the concentration and time of exposure to isopetasin. Pre-exposure to isopetasin of cultured TG neurons or their central terminals in the dorsal brainstem reduced AITC-evoked currents or CGRP release respectively. Desensitization of sensory nerve terminals by isopetasin was not limited to trigeminal innervation, as it was observed in nerve terminals of the rat urinary bladder.

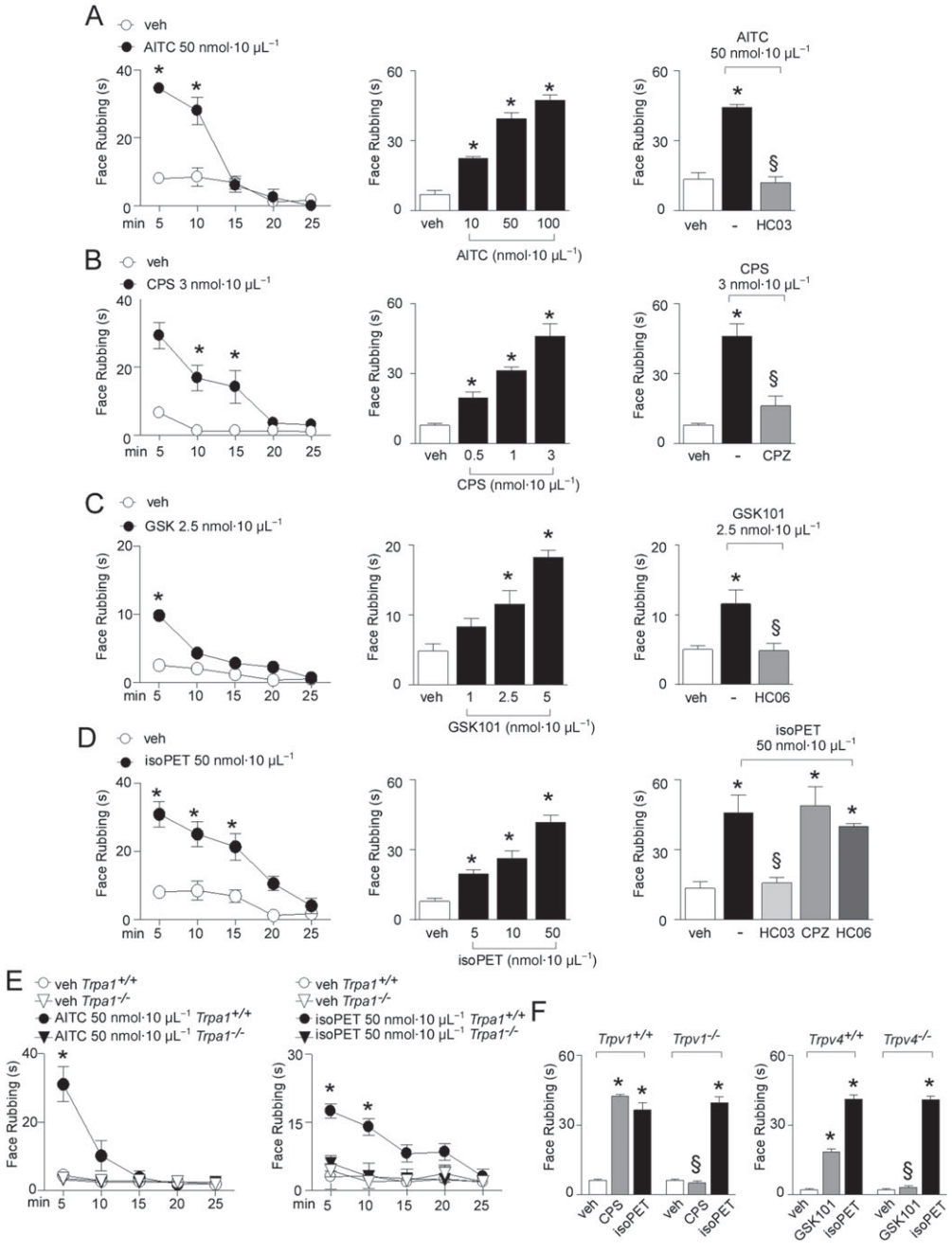

Figure 4

Local administration of isopetasin (isoPET) evokes irritant pain. (A) Time course of facial-rubbing in C57BL/6 mice after s.c.injection (10 μL) of the selective TRPA1 agonist, AITC (50 nmol) or its vehicle (veh) into the whisker pad. Time that mice spent rubbing is plotted for each 5 min block over 25 min. The irritant pain evoked by AITC, quantified as the total time spent rubbing in the first 10 min, is dose-dependent and abolished in mice pretreated with the selective TRPA1 antagonist HC-030031 (HC03; 100 mg·kg^{-1} i.p., 1 h before). Similar to AITC, s.c. injection of (B) the selective TRPV1 agonist, capsaicin (CPS) or (C) the selective TRPV4 channel agonist, GSK1016790A (GSK101), shows dose-dependent irritant pain behaviours which are blocked by pretreatment with their respective antagonist, capsazepine (CPZ; 4 mg·kg^{-1}, i.p. 30 min before) or HC-067047 (HC06; 10 mg·kg^{-1}, i.p., 30 min before). (D) Local injection (10 μL, s.c.) of isoPET into the whisker pad of C57BL/6 mice elicits dose-dependent irritant behaviours. The effect evoked by isoPET (50 nmol) was blocked in mice pretreated with HC03 but unaffected in HC06 or CPZ pretreated mice. (E) Time course of the facial-rubbing activity evoked by both AITC (50 nmol in 10 μL, s.c.) and isoPET (50 nmol in 10 μL, s.c.), or their respective veh in *Trpa1*$^{+/+}$ mice. No effect is observed in injected *Trpa1*$^{-/-}$ mice. (F) isoPET injection (50 nmol in 10 μL, s.c.) into the whisker pad of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice elicits a similar irritant effect, while CPS (3 nmol in 10 μL, s.c.) produces an irritant response only in *Trpv1*$^{+/+}$ mice. Similarly, *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ show a similar irritant response to isoPET, while GSK101 (2.5 nmol in 10 μL, s.c.) produces an irritant response only in *Trpv4*$^{+/+}$ mice. Values are mean ± SEM of at least five mice from three independent experiments. $^{*}P < 0.05$, significantly different from veh or veh *Trpa1*$^{+/+}$ or veh *Trpv1*$^{+/+}$ or veh *Trpv4*$^{+/+}$, $^{§}P < 0.05$ significantly different from AITC or CPS or GSK101 or isoPET or CPS *Trpv1*$^{+/+}$ or GSK101 *Trpv4*$^{+/+}$, ANOVA followed by Bonferroni test or Student's *t*-test.

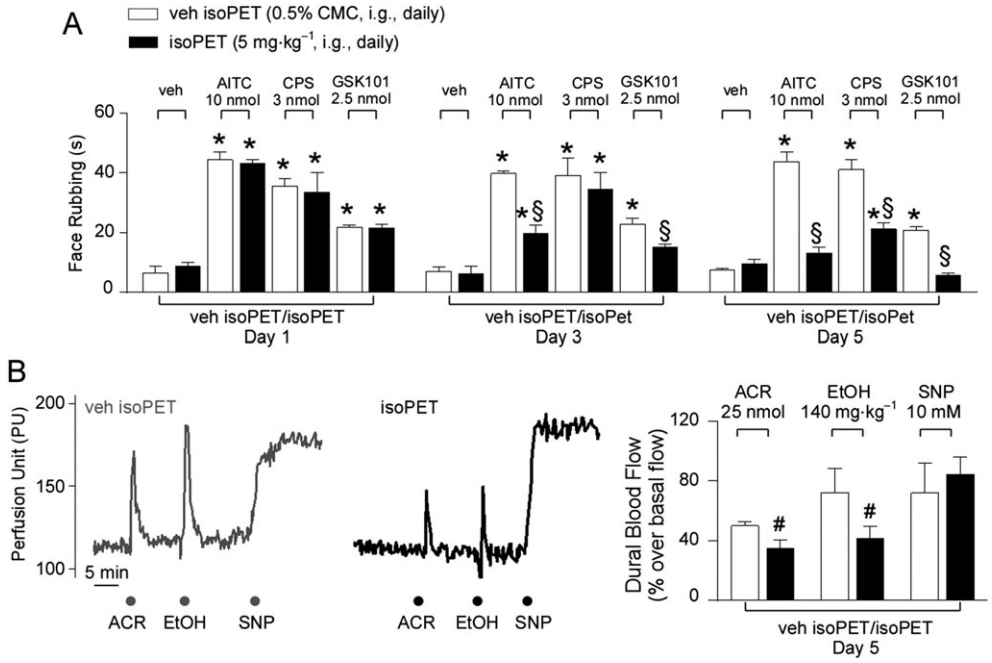

Figure 5

Chronic administration of isopetasin (isoPET) desensitizes sensory nerve endings. (A) Daily i.g. administration of isoPET (5 mg·kg^{-1}) in naïve C57BL/6 mice gradually reduces the nociceptive response evoked by s.c. injection (10 μL) of AITC (10 nmol), capsaicin (CPS; 3 nmol) or GSK1016790A (GSK101; 2.5 nmol) in mouse whisker pad and measured as the facial-rubbing activity observed in the first 15 min after the injection. Veh is the vehicle of the various stimuli. Values are mean ± SEM of at least five mice per group from three independent experiments. $^{*}P < 0.05$, significantly different from veh, $^{\S}P < 0.05$, significantly different from veh-isoPET, ANOVA followed by Bonferroni test. (B) Representative traces and pooled data of the increases in dural blood flow evoked by intranasal acrolein (ACR, 50 nmol in 50 μL), intravenous ethanol (EtOH, 140 μL·kg^{-1}) or dural application (100 μL) of sodium nitroprusside (SNP; 10 mM) in rat treated for 5 days with systemic isoPET (5 mg·kg^{-1}, i.g. per day) or its vehicle (0.5% CMC 10 mL·kg^{-1}, i.g. per day). Chronic treatment with isoPET significantly reduced responses to ACR and EtOH, but not to SNP. Values are mean ± SEM of at least five rats per group from three independent experiments. $^{\#}P < 0.05$, significantly different from veh-isoPET; Student's *t*-test.

In this preparation, neurogenic SP-mediated smooth muscle contractions evoked by AITC and capsaicin (Andrade *et al.*, 2006) were markedly reduced by pre-exposure to isopetasin. Inhibition of neurogenic inflammation by isopetasin in extra-trigeminal innervation may help explain the reported beneficial effects of butterbur in painful and inflammatory conditions, such as arthritis (Arnold *et al.*, 2015) and allergic rhinitis (Lee *et al.*, 2003; Schapowal, 2002).

Data obtained *in vitro* were reproduced *in vivo*. The facial rubbing evoked by s.c. AITC was characterized as an effect mediated by TRPA1 channel, while capsaicin or GSK1016790A were effective *via* TRPV1 or TRPV4 channels respectively. To mimic the clinical use of butterbur in migraine treatment, isopetasin was given *via* i.g. administration. Results showed a time-dependent increase in the desensitizing effect of i.g. isopetasin that initially involved only TRPA1 channel but, later, involved TRPV4 and TRPV1 channels. Thus, after a single isopetasin dose, the rubbing responses to AITC, capsaicin and GSK1016790A were unaffected, significant inhibition of AITC- or GSK1016790A-mediated responses was observed following a 3 day cycle of isopetasin administration. After a 5 day cycle, the capsaicin-mediated nociceptive response was also attenuated. This finding suggests that a more prolonged treatment schedule, providing a non-selective (heterologous) desensitization of peptidergic nerve terminals with TRPV1/TRPA1/TRPV4 channels, might be beneficial in migraine prophylaxis.

The molecular bases of the process that results in channel and neuronal desensitization have been investigated more extensively following activation of TRPV1 channel. Exposure to capsaicin or resiniferatoxin results in a time- and concentration-dependent massive Ca^{2+} influx into the neurons that results in a neurotoxic cation overload, thus attenuating nociceptor functioning. Although neuronal desensitization produced by TRPA1 channel has been explored in less detail, the AITC-evoked heterologous desensitization of CGRP release from the rat hind paw skin is Ca^{2+}-dependent, similar to that produced by capsaicin (Ruparel *et al.*, 2008). Furthermore, in meningeal afferents, selective stimulation of TRPA1 channel increased activation threshold and promoted CGRP release but did not cause propagated action potentials, suggesting that co-expression of TRPV1 channel in peripheral nociceptors is required for TRPA1-mediated pro-nociceptive function (Denner *et al.*, 2017). Our present experiments do not contradict this proposal because, as indicated by desensitization studies, isopetasin-evoked responses (bladder contraction, rubbing behaviour, CGRP release and increase in dural blood flow) were all mediated by TRPA1 channel apparently expressed in TRPV1-positive primary afferents.

Clinical studies with low MW compounds that selectively block the CGRP receptor for the acute and preventive treatment of the migraine attack (Edvinsson, 2015a; Ho *et al.*, 2008; Olesen *et al.*, 2004) or, more recently, with anti-CGRP monoclonal antibodies as preventive anti-migraine medicines (Bigal *et al.*, 2015), have strengthened the hypothesis of a pivotal role of this neuropeptide in the mechanism of migraine headaches. Although the precise CGRP-dependent pathway that results in the pain and associated symptoms of migraine attack has not been completely identified, peptidergic neurons of the trigeminal ganglion appear to play a major role in migraine pain (Edvinsson, 2015a). CGRP released from sensory nerve terminals in rodents and other mammals, including humans, dilates arterial vessels (Sinclair *et al.*, 2010), including meningeal arteries (Dux *et al.*, 2016; Kunkler *et al.*, 2011; Nicoletti *et al.*, 2008). Thus, CGRP-mediated meningeal vasodilatation following stimulation of TG neurons is considered a good approximation of the neurochemical events that occur during the migraine attack. Previous studies have shown that meningeal vasodilation evoked by administration of either acrolein (intranasal) or ethanol (i.p.) is mediated by CGRP released from perivascular trigeminal nerve endings triggered by activation of TRPA1 or TRPV1 channels respectively (Kunkler *et al.*, 2011; Nicoletti *et al.*, 2008). The present observation that 5 days of isopetasin, given i.g., attenuated the increase in rat dural blood flow evoked by both acrolein and ethanol suggests that isopetasin, possibly by targeting TRPA1 channels, desensitized trigeminal nociceptors so they were no longer able to release the migraine mediator, CGRP. This novel property of isopetasin may add to the mechanisms previously described (Ko *et al.*, 2001; Thomet *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002) to explain the action of chronically administered butterbur to prevent migraine (Grossmann and Schmidramsl, 2000; Lipton *et al.*, 2004; Pothmann and Danesch, 2005).

Migraine therapy consists of different classes of drugs. While acute treatment of attacks is confined to analgesics, non-steroidal antiinflammatory drugs and triptans, the prevention of attacks is primarily based on β-blockers, antiepileptics, anti-depressants and 5-HT antagonists. It is possible that in the near future, low MW CGRP receptor antagonists (gepants) will be used for acute treatment and anti-CGRP or anti-CGRP receptor monoclonal antibodies will be used for prophylaxis. Our present findings support the view that some drugs and herbal medicines currently prescribed for the acute or prophylactic treatment of migraine can be grouped into a novel class of therapeutics, namely, TRPA1 channel inhibitors. These comprise compounds that target the channel with different modalities. The universally used acetaminophen (paracetamol) *via* its reactive metabolite, N-acetylbenzoquinoneimine, activates (Nassini *et al.*, 2010) and then desensitizes (Andersson *et al.*, 2011) TRPA1 channel. The well-established, but still widely and successfully prescribed pyrazolone derivatives (Ramacciotti *et al.*, 2007), dipyrone (metamizole) (Bigal *et al.*, 2002) and propyphenazone, are selective TRPA1 channel antagonists (Nassini *et al.*, 2015). The active component of feverfew, parthenolide, moderately stimulates TRPA1 channel both *in vitro* and *in vivo*, causing channel desensitization and neuronal dysfunction (Materazzi *et al.*, 2013). Butterbur is indicated as per the American Headache Society guidelines with a level A recommendation for migraine prophylaxis (Holland *et al.*, 2012). The present findings would add the butterbur constituent, isopetasin (Aydin *et al.*, 2013), to the list of TRPA1-tropic agents, which, like parthenolide, stimulate the channel, thereby causing desensitization of peptidergic trigeminal nerve terminals, attenuating their ability to release CGRP and to signal pain. Successful treatment and prevention of migraine by targeting of TRPA1 cation channels and the ensuing peptidergic sensory neuron dysfunction by parthenolide and isopetasin provides a solid basis for future basic and translational investigations of migraine treatments based on actions at TRPA1 channels.

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Author contributions

S.B., F.D.L., G.A., P.G., S.M. and R.N. designed experiments and interpreted results. F.D.L. and F.P. performed chemical *Petasites* extraction and purification. S.L.P. and S.M. performed calcium experiments, S.L.P. and E.C. performed electrophysiological experiments, F.D.L., F.U. and S.M. performed neurochemical *in vitro* assays, F.D.L., I.M.M and S.M. performed *in vivo* experiments, and S.B., F.D.L., W.G., F.P., G.A., P.G., S.M. and R.N. 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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Supporting Information

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

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Figure S1 (A) Chemical structures of isopetasol, angelic acid and isopetasin (isoPET). (B) Challenge with isoPET (50 μM) does not elicit calcium responses or ion currents in untransfected HEK293 cells which instead responded to the activating peptide for human proteinase activated receptor 2 (hPAR2-AP, 100 μM) or KCl (100 μM). (C) Representative traces, concentration–response curve and pooled data of the calcium response evoked by isopetasol in HEK293 cells transfected with the cDNA coding for human TRPA1 channels (hTRPA1-HEK293). isoPET induces a similar concentration–response curve in hTRPA1-HEK293 cells with a higher maximum effect at the highest concentration used. Angelic acid does not produce calcium response at any of the concentrations tested (0.5 μM – 3 mM). The calcium response evoked by isopetasol (100 μM) is abolished in the presence of the selective TRPA1 channel antagonist HC-030031 (HC03; 30 μM) and is absent in (D) untransfected HEK293

cells. Concentration–response curves of the calcium response evoked by isoPET in human fetal lung fibroblasts, IMR90 (E), and in rat cultured trigeminal ganglion (TG) neurons (F). Veh is the vehicle of isopetasol. Each column or point represents the mean ± SEM of $n > 20$ cells or neurons from 3–6 independent experiments. Dash indicates vehicle of HC03. *$P < 0.05$ *vs.* veh, §$P < 0.05$ *vs.* isopetasol; ANOVA followed by Bonferroni test.

Figure S2 (A) Isopetasin (isoPET; 300 μM) evokes a contractile response in strips of mouse urinary bladder isolated form *Trpa1*$^{+/+}$ mice, an effect that is absent in urinary bladder from *Trpa1*$^{-/-}$ mice. (B) Exposure (20 min, twice) to a high concentration of isoPET (300 μM) markedly reduces the contractile response evoked by the selective TRPV1 channel agonist, capsaicin (CPS; 0.3 μM), in longitudinal strips of mouse urinary bladder isolated from *Trpa1*$^{+/+}$ mice, but not in tissues from *Trpa1*−/− mice. isoPET (300 μM) does not affect the contractile response to carbachol (CCh; 1 μM) in either *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mouse urinary bladders. Each column represents the mean ± SEM of $n = 5$ from 3 independent experiments. Veh is the vehicle of isoPET. *$P < 0.05$ *vs.* *Trpa1*$^{+/+}$ veh or *Trpa1*$^{+/+}$ veh isoPET; ANOVA followed by Bonferroni test. (C) In *Trpa1*$^{-/-}$ mice daily intragastric (i.g.) administration of isoPET (5 mg·kg^{-1}) does not affect nociceptive response elicited by s.c. injection (10 μL) of capsaicin (CPS; 3 nmol) or GSK1016790A (GSK101; 2.5 nmol) into mouse whisker pad and measured as the facial-rubbing activity observed in the first 15 min after injection. Veh is the vehicle of the various stimuli. Values are mean ± SEM of at least 4 mice per group from 3 independent experiments. *$P < 0.05$ *vs.* veh, ANOVA followed by Bonferroni test. (D) Representative trace of the effect in rat dural blood flow evoked by intraperitoneal isoPET (5 mg·kg^{-1}), intranasal acrolein (ACR, 50 nmol in 50 μL), intravenous ethanol (EtOH, 140 μL·kg^{-1}) or dural application (100 μL) of sodium nitroprusside (SNP; 10 mM,).