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RESEARCH PAPER

The TRPA1 channel mediates the analgesic action of dipyrone and pyrazolone derivatives

Romina Nassini[1]*, Camilla Fusi[1]*, Serena Materazzi[1]*, Elisabetta Coppi[1], Tiziano Tuccinardi[2], Ilaria M Marone[1], Francesco De Logu[1], Delia Preti[3], Raquel Tonello[1,4], Alberto Chiarugi[1], Riccardo Patacchini[5], Pierangelo Geppetti[1] and Silvia Benemei[1]

[1]*Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Florence, Italy,* [2]*Department of Pharmacy, University of Pisa, Pisa, Italy,* [3]*Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy,* [4]*Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil, and* [5]*Department of Corporate Drug Development, Chiesi Farmaceutici SpA, Parma, Italy*

Correspondence
Pierangelo Geppetti, Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. E-mail: geppetti@unifi.it

*These authors contributed equally to this study.

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BACKGROUND AND PURPOSE

Although still used by hundreds of millions of people worldwide, the mechanism of the analgesic action of the pyrazolone derivatives (PDs), dipyrone, propyphenazone and antipyrine remains unknown. The transient receptor potential ankyrin 1 (TRPA1) channel, expressed by nociceptors, is emerging as a major pain transduction pathway. We hypothesized that PDs target the TRPA1 channel and by this mechanism produce their analgesic effect.

EXPERIMENTAL APPROACH

Calcium responses and currents were studied in cultured TRPA1-expressing rodent dorsal root ganglion neurons and human cells. Acute nociception and mechanical hypersensitivity were investigated in naïve and genetically manipulated mice.

KEY RESULTS

Pyrazolone and PDs selectively inhibited calcium responses and currents in TRPA1-expressing cells and acute nocifensor responses in mice evoked by reactive channel agonists (allyl isothiocyanate, acrolein and H_2O_2). In line with recent results obtained with TRPA1 antagonists and TRPA1 gene deletion, the two most largely used PDs, dipyrone and propyphenazone, attenuated TRPA1-mediated nociception and mechanical allodynia in models of inflammatory and neuropathic pain (formalin, carrageenan, partial sciatic nerve ligation and the chemotherapeutic drug, bortezomib). Notably, dipyrone and propyphenazone attenuated carrageenan-evoked mechanical allodynia, without affecting PGE_2 levels. The main metabolites of PDs did not target TRPA1 and did not affect TRPA1-dependent nociception and allodynia.

CONCLUSIONS AND IMPLICATIONS

Evidence that in rodents the nociceptive/hyperalgesic effect produced by TRPA1 activation is blocked by PDs suggests that a similar pathway is attenuated by PDs in humans and that TRPA1 antagonists could be novel analgesics, devoid of the adverse haematological effects of PDs.

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Abbreviations

AITC, allyl isothiocyanate; AntiP, antipyrine; CI, confidence interval; Db-cAMP, dibutyryl cAMP; Dip, dipyrone; dm-PPh, *N*-demethylpropyphenazone; DRG, dorsal root ganglia; Edar, edaravone; EEC, European Communities Council; IMR90, human embryonic lung fibroblasts; MAA, 4-methylaminoantipyrine; NSAIDs, non-steroidal anti-inflammatory drugs; PAR2, proteinase activated receptor 2; PDs, pyrazolone derivatives; PPh, propyphenazone; Pyr, pyrazolone; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1

Tables of Links

TARGETS	
GPCRs[a]	**Ion channels**[b]
PAR2	TRPA1
Enzymes[c]	TRPV1
COX-1	TRPV4
COX-2	

LIGANDS		
Acrolein	Dibutyryl cAMP	Icilin
Allyl isothiocyanate (AITC)	Formalin	Indomethacin
Bortezomib	GSH	Menthol
Capsaicin	GSK1016790A	PGE_2
Capsazepine	H_2O_2	Pyrazolone
	HC-030031	SLIGKV-NH_2

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 (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

The pyrazolone (Pyr) derivatives (PDs), antipyrine (AntiP), dipyrone (Dip) and propyphenazone (PPh), introduced in 1883, 1922 and 1951, respectively, have been successfully used for more than a century by hundreds of millions of people worldwide in a series of painful diseases, including migraine, colic and post-surgical and neuropathic pain (Babej-Dolle *et al.*, 1994; Brune, 1997; Ramacciotti *et al.*, 2007; Vallano *et al.*, 2007; Derry *et al.*, 2010). However, despite this long-term therapeutic success, the mechanism of the analgesic action of PDs remains to be understood.

Painkillers, such as non-steroidal antiinflammatory drugs (NSAIDs) or coxibs, relieve pain by inhibiting COX-1 and COX-2 respectively (Simmons *et al.*, 2004). COX inhibition by PDs is, however, weak, resulting in poor anti-inflammatory effects, which do not match their potent analgesic action (Chandrasekharan *et al.*, 2002; Simmons *et al.*, 2004; Hinz *et al.*, 2007; Malvar Ddo *et al.*, 2011). Indeed, while indomethacin comparably inhibited carrageenan-evoked hyperalgesia/oedema, dipyrone was an effective anti-hyperalgesic and a poor anti-inflammatory agent (Lorenzetti and Ferreira, 1985). Dipyrone failed to reduce PG levels in rat tissues and, accordingly, dipyrone gastrointestinal toxicity is negligible (Weithmann and Alpermann, 1985; Brogden, 1986; Sanchez *et al.*, 2002; Berenguer *et al.*, 2004). Thus, the pharmacological actions of PDs do not replicate the COX-dependent effects of NSAIDs/coxibs and additional mechanisms (Lorenzetti and Ferreira, 1996; Sachs *et al.*, 2004) have not received further support.

The transient receptor potential ankyrin 1 (TRPA1) channel is co-expressed by a subset of primary sensory neurons with cell bodies in dorsal root ganglia (DRG), which express the capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1) channel, the hypotonic solution-sensitive channel (TRPV4) and other TRP channels (Andrade *et al.*, 2012; Nassini *et al.*, 2014). Both pharmacological and genetic findings indicate that TRPA1 contributes to inflammatory and neuropathic pain models, including those evoked by formalin, spinal nerve ligation and chemotherapeutics (McNamara *et al.*, 2007; Andrade *et al.*, 2012; Trevisan *et al.*, 2013b; Nassini *et al.*, 2014).

Besides allyl isothiocyanate (AITC) and cinnamaldehyde (Bandell *et al.*, 2004; Bautista *et al.*, 2006), reactive/electrophilic by-products of oxidative stress, such as H_2O_2, 4-hydroxynonenal and acrolein, activate TRPA1 via a Michael addition or oxidation reactions of specific amino acid residues (Hinman *et al.*, 2006; Macpherson *et al.*, 2007; Trevisani *et al.*, 2007; Taylor-Clark *et al.*, 2009). We hypothesized that PDs inhibit TRPA1 and hence produce analgesia. The results indicate that PDs selectively antagonize TRPA1 activation by reactive channel agonists, thereby producing antinociceptive and antihyperalgesic effects.

Methods

Further information can be found in Supporting Information Appendix S1.

Animals

The animal experiments carried out conformed to the European Communities Council (ECC) guidelines for animal care procedures and the Italian legislation (DL 116/92) application of the ECC directive 86/609/EEC. Studies were conducted under the University of Florence research permit number 204/2012-B. Male C57BL/6 (25–30 g) (Harlan Laboratories, Milan, Italy), wild-type, *Trpa1*$^{+/+}$ or TRPA1-deleted *Trpa1*$^{-/-}$ (25–30 g) mice generated by heterozygotes on a C57BL/6

background (B6;129P-Trpa1tm1Kykw/J; Jackson Laboratories, Bar Harbor, ME, USA) (Kwan *et al.*, 2006) or Sprague Dawley rats (35–50 g, male, Harlan Laboratories) were used. For each behavioural experiment, we used groups of six mice. For the *in vitro* experiments as a whole, we used 30 rats and 28 mice. Animals were housed in a temperature- and humidity-controlled vivarium (12 h dark/light cycle, free access to food and water). Behavioural experiments were performed in a quiet, temperature-controlled (20 to 22°C) room between 0900 and 1700 h. Animals were killed with a high dose of i.p. sodium pentobarbital (200 $mg \cdot kg^{-1}$). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Reagents

HC-030031 has been synthesized as described previously (Andre *et al.*, 2008). The activating peptide and its reverse peptide for human proteinase-activated receptor 2 (PAR2) (SLIGKV-NH2 and VKGILS-NH2, respectively) were dissolved in distilled water. If not otherwise indicated, all other reagents were from Sigma-Aldrich (Milan, Italy).

Cell culture and isolation of primary sensory neurons

HEK293 naïve cells or HEK293 cells stably transfected with the cDNA of human TRPV1 (hTRPV1-HEK293) or with the cDNA of human TRPV4 (hTRPV4-HEK293) were used and cultured as previously described (Nassini *et al.*, 2012). HEK293 cells were transiently transfected with the cDNAs for wild-type or mutant 3C/K-Q (C619S, C639S, C663S, K708Q) human TRPA1 (Hinman *et al.*, 2006; Trevisani *et al.*, 2007). Human embryonic lung fibroblasts (IMR90; American Type Culture Collection, Manassas, VA, USA), which express the native TRPA1 channel (Nassini *et al.*, 2012), were also used. DRG neurons were isolated from Sprague Dawley rats or C57BL/6 mice and cultured as previously described (Materazzi *et al.*, 2012).

Cellular recordings

Single cell intracellular calcium levels were measured in transfected and untransfected HEK293 cells, IMR90 cells or in rat and mouse DRG neurons as previously reported (Materazzi *et al.*, 2013). Whole-cell patch-clamp recordings were performed as previously reported in rat DRG neurons or in IMR90 cells. TRPA1 or TRPV1 currents were detected as inward currents activated upon cell superfusion with the different stimuli. Peak current was normalized with respect to cell membrane capacitance and expressed as mean of the current density (pA/pF) as previously reported (Nassini *et al.*, 2012).

Behavioural experiments

For behavioural experiments, after habituation, C57BL/6 and $Trpa1^{+/+}$ or $Trpa1^{-/-}$ mice were randomized into treatment groups and an investigator blinded to treatments recorded the responses. AITC, acrolein, H_2O_2, dibutyryl cAMP (Db-cAMP), capsaicin, zinc acetate, icilin and hypotonic solution (0.27% NaCl) were given by intraplantar (i.pl.) injection to provoke acute nociception and hyperalgesia. Formalin (McNamara *et al.*, 2007) and carrageenan (i.pl.) (Bonet *et al.*, 2013) were used as models of inflammatory pain. Partial sciatic nerve ligation (Zhou *et al.*, 2013) and bortezomib (i.p.) (Trevisan *et al.*, 2013b) were used as models of neuropathic pain. pyrazolone, propyphenazone, dipyrone, antipyrine and their metabolites, 4-methylaminoantipyrine (MAA), *N*-demethylpropyphenazone (dm-propyphenazone) and edaravone (Edar), respectively, HC-030031 or indomethacin were injected i.pl. or i.p. to reduce agonist-evoked responses.

Acute nociceptive behaviour was measured by assessing the total time spent in lifting/licking of the injected hind paw (Trevisani *et al.*, 2007). Mechanical allodynia was measured by the up-and-down paradigm using Von Frey hairs (Chaplan *et al.*, 1994). Cold hypersensitivity was assessed by measuring the acute nocifensive response to acetone (Trevisan *et al.*, 2013b). Paw oedema was measured using an engineer's micrometre (Trevisan *et al.*, 2013a).

PGE_2 assay

PGE_2 levels were measured by enzyme immunoassay in the paw homogenate as previously reported (Ulmann *et al.*, 2010; Ma *et al.*, 2013).

Molecular modelling

A high resolution structure of the TRPA1 channel has yet to be elucidated. Therefore, in order to explore a possible binding mode of the PDs into the channel, a homology model of the human TRPA1 was developed.

Statistical analysis

Data represent mean ± SEM or 95% confidence interval (CI). Statistical analysis was performed by use of Student's unpaired two-tailed *t*-test for comparisons between two groups or ANOVA, followed by Bonferroni's *post hoc* test for comparisons of multiple groups. Potency of antagonists was expressed as IC_{50}, that is, the molar concentration of the antagonist required for 50% inhibition of the maximum effect evoked by the agonist. $P < 0.05$ was considered statistically significant. GraphPadPrism version 5.00 (GraphPad Software, San Diego, CA, USA) was used.

Results

PDs are selective TRPA1 antagonists

Pyrazolone or PDs, without producing any stimulating effect (Figure 1A and B), inhibited calcium responses evoked by AITC in rat and mouse DRG neurons and in human embryonic lung fibroblasts (IMR90, i.e. the cell type from which TRPA1 was originally cloned) (Jaquemar *et al.*, 1999; Figure 1A–E). They did not affect responses to the selective TRPV1 agonist, capsaicin, high KCl or the PAR2 agonist, SLIGKV-NH_2, thus indicating selectivity (Figure 1D–F). IC_{50}s were similar for the two systemically used PDs, propyphenazone and dipyrone (~60 μM) and higher for antipyrine (~600 μM) (Figure 1C). Pyrazolone and the PDs did not affect responses to selective TRPV1 and TRPV4 agonists (capsaicin or GSK1016790A, respectively) in channels expressed in recombinant systems (Supporting Information Fig. S1A).

While the selective TRPA1 antagonist, HC-030031 (McNamara *et al.*, 2007), inhibited responses evoked by all TRPA1 agonists, PDs only reduced responses evoked by electrophilic or reactive agonists, such as AITC, acrolein or H_2O_2

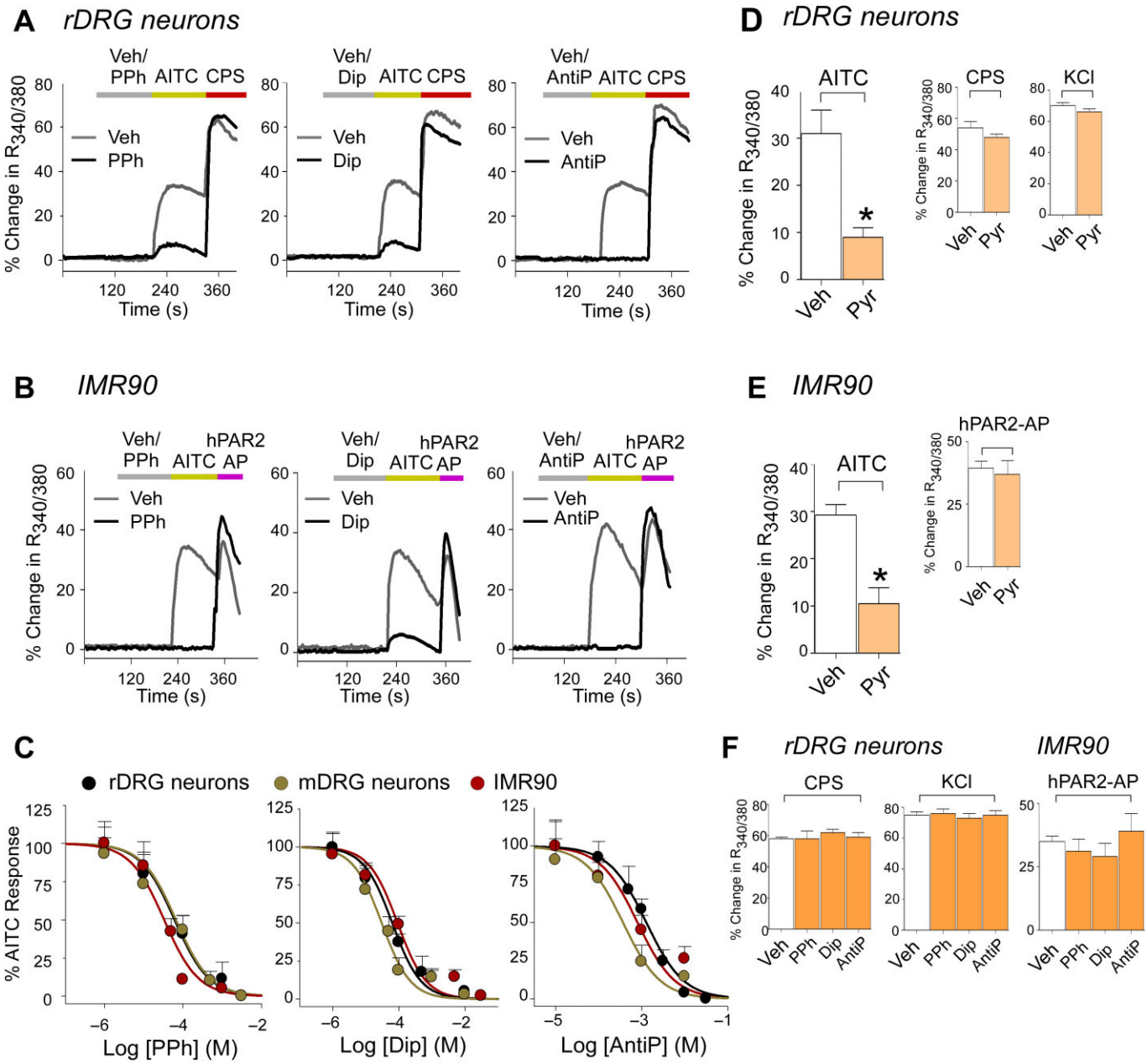

Figure 1

Pyrazolone (Pyr) and its derivatives selectively inhibit the calcium response evoked by TRPA1 stimulation. (A and B) Typical traces of the inhibitory effect of pre-exposure (10 min) to the PDs, PPh (100 μM), Dip (100 μM) and AntiP (5 mM) on the calcium response evoked by the TRPA1 agonist, AITC (10 μM), in rat cultured DRG (rDRG) neurons and IMR90. (C) Concentration-response curves for the inhibitory effect of PPh (IC_{50}s 33–66 μM), Dip (IC_{50}s 30–91 μM) and AntiP (IC_{50}s 360–1300 μM) on the calcium response evoked by AITC in rDRG neurons, mouse DRG (mDRG) neurons and IMR90 cells (AITC concentrations are 10, 10 and 1 μM respectively). (D and E) Pyr (100 μM) inhibits the calcium response evoked in rDRG neurons and IMR90 cells by AITC (10 μM and 1 μM respectively). (D–F) PPh (100 μM), Dip (100 μM), AntiP (5 mM) and Pyr (100 μM) do not affect the responses evoked by capsaicin (CPS, 0.1 μM) or high KCl (50 mM) in rDRG neurons and by the activating peptide of the human PAR2 receptor (hPAR2-AP, 100 μM) in IMR90 cells. Values are mean ± SEM of $n > 25$ cells from at least three different experiments for each condition. *$P < 0.05$ versus vehicle (Veh).

(Figure 2A), with no effect against the non-reactive agonists, $ZnCl_2$ and icilin (Figure 2B), which act independently from binding key cysteine residues of TRPA1 (McKemy *et al.*, 2002; Hu *et al.*, 2009). In a mutated channel, which is without the cysteine and lysine residues, required for channel activation by reactive agonists (3C/K-Q, TRPA1-3C/K-Q) (Hinman *et al.*, 2006; Macpherson *et al.*, 2007; Trevisani *et al.*, 2007), calcium responses to non-reactive stimuli, menthol (100 μM) or icilin (30 μM), were reduced by HC-030031 but unaffected by pyrazolone and PDs (Figure 2C).

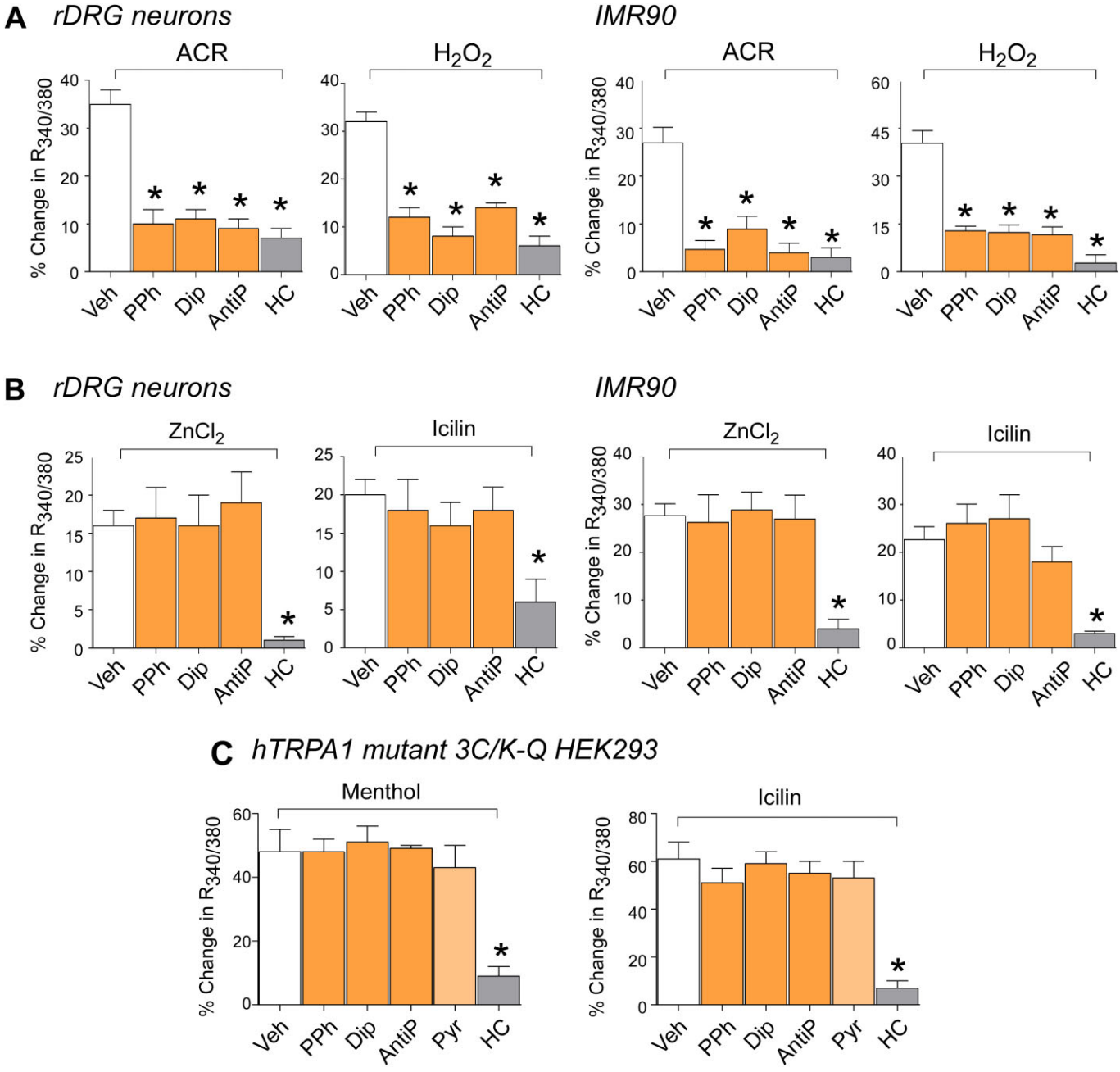

Figure 2

Pyrazolone (Pyr) and its derivatives selectively inhibit the calcium response evoked by reactive TRPA1 agonists. (A and B) PPh (100 μM), Dip (100 μM) and AntiP (5 mM) inhibit the calcium response evoked by acrolein (ACR, 10 μM) or H_2O_2 (500 μM) and do not affect the response induced by the non-reactive TRPA1 agonists, $ZnCl_2$ (1 μM) and icilin (30 μM) in rat DRG (rDRG) neurons or in IMR90 cells. The selective TRPA1 antagonist, HC-030031 (HC, 30 μM) inhibits responses to both reactive and non-reactive agonists. (C) PPh, Dip, AntiP and Pyr (all, 100 μM) do not affect the calcium response of cells transfected with the cDNA codifying for the mutant human TRPA1 channel (3C/K-Q) responding to the non-electrophilic agonists, menthol (100 μM) and icilin (30 μM). Values are mean ± SEM of $n > 25$ cells from at least three different experiments for each condition. *$P < 0.05$ versus vehicle (Veh).

Metabolites of PDs are inactive

We also tested whether the metabolites of dipyrone, propyphenazone and antipyrine, MAA, dm-propyphenazone and edaravone, respectively, antagonize TRPA1 or scavenge TRPA1 reactive agonists. Ten minutes of pre-exposure to PD metabolites or the aldehyde and reactive oxygen species scavenger, GSH, failed to affect AITC- acrolein- or H_2O_2-evoked calcium responses (Supporting Information Fig. S1B). In contrast, 30 min coincubation of AITC, acrolein or H_2O_2 with GSH, but not with each individual PD or metabolite, reduced

calcium responses as compared with responses produced by co-incubation with respective vehicles (Supporting Information Fig. S1C).

Mode of TRPA1 targeting by propyphenazone

To investigate whether PDs interact with specific cysteine residues, we used propyphenazone, given that some of its analogues exhibit moderate electrophilic properties (Li *et al.*, 1998). By means of *in silico* analysis, we found that propyphenazone could interact with cysteine 608, so that its scaffold orientates in such a way that the oxygen atom of the pyrazolidinone ring forms an H-bond with the hydroxyl group of serine 582. The binding pose was further stabilized by the insertion of a propyphenazone phenyl ring within a lipophilic pocket delimited by hydrophobic residues (Supporting Information Fig. S2).

PDs inhibit currents evoked by AITC

In cultured rat DRG neurons, AITC and capsaicin evoked inward currents, which were reduced by HC-030031 and capsazepine respectively. Responses to AITC, but not to capsaicin, were markedly attenuated by pyrazolone, dipyrone, propyphenazone and antipyrine (Figure 3A). In IMR90 cells, AITC-evoked currents were attenuated by pre-exposure (Figure 3B) and reversed in about 1 min by the subsequent administration of HC-030031, dipyrone and propyphenazone (Figure 3C). Pyrazolone or the PDs did not produce any stimulating effect (Figure 3A,B).

PDs selectively block nocifensor responses evoked by reactive TRPA1 agonists

Systemic (i.p.) administration of PDs reduced in a dose-dependent manner acute nociceptive responses evoked by i.pl. AITC injected 30 min after PDs (Figure 4A), for example, when these drugs exerted their maximum antinociceptive effect (Figure 4B). Systemic (i.p.) administration of dm-propyphenazone, MAA and edaravone failed to affect the nociceptive response evoked by i.pl. AITC, injected 30 after the PD metabolites (Figure 4C). The nociceptive effect evoked by i.pl. AITC was markedly reduced by systemic HC-030031, given 60 min before AITC (Figure 4C). PDs did not affect nociception evoked by capsaicin or by a hypo-osmotic stimulus (TRPV1- or TRPV4-mediated responses respectively) (Figure 4D). Nocifensor behaviours by acrolein or H_2O_2 (i.pl.) were inhibited by HC-030031 (Figure 5A) and reduced in TRPA1-deleted mice (Figure 5A, insets). Responses induced by acrolein and H_2O_2 were markedly attenuated by PDs (Figure 5A). In contrast, nocifensor responses evoked by the non-reactive agonists, Zn acetate or icilin (i.pl.), which are markedly attenuated in TRPA1-deleted mice and by HC-030031, were completely unaffected by PDs (Figure 5B).

In accord with previous findings (Lorenzetti and Ferreira, 1985), the PDs did not reduce Db-cAMP (i.pl.)-evoked mechanical allodynia, a response which is maintained in TRPA1-deleted mice (Figure 5C). To test whether PDs target TRPA1 in peripheral terminals of nociceptors, PDs were injected in the same paw as AITC (*ipsi*) or in the contralateral paw (*contra*). Nociception was inhibited only in the *ipsi* paw (Figure 5D); dm-propyphenazone or MAA failed to attenuate AITC-evoked responses in the *ipsi* paw (Figure 5D).

PDs selectively reduce TRPA1-dependent nociception and hyperalgesia in models of inflammatory pain

In agreement with earlier reports, HC-030031 (McNamara *et al.*, 2007), but not indomethacin (Malmberg and Yaksh, 1992), inhibited Phase I of the response to formalin, thus confirming and negating the role of TRPA1 and COX metabolites respectively (Figure 6). Propyphenazone and dipyrone reduced the nociception of Phase I, whereas their metabolites were ineffective (Figure 6). As previously reported (Malmberg and Yaksh, 1992; McNamara *et al.*, 2007), the phase II response to formalin was inhibited by a variety of drugs, including, propyphenazone, dipyrone, indomethacin and MAA (Figure 6).

Carrageenan-evoked pain-like symptoms were markedly reduced in TRPA1-deleted mice (Figure 7A, insets). HC-030031, propyphenazone and dipyrone, but not MAA and dm-propyphenazone, attenuated both mechanical and cold hyperalgesia induced by i.pl. carrageenan (Figure 7A). While the antihyperalgesic and anti-inflammatory actions of indomethacin were associated with abolition of the carrageenan-evoked increase in PGE_2 tissue levels, the antihyperalgesic effect of PDs and HC-030031 was associated with no inhibition of the increased oedema and PGE_2 levels (Figure 7B,C).

PDs selectively reduce TRPA1-dependent nociception and hyperalgesia in models of neuropathic pain

A single administration in mice of the chemotherapeutic agent, bortezomib, has been previously shown to produce mechanical and cold hypersensitivity, which was completely TRPA1-dependent (Trevisan *et al.*, 2013b). Here, we confirmed that bortezomib-evoked mechanical and cold hypersensitivity was HC-030031-dependent and we found that it was completely indomethacin-insensitive (Supporting Information Fig. S3) and markedly attenuated by propyphenazone and dipyrone, but not by dm-propyphenazone and MAA (Figure 8A). Mechanical allodynia evoked by spinal nerve ligation, a response attenuated in TRPA1-deleted mice (Figure 8B), was reduced by PDs and HC-030031, but not by PD metabolites or indomethacin (Figure 8C). Notably, PDs and HC-030031 were unable to further affect the reduced allodynia observed in TRPA1-deleted mice (Figure 8C). Identical results were obtained when cold hypersensitivity was assessed (Figure 8C).

Discussion

Since the seminal discovery of antipyrine by Ludwig Knorr in 1883, PDs have been one of the most successful classes of drugs in pain pharmacotherapy. However, the question of the mechanism of their analgesic action has remained unresolved. Here, we demonstrated that pyrazolone and its

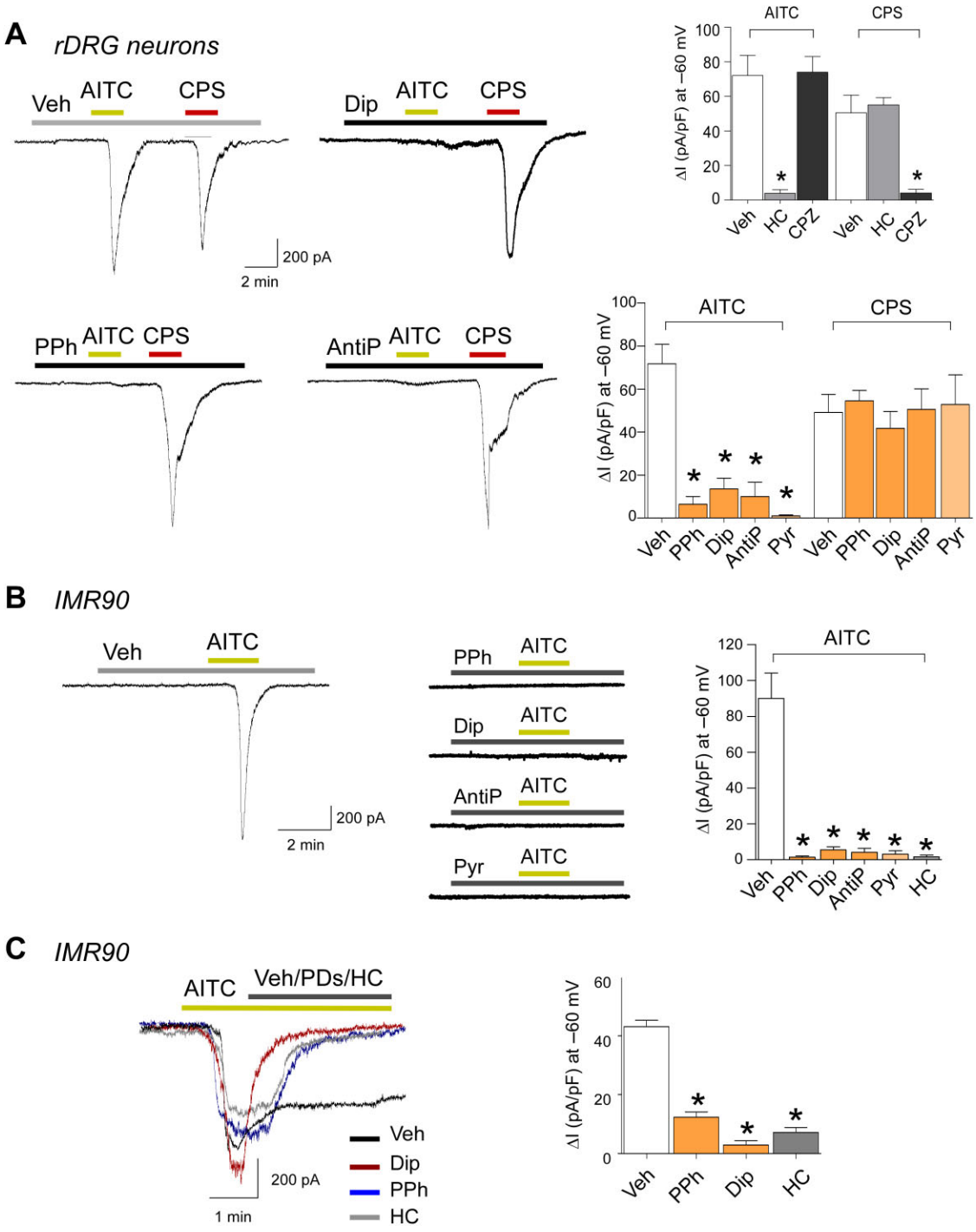

Figure 3

Pyrazolone (Pyr) and its derivatives selectively inhibit ion currents evoked by TRPA1 stimulation. (A) Original current traces and pooled data obtained by whole-cell patch-clamp recordings in rat DRG (rDRG) neurons. Application of AITC (100 μM) or capsaicin (CPS, 1 μM) elicits inward currents at –60 mV, which are blocked by the TRPA1 selective antagonist, HC-030031 (HC, 50 μM) and the TRPV1 selective antagonist capsazepine (CPZ, 10 μM) respectively. PPh (100 μM), Dip (100 μM), AntiP (1 mM) and Pyr (100 μM) prevent AITC-induced currents, but do not affect the currents evoked by CPS. (B) Original current traces and pooled data recorded in IMR90. Under control conditions, AITC (20 μM) activates inward currents at –60 mV, which are completely blocked by PPh (50 μM), Dip (50 μM), AntiP (100 μM), Pyr (50 μM) and HC (50 μM). (C) Original current traces and pooled data of the effect of PPh (50 μM), Dip (50 μM) and HC (50 μM) given after the application of AITC (20 μM) in IMR90 cells. PDs and HC reverse the effect of the agonist. Values are mean ± SEM of at least five cells for each experimental condition. *$P < 0.05$ versus vehicle (Veh).

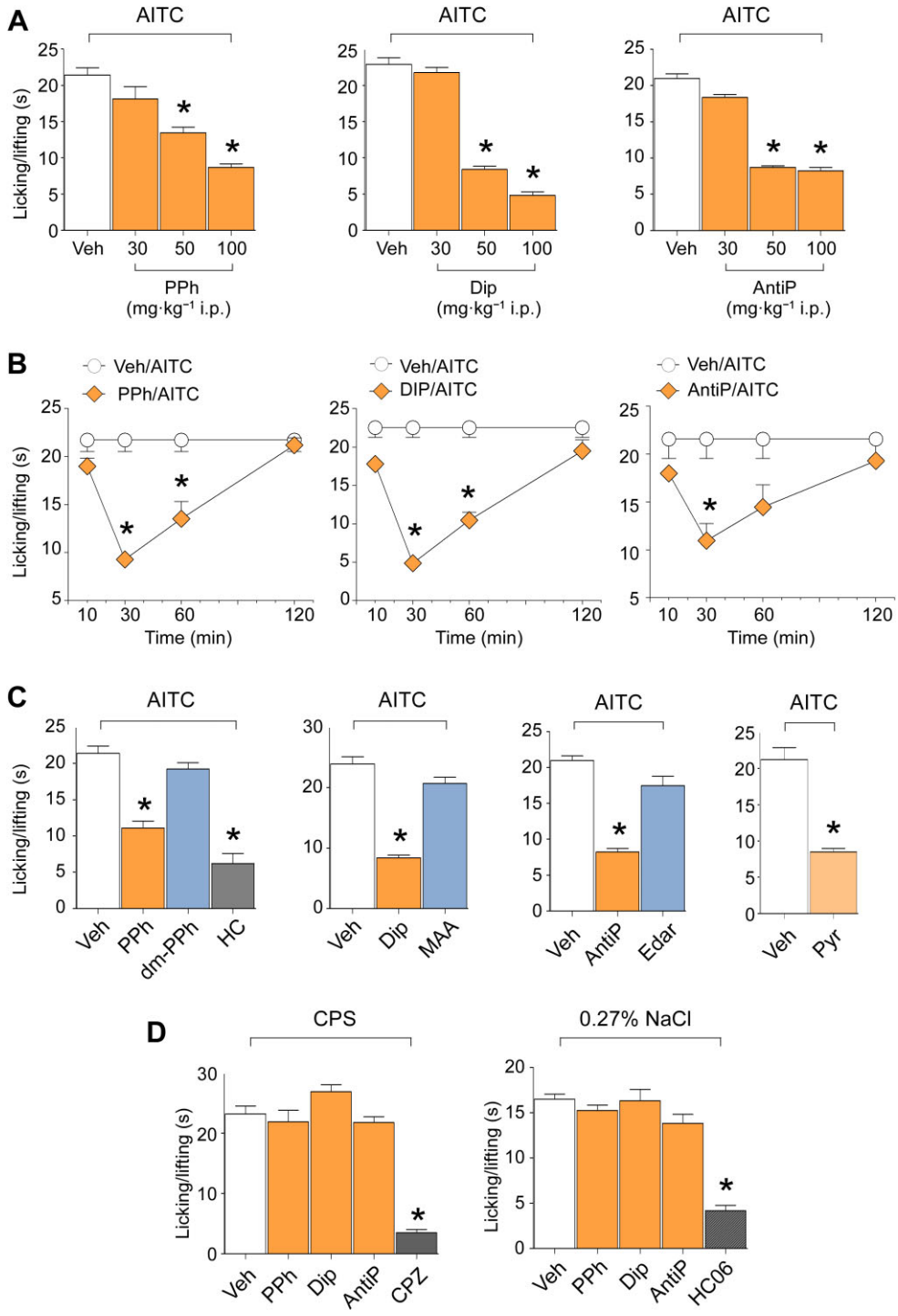

Figure 4

Pyrazolone (Pyr) and its derivatives selectively inhibit nociceptive responses evoked by the TRPA1 agonist, AITC, in mice. (A) Effect of increasing doses (30, 50 and 100 mg·kg^{-1}) of i.p. PPh, Dip and AntiP on the nociceptive response evoked by the i.pl. injection (20 μL, i.pl.) of AITC (10 nmol per paw) in C57BL/6 mice. Measurements were performed 30 min after the i.p. administration of PDs. (B) Time course of the inhibitory effect of PPh, Dip and AntiP (all 50 mg·kg^{-1}, i.p.) on the nociceptive response evoked by AITC (10 nmol per paw, 20 μL i.pl.) measured 10, 30, 60 and 120 min after the i.p. administration of PDs. (C) I.p. administration of Pyr, PPh, Dip, AntiP and the selective TRPA1 antagonist, HC-030031 (HC, 100 mg·kg^{-1}) but not the PD metabolites, dm-PPh, MAA and edaravone (all 50 mg·kg^{-1}), inhibit the nociceptive response evoked by i.pl. injection (20 μL) of AITC (10 nmol per paw) in mice. (D) PPh, Dip and AntiP (all 50 mg·kg^{-1}, i.p.) do not affect nociceptive responses produced by capsaicin (0.1 nmol per paw, i.pl.) or hypotonic solution (0.27% NaCl, i.pl.), which are, however, inhibited by the TRPV1 antagonist, capsazepine (CPZ, 10 mg·kg^{-1}, i.p.) and TRPV4 antagonist, HC-067047 (HC06 10 mg·kg^{-1}, i.p.) respectively. Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates vehicle of PDs, their metabolites, HC, CPZ and HC06. *P < 0.05 versus Veh.

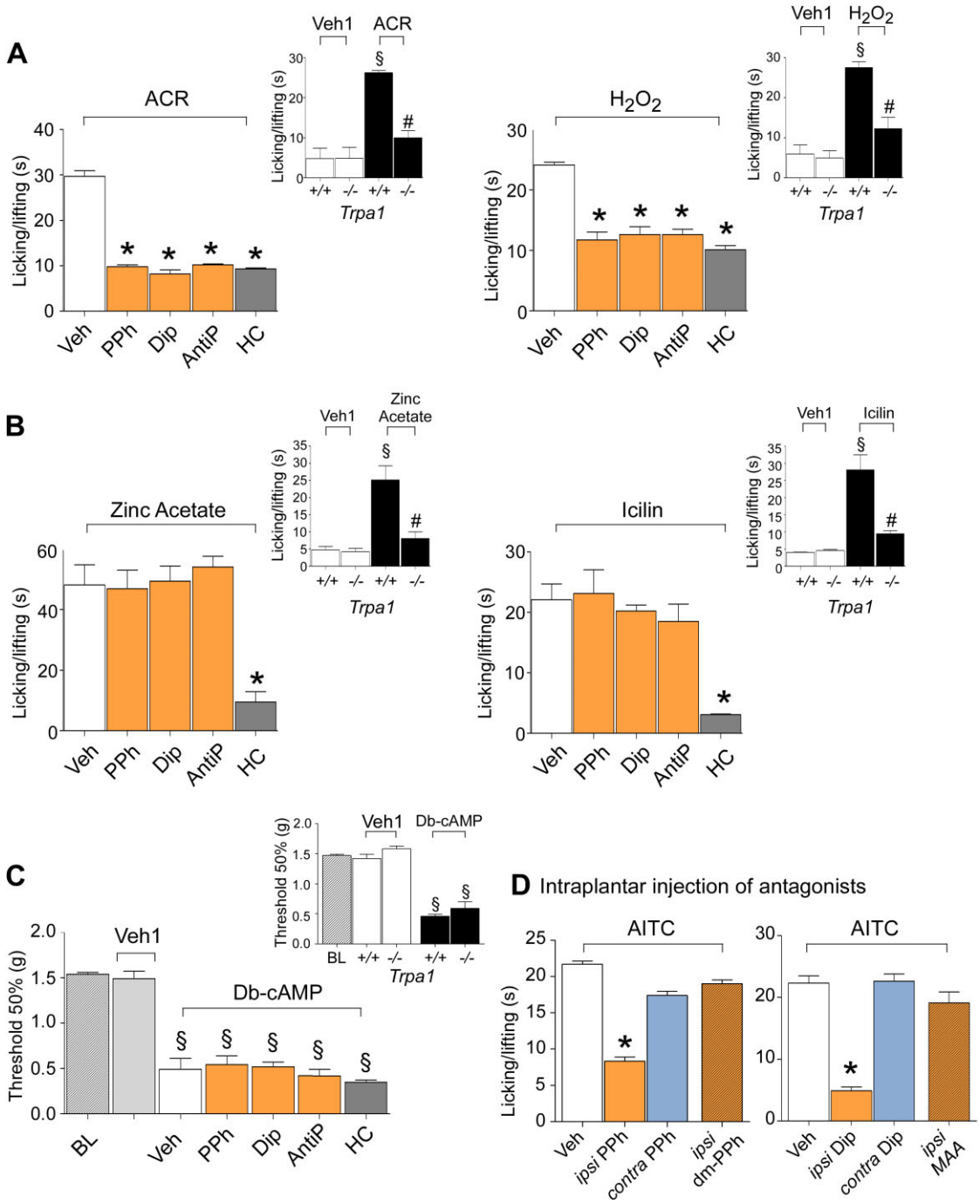

Figure 5

Pyrazolone (Pyr) derivatives selectively inhibit nociceptive responses evoked by reactive TRPA1 agonists in mice. (A and B) PPh, Dip and AntiP (all 50 mg·kg^{-1}, i.p.) inhibit nociceptive responses evoked by the injection (20 μL, i.pl.) of acrolein (ACR, 10 nmol per paw) or H_2O_2 (1 μmol per paw) but not responses evoked by both zinc acetate and icilin. HC-030031 (HC, 100 mg·kg^{-1}, i.p.) inhibits nociceptive responses evoked by both reactive and non-reactive TRPA1 agonists. (A and B, insets) Responses to both reactive and non-reactive agonists are markedly attenuated in *Trpa1*$^{-/-}$ mice. (C) Delayed allodynia observed 120 min after (20 μL, i.pl.) Db-cAMP (0.2 μmol per paw), which is similar in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice, is unaffected by PPh, Dip or AntiP (all 50 mg·kg^{-1}, i.p.) (BL, basal level threshold). (D) In mice, ipsilateral (*ipsi*) injection (20 μL, i.pl.) of a mixture of AITC (10 nmol per paw) with PPh (1 μmol per paw) or Dip (10 μmol per paw), but not with dm-PPh (1 μmol per paw) or MAA (10 μmol·paw^{-1}), reduces the nociceptive response evoked by the ipsilateral injection of the mixture of AITC (10 nmol per paw) with the vehicle (Veh). Nociceptive responses by AITC (10 nmol per paw) are not reduced by (20 μL, i.pl.) PPh (1 μmol per paw) or Dip (10 μmol per paw) injected in the contralateral (*contra*) paw. Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates vehicle of PDs, their metabolites and HC. Veh1 indicates vehicle of ACR, H_2O_2, zinc acetate, icilin or Db-cAMP. *$P < 0.05$ versus Veh; §$P < 0.05$ versus Veh1; #$P < 0.05$ versus *Trpa1*$^{+/+}$.

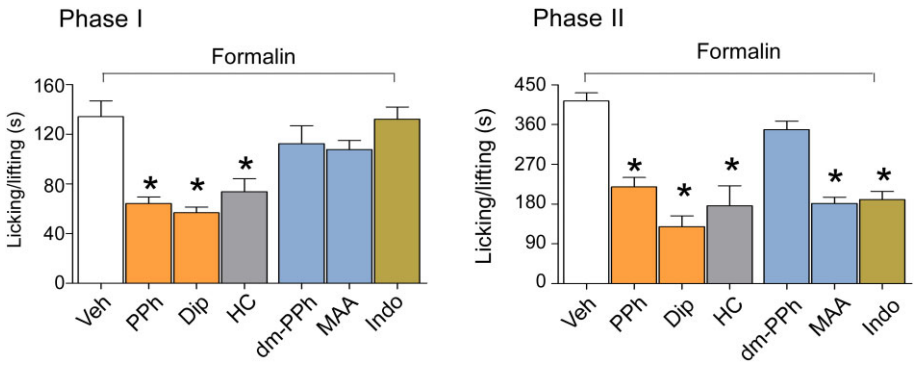

Figure 6

Pyrazolone (Pyr) derivatives produce antinociception and antihyperalgesia in the formalin model of inflammatory pain. I.p. administration of PPh or Dip (both 50 mg·kg^{-1}) and the selective TRPA1 antagonist, HC-030031 (HC, 100 mg·kg^{-1}, i.p.), inhibit phase I and phase II of the formalin test. The metabolites of PPh and Dip, dm-PPh and MAA (both 50 mg·kg^{-1}) do not affect phase I. MAA, but not dm-PPh, inhibits phase II of the formalin test. Indomethacin (Indo, 30 mg·kg^{-1}, i.p.) inhibits phase II, but does not affect phase I. Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates the vehicle of PDs, their metabolites, HC and Indo. *$P < 0.05$ versus Veh.

derivatives, dipyrone, propyphenazone and antipyrine, are selective antagonists of the TRPA1 channel, with no agonistic effects on TRPA1. PDs, such as NSAIDs/coxibs, have been proposed to act through inhibition of PG synthesis (Simmons *et al.*, 2004; Hinz *et al.*, 2007; Malvar Ddo *et al.*, 2011). However, this hypothesis was soon challenged by a series of preclinical and clinical findings. Pain models responsive to dipyrone are clearly distinct from those inhibited by classical COX inhibitors (Brune and Alpermann, 1983; Lorenzetti and Ferreira, 1985). IC_{50}s of dipyrone to inhibit COX-1 and COX-2 (0.35 and 1 mM, respectively) (Chandrasekharan *et al.*, 2002) markedly exceed the plasma concentrations attainable with clinical doses (Volz and Kellner, 1980). The higher efficiacy of dipyrone versus NSAIDs in reducing pain in neuropathic pain conditions, such as sciatic pain or post-surgical pain (Babej-Dolle *et al.*, 1994; Derry *et al.*, 2010) as well as poor gastrointestinal, cardiovascular and renal toxicity (Pogatzki-Zahn *et al.*, 2014), further differentiate PDs from NSAIDs with regard to their pharmacological mechanism of action. However, it should be noted that while the role of PDs in inflammatory pain is established over more than a century by a number of studies, little evidence has been gathered regarding the efficacy of PDs in neuropathic pain. This discrepancy may be due to the safety issues (agranulocytosis), which arose years before (Edwards and McQuay, 2002) the scientific community had reached a common definition and identification of neuropathic pain (Merskey and Bogduk, 1994). Thus, the ability to explore the effect of PDs in neuropathic pain has most likely been hampered by the recently acquired awareness of their poor safety profile. Nevertheless, the efficacy of dipyrone has been shown in acute lumbago or sciatic pain (Babej-Dolle *et al.*, 1994) and in conditions of mixed pain, such as post-operative pain (Grundmann *et al.*, 2006; Pogatzki-Zahn *et al.*, 2014).

In this study, we provide robust evidence against the role of PG inhibition in the analgesic action of PDs. Although PDs and HC-030031 reduced carrageenan-evoked hyperalgesia similarly to indomethacin, the associated increase in oedema and PGE_2 levels was attenuated only by indomethacin and not affected by PDs and HC-030031. Thus, the marked lack of association between the antihyperalgesic and anti-inflammatory action and of PDs contrasts with the entirely COX-dependent effects of indomethacin. The present observations, consistent with the poor ability of PDs to inhibit COXs (Chandrasekharan *et al.*, 2002; Simmons *et al.*, 2004), casts further doubts on COX inhibition as the main mechanism responsible for the analgesic action of PDs. In addition, the remarkable selectivity of PDs at inhibiting TRPA1 activation should be outlined. Notably, PDs only attenuated nociception evoked by the injection in the paw of the selective TRPA1 agonist, AITC, without affecting the action of the selective TRPV1 agonist, capsaicin, or a hypoosmotic stimulus which activates TRPV4.

Dipyrone, through the action of its main metabolite, MAA, has been reported to possess COX-inibitory, antioxidant and scavenging properties (Costa *et al.*, 2006; Hinz *et al.*, 2007; Pierre *et al.*, 2007; Aldini *et al.*, 2010), which early on were claimed to contribute to PD-evoked analgesia (Weithmann and Alpermann, 1985). MAA, through an iron-dependent mechanism, may sequestrate radicals that are necessary to initiate COX catalytic cycle (Pierre *et al.*, 2007). Therapeutic effects of edaravone have also been associated with its scavenging activity (Mao *et al.*, 2009). The finding that PDs selectively inhibit TRPA1 channel activation by reactive agonists raises the plausible hypothesis that PD metabolites may contribute to the analgesic action of dipyrone, propyphenazone and antipyrine by sequestering reactive electrophilic agonists. However, PD metabolites were unable to reduce nociception or hyperalgesia in all the TRPA1-dependent pain-like responses investigated in the present study. In addition, only prolonged incubation with the classical scavenger molecule, GSH, abolished TRPA1 activation by eletrophifilic or reactive agonists, AITC, acrolein or H_2O_2, while MAA, dm-propyphenazone and edaravone were ineffective. Finally, although propyphenazone shares all the antinociceptive and antihyepralgesic effects of dipyrone (Costa *et al.*, 2006), propyphenazone and dm-propyphenazone did not show any antioxidant properties (Costa *et al.*, 2006).

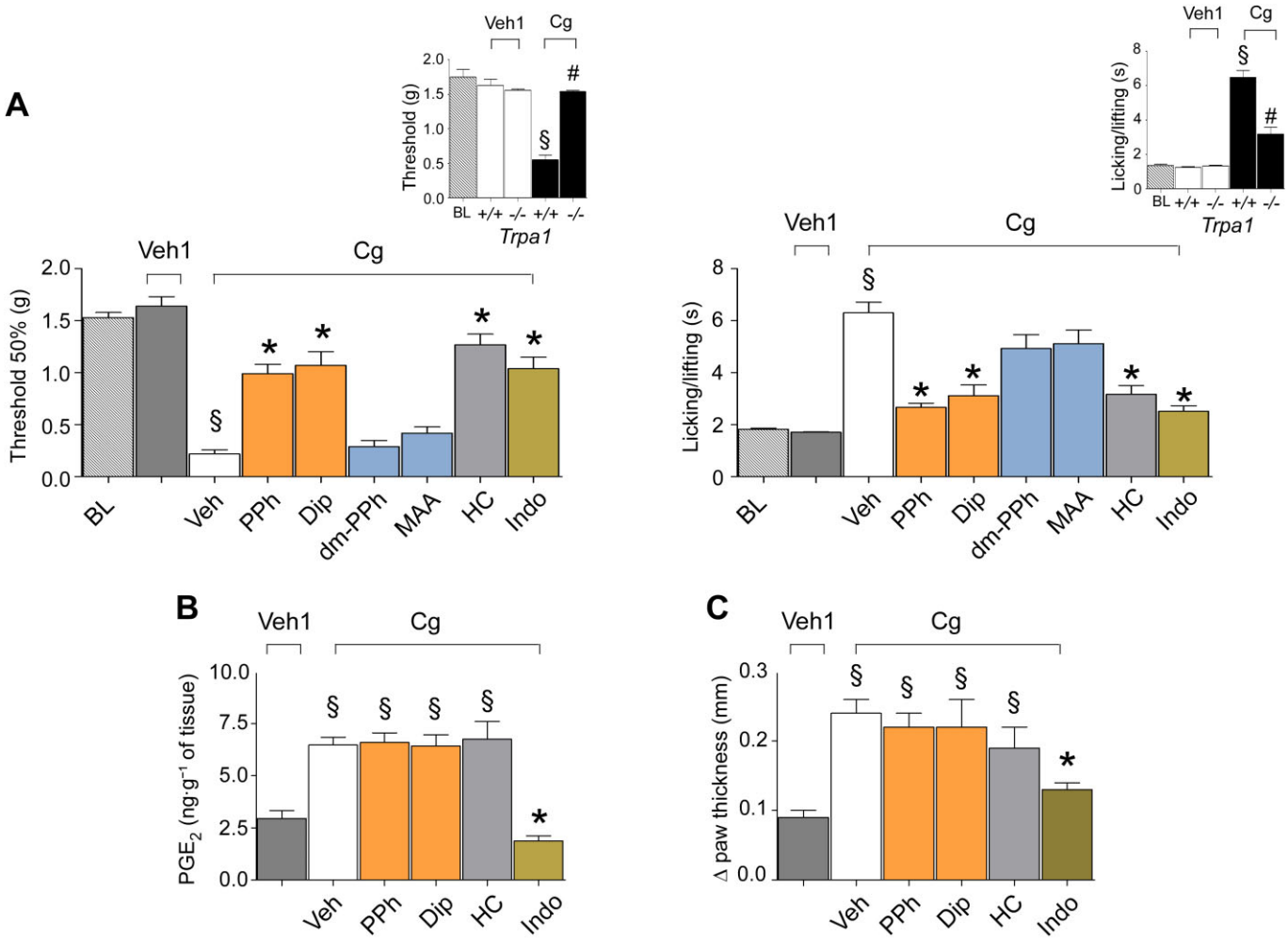

Figure 7

PDs produce antinociception and antihyperalgesia in the carrageenan model of inflammatory pain. (A) Mechanical allodynia and cold hypersensitivity evoked by i.pl. (20 μL) injection of carrageenan (Cg, 300 μg per paw) in *Trpa1*$^{+/+}$ are markedly attenuated in *Trpa1*$^{-/-}$ mice and inhibited by PPh or Dip (both 50 mg·kg^{-1}), the selective TRPA1 antagonist, HC-030031 (HC, 100 mg·kg^{-1}, i.p.), and indomethacin (Indo, 30 mg·kg^{-1}, i.p.) but not by the PD metabolites, dm-PPh and MAA (both 50 mg·kg^{-1}, i.p.) (BL, basal level threshold). (B) PGE_2 levels in paw homogenate are increased 180 min after Cg injection (300 μg·20 μL^{-1}, i.pl.). PPh, Dip (both 50 mg·kg^{-1}, i.p.) and HC (100 mg·kg^{-1}, i.p.) do not affect the increase in PGE_2, which is, however, prevented by Indo (30 mg·kg^{-1}, i.p.). (C) Indo (30 mg·kg^{-1}, i.p.), but not PPh, Dip (both 50 mg·kg^{-1}, i.p.) and HC (100 mg·kg^{-1}, i.p.), inhibits paw oedema induced by i.pl. (20 μL) injection of Cg (300 μg per paw). Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates the vehicle of PDs, their metabolites, HC and Indo. Veh1 indicates the vehicle of Cg. *$P < 0.05$ versus Veh; $^{§}P < 0.05$ versus Veh1; $^{\#}P < 0.05$ versus *Trpa1*$^{+/+}$.

Thus, the potential antioxidant and scavenging properties of MAA and edaravone do not seem to be responsible for the TRPA1-dependent analgesic action of their precursors.

In summary, our findings showed that: (i) PDs, but not their metabolites, are selective TRPA1 antagonists; (ii) the TRPA1-dependent analgesic profile of PDs is not shared by their metabolites; (iii) attenuation by PDs of pain-like effects in the carrageenan test is completely unrelated to PG inhibition; and (iv) in all pain models, the ability of dipyrone and propyphenazone to produce antinociceptive or antihyperalgesic effects *in vivo* is exquisitely TRPA1-dependent. Thus, we propose that the analgesic activity of PDs resides substantially in their ability to directly target the TRPA1 channel. This hypothesis is corroborated by the observation that the IC_{50}s required to inhibit the rodent and human native TRPA1 (~60 μM) are close to those found in humans after therapeutic doses of dipyrone or propyphenazone, the two PDs applied by a systemic route of administration (Volz and Kellner, 1980).

Emerging evidence indicates that oxidative and nitrative stress and the ensuing lipid peroxidation by-products produce nociception and hyperalgesia by targeting TRPA1 (Bautista *et al.*, 2006; Trevisani *et al.*, 2007; Taylor-Clark *et al.*, 2009). This novel pathway has been reported to contribute to models of both inflammatory and neuropathic pain (McNamara *et al.*, 2007; Andrade *et al.*, 2012; Trevisan *et al.*, 2013b; Nassini *et al.*, 2014). The selective antagonism against potentially reactive endogenous agonists (acrolein and H_2O_2),

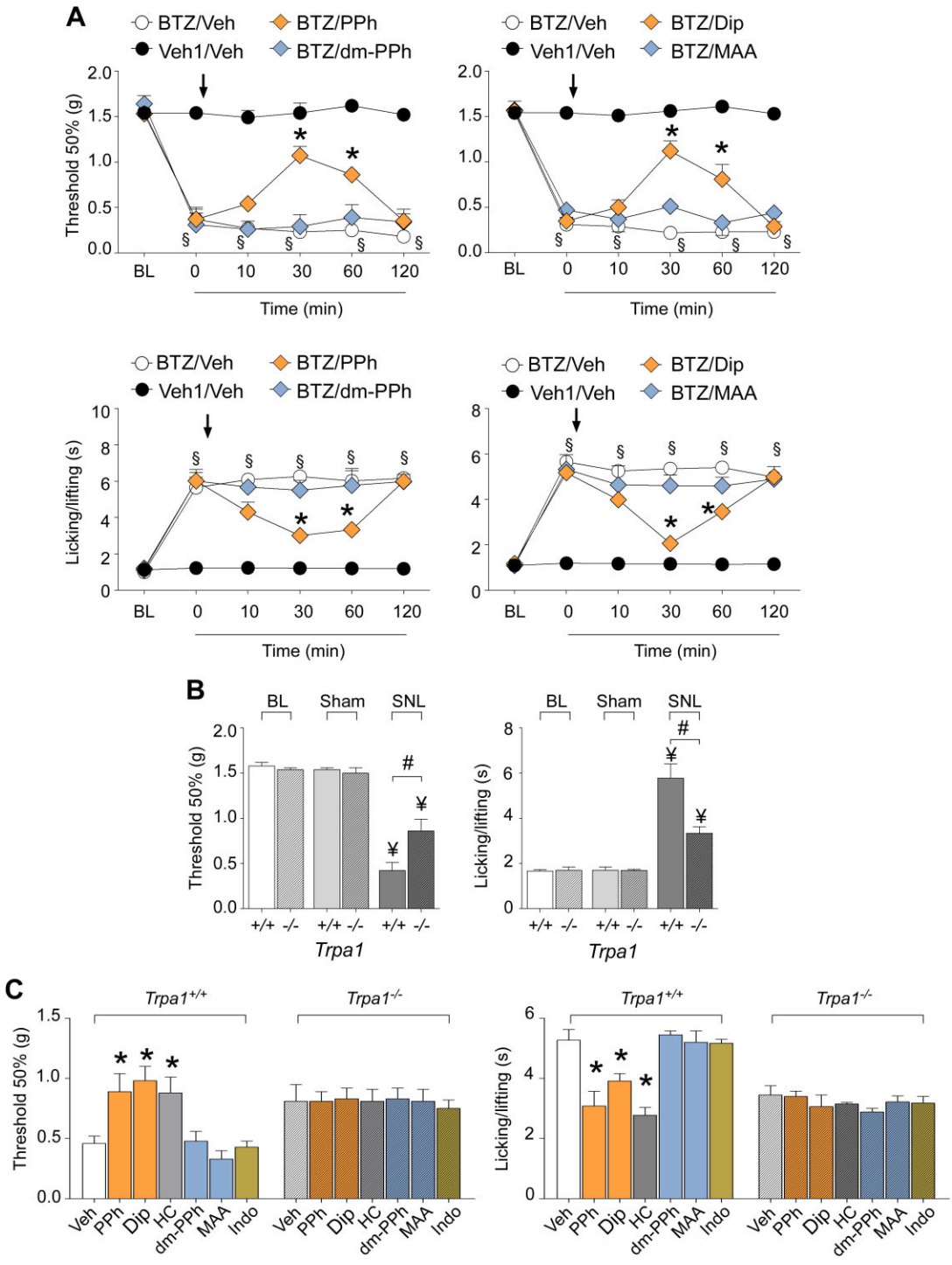

Figure 8

PDs produce antihyperalgesic effects in models of neuropathic pain. (A) At day 7 after treatment with bortezomib (BTZ, 1 mg·kg^{-1}, i.p.) mechanical allodynia and cold hypersensitivity are increased (BL, basal level threshold at day 0 before BTZ). At day 7, injection (arrow) of PPh or Dip (both 50 mg·kg^{-1}, i.p.), but not of their metabolites, dm-PPh and MAA (both 50 mg·kg^{-1}, i.p.), inhibits mechanical allodynia and cold hypersensitivity induced by BTZ. (B) At day 10 after partial sciatic nerve ligation (SNL), *Trpa1*$^{+/+}$ mice develop mechanical allodynia and cold hypersensitivity, which are partially reduced in *Trpa1*$^{-/-}$ mice and not present in mice that underwent the sham procedure (Sham). (C) At day 10 after SNL, injection of PPh, Dip (both 50 mg·kg^{-1}, i.p.) and HC (100 mg·kg^{-1}, i.p.) reverses the mechanical allodynia and cold hypersensitivity in *Trpa1*$^{+/+}$ mice, but does not provide further protection in *Trpa1*$^{-/-}$ mice. dm-PPh and MAA (both 50 mg·kg^{-1}, i.p.) or indomethacin (Indo, 30 mg·kg^{-1}, i.p.) do not affect mechanical allodynia and cold hypersensitivity in either *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice. Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates the vehicle of PDs, their metabolites, HC and Indo. Veh1 indicates the vehicle of BTZ. $^{§}P < 0.05$ versus Veh1/Veh; $^{*}P < 0.05$ versus BTZ/Veh (A) or Veh (C); $^{¥}P < 0.05$ versus respective BL and Sham; $^{\#}P < 0.05$ versus *Trpa1*$^{+/+}$.

corroborated by mutagenesis studies and *in silico* analysis, strengthens the proposal that PDs target TRPA1 by binding to cysteine residues required for TRPA1 channel activation by reactive endogenous agonists. A consequence of such a hypothesis is that, in models of inflammatory and neuropathic pain, PDs block the signalling pathway activated by oxidative stress by-products, via stimulation of the TRPA1 channel, expressed in primary sensory neurons (Andrade *et al.*, 2012; Nassini *et al.*, 2014). Attenuation of the pain-producing TRPA1-dependent pathway activated by oxidative stress by-products might also contribute to the analgesic action of PDs in various types of pain in humans (Babej-Dolle *et al.*, 1994; Ramacciotti *et al.*, 2007; Derry *et al.*, 2010).

In spite of their generally good tolerability, the use and further development of PDs has been hampered by their adverse haematological effects, as indicated by the reported increased incidence of blood dyscrasias and agranulocytosis (Hedenmalm and Spigset, 2002), which has led to the withdrawal of dipyrone and propyphenazone in several countries. More recently, pharmacovigilance reports failed to detect a strong association between dipyrone use and agranulocytosis (Ibanez *et al.*, 2005). Nevertheless, a substantial portion of the world population can no longer use a group of effective and otherwise well-tolerated pain killers because of this severe adverse haematological reaction. The present findings strongly support the rationale for the development of TRPA1 antagonists as new analgesics for the treatment of inflammatory and neuropathic pain. The new chemical entities with TRPA1 antagonistic properties, while maintaining good efficacy in pain treatment and general safety profile of dipyrone or propyphenazone, should be devoid of the life-threatening adverse haematological reactions, presumably associated with the chemical structure of PDs.

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Author contributions

R. N., S. M., P. G., R. P., S. B. and A. C. designed the experiments and interpreted the results. C. F., S.M. and F. D. L. performed the calcium experiments. E. C. performed the electrophysiological experiments. R. N., I. M. M., R. T. and D. P. performed the *in vivo* experiments and T. T. performed the docking studies.

Conflict of interest

R. P. is full-time employee at Chiesi Farmaceutici S.p.A. Other authors state no conflict of interest.

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Supporting information

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Appendix S1 Supplemental methods.

Figure S1 (A) PPh, Dip, AntiP and Pyr (all, 100 μM) do not affect the calcium response evoked by capsaicin (CPS, 0.1 μM) in HEK293 cells transfected with the cDNA of the human TRPV1 (hTRPV1-HEK), a response blocked by capsazepine (CPZ, 10 μM) and the calcium response evoked by the TRPV4 agonist, GSK1016790A (GSK, 0.1 μM), in HEK293 cells transfected with the cDNA of the human TRPV4 (hTRPV4-HEK), a response blocked by the TRPV4 antagonist, HC-067047 (HC06, 10 μM). (B) Pre-exposure (10 min before) to metabolites of PPh, Dip and AntiP, dm-PPh (500 μM), MAA (1 mM) and Edar (1 mM), respectively, and to the aldehyde and reactive oxygen species scavenger, GSH (1 mM) do not affect the calcium response to AITC in rat DRG neurons (10 μM) and IMR90 cells (1 μM). (C) In another series of experiments, acrolein (ACR, 3 μM) or H2O2 (500 μM) were co-incubated for 30 min with PPh (100 μM), Dip (1 mM), AntiP (5 mM), Pyr (100 μM), GSH (1 or 5 mM against ACR or H2O2, respectively), dm-PPh (500 μM), MAA (1 mM), edaravone (1 mM) or their vehicles (Veh). IMR90 cells were then challenged with the mixtures. Responses produced by the mixture of ACR or H2O2 and Vehs are not affected by Pyr, PDs or their metabolites, but are abated by GSH. Pre-exposure (10 min prior stimuli) of IMR90 cells to GSH (1 or 5 mM against ACR or H2O2, respectively) does not affect the calcium response evoked by ACR (3 μM) or H2O2 (500 μM). Values are mean ± SEM of at least $n > 25$ cells from at least three different experiments for each experimental condition. Veh indicates the combination of the vehicles of the various compounds. *$P < 0.05$ versus Veh.

Figure S2 Hypothetical interaction of PPh with TRPA1. Receptor (left) and binding site (right) visualization. The TRPA1 electron density map is also reported in the receptor visualization. The interaction between PPh and C608 allows the formation of an H-bond between the hydroxyl group of S582 and the oxygen of the pyrazolidinone ring of PPh. The isopropyl substituent appears to be solvent exposed and the methyl group interacts with L609. Finally, the phenyl ring is inserted into a lipophilic pocket mainly delimited by L584, L588, V596 and I600.

Figure S3 Pharmacological interventions in mouse models of neuropathic pain. At day 7 after treatment with bortezomib (BTZ, 1 mg·kg^{-1}, i.p.), mechanical allodynia and cold hypersensitivity are increased (BL, baseline at day 0 before BTZ). At day 7, injection (arrow) of HC-030031 (HC, 100 mg·kg^{-1}, i.p.), but not indomethacin (Indo, 30 mg·kg^{-1}, i.p.), inhibits mechanical allodynia and cold hypersensitivity induced by BTZ. Values are mean ± SEM of at least six mice for each experimental condition. Veh indicates the vehicle of HC or Indo. Veh1 indicates the vehicle of BTZ. $^{§}P < 0.05$ versus Veh1/Veh; *$P < 0.05$ versus BTZ/Veh.