Bioorganic & Medicinal Chemistry 20 (2012) 1690–1698

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

7-Substituted-pyrrolo[3,2-*d*]pyrimidine-2,4-dione derivatives as antagonists of the transient receptor potential ankyrin 1 (TRPA1) channel: A promising approach for treating pain and inflammation

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ARTICLE INFO

Article history:
Received 3 November 2011
Revised 9 January 2012
Accepted 11 January 2012
Available online 24 January 2012

Keywords:
TRPA1 channel
Pain
Inflammation
Asthma
TRPA1 antagonists

ABSTRACT

The transient receptor potential ankyrin 1 (TRPA1) channel is activated by a series of by-products of oxidative/nitrative stress, produced under inflammatory conditions or in the case of tissue damage, thus generating inflammatory and neuropathic pain and neurogenic inflammatory responses. These findings have identified TRPA1 as an emerging opportunity for the design and synthesis of selective inhibitors as potential analgesic and antiinflammatory agents. Herein we present the synthesis and functional evaluation of a new series of 7-substituted-1,3-dimethyl-1,5-dihydro-pyrrolo[3,2-*d*]pyrimidine-2,4-dione derivatives designed as TRPA1 antagonists. A small library of compounds has been built by the introduction of differently substituted N^7-phenylacetamide or N^7-[4-(substituted-phenyl)-thiazol-2-yl]-acetamide chains. All the synthesized compounds were assayed to evaluate their ability to block acrolein-mediated activation of native human and rat TRPA1 channels employing a fluorometric calcium imaging assay. Our study led us to the identification of compound **3h** which showed considerably improved potency (IC_{50} = 400 nM) against human TRPA1 with regard to some of the most representative antagonists previously reported and integrated in our screening program as reference compounds. In addition, **3h** proved to maintain its efficacy toward rTRPA1, which designates it as a possible candidate for future evaluation of in vivo efficacy in rodent animal model of inflammatory and neuropathic pain.

1. Introduction

The transient receptor potential ankyrin 1 (TRPA1) belongs to the largest family of TRP ion channels, which exert pleiotropic functions in a variety of cells and pathophysiological conditions.[1] The superfamily of TRP channels in mammals consists of 28 different proteins behaving as nonselective Ca^{2+}-permeable cation channels with a variable permeability ratio (Ca^{2+}/Na^{+}) between different members of the superfamily, and also among the individual members of each subfamily. TRPA1 channel, first cloned from human fetal lung fibroblasts,[2] is the sole representative of the A subfamily; it exhibits 14 NH_2-terminal ankyrin repeats,[3] an unusual structural feature, from which it derives its name.[4,5] TRPA1 localizes to primary sensory neurons of the trigeminal (TG), vagal (VG), dorsal root (DRG) ganglia, and in hair cells.[3,5–7] More specifically, TRPA1 co-localizes with the vanilloid, TRPV1, channel in a subset of somatosensory neurons with non-myelinated or thinly myelinated fibers and with nociceptive and pain producing functions.[3] Most of the TRPA1-expressing neurons contain and release proinflammatory neuropeptides which mediate nociceptive responses and neurogenic inflammation.[8]

Growing evidence has been accumulated in the last 5 years with respect to the contribution of TRPA1 to nociceptive responses evoked by a host of xenobiotics,[9] including ingredients of various spicy foods, such as wasabi and mustard oil (allyl isothiocyanate), cinnamon (cinnamaldehyde), garlic (allicin), and other spices[9] or irritant compounds, such as crotonaldehyde (contained in cigarette

Abbreviations: AITC, allyl isothiocyanate; BSA, bovine serum albumin; CA, cinnamaldehyde; CHO, Chinese hamster ovary cells; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modified eagle medium; DRG, dorsal root ganglia; EDAC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole hydrate; IMR90, fetal human lung fibroblasts; NGF, nerve growth factor; ROS, reactive oxygen species; RNS, reactive nitrogen species; TG, trigeminal ganglia; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; VG, vagal ganglia.

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doi:10.1016/j.bmc.2012.01.020

smoke),[6] formaldehyde,[10] and general anesthetics.[11] This action accounts for their irritant and pain producing action. Endogenous TRPA1 activators generally include reactive oxygen species (ROS), reactive nitrogen species (RNS), and their by-products, and specifically acrolein, 4-hydroxy-2-nonenal, hydrogen peroxide, peroxinitrite anion, hypochlorite, nitrooleic acid, lipid peroxidation by-products and cyclopentenone prostaglandins, and isoprostanes.[9,12–14] All these agents, formed in large amounts and ubiquitously at sites of inflammation or tissue injury, are now identified as mediators which signal a major pain generating system. The possibility that by-products of oxidative/nitrative stress, produced under inflammatory conditions or in case of tissue damage, target TRPA1, thus contributing to inflammatory and neuropathic pain, furnished the rational for the design and synthesis of selective inhibitors as potential analgesic and antiinflammatory agents. Despite the fact that in recent years much effort has been dedicated to the discovery of better and safer analgesics, the medical need for this type of drugs remains substantially unmet. In particular, compounds capable of targeting both nociceptive and neuropathic pain are lacking. Thus, the contribution of neuronal TRPA1 in the orchestration of the inflammatory response and its emerging role in the mechanism of neuropathic pain indicates TRPA1 antagonists as novel analgesic drugs. Moreover, there is increasing evidence that selective inhibitors of TRPA1 channels may be considered potential therapeutic agents in treating respiratory diseases characterized by airways inflammation with beneficial effects in asthma and COPD, and maybe, chronic cough.[15,16]

In agreement with the hypothesis that TRPA1 blockade may be beneficial in the treatment of pain, the TRPA1 antagonist 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-*N*-(4-isopropyl-phenyl)-acetamide **1** developed at Hydra Biosciences (HC-030031, Fig. 1),[10] proved to inhibit mechanical hypersensitivity caused by both inflammatory and neuropathic pain models.[17] In addition, the *N*′-(4-*sec*-butyl-phenyl) analog of **1** (namely Chembridge-5861528) synthesized by ChemBridge Corporation is under preclinical investigation for the treatment of diabetic neuropathy.[18] Different bicycle/tricycle congeners of **1** have recently been developed at Glenmark Pharmaceuticals as TRPA1 antagonists: substituted phthalimide,[19] imidazo[2,1-*b*]purine,[20] thieno-pyrimidine dione,[21] furo-pyrimidinedione,[22] isothiazolo-pyrimidinedione,[23] quinazolinedione[24] derivatives. Thanks to a high-throughput screening approach, some other molecules have been identified, such as a small series of trichloro(sulfanyl)ethyl benzamides[25] and a series of (heteroaryl)alkenone oxime derivatives[26], as TRPA1 antagonists. Even though the current status of this research field shows stimulating pioneering aspects, the applicative value of the area emerges from the recent announcement by Glenmark Pharmaceuticals of the identification of the first TRPA1 selective antagonist (GRC-17536) planned to enter phase I human clinical trials for the treatment of pain and respiratory disorders in January 2011.

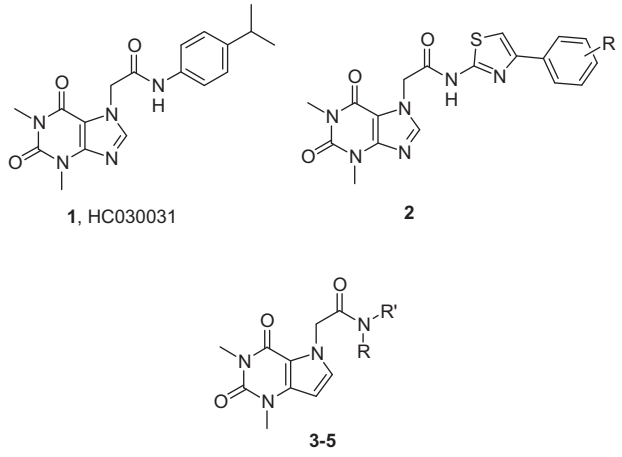


Figure 1. General structures of the newly designed compounds (**3–5**) and their 9-aza analogues **1** and **2** reported in literature.

Herein we present our efforts aimed at the identification of new TRPA1 antagonists starting from the structure of HC-030031. This compound, displaying μM potency in blocking TRPA1, shows a central xanthine core functionalized at the 7-position with a phenyl-acetamide chain. In order to improve HC-030031 potency, we synthesised and tested a new series of 7-substituted-1,3-dimethyl-1,5-dihydro-pyrrolo[3,2-*d*]pyrimidine-2,4-dione derivatives (general structures **3–5**, Fig. 1) conceived as 9-deaza-analogs of the xanthine-based reference compounds with general structures **1** and **2**.[27] This approach allowed us to indirectly evaluate the importance of N^9 for receptor interaction. Thanks to the optimization of a helpful synthetic approach (Scheme 1), we were able to build a small library of compounds introducing an N^7-(substituted)phenylacetamide (in accordance to HC) or an N^7-[4-(substituted-phenyl)-thiazol-2-yl]-acetamide (in accordance to **2**) chain. Different substituents have been introduced at the phenyl moiety of the N^7-phenyl/thiazol-2-yl-acetamide chain such as halogens or bulky cyclo/arylalkyl groups. During the progress of our work, Glenmark Pharmaceuticals claimed an analogous series of fused pyrimidinedione derivatives as TRPA1 modulators.[28]

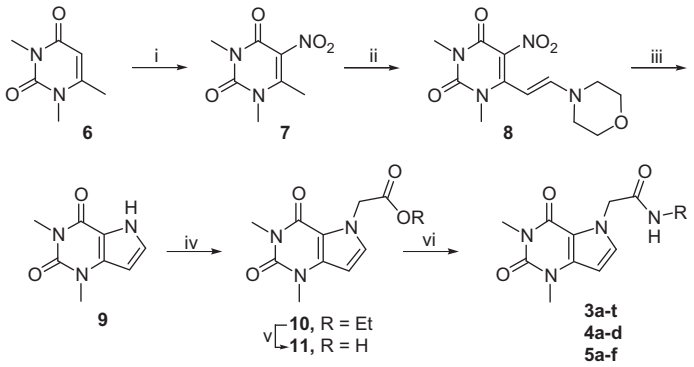
2. Results and discussion

2.1. Synthesis

1,3-Dimethyl-1,5-dihydro-pyrrolo[3,2-*d*]pyrimidine-2,4-dione **9** (Scheme 1) was prepared in good yield starting from standard nitration at the 5-position of the commercially available 1,3,6-trimethyl-uracil **6** followed by reaction with an excess of triethyl orthoformate in the presence of morpholine to give the 6-morpholino vinyl derivative **8** and final reductive cyclization with zinc in acetic acid, according to previously reported procedures.[29] Alkylation at the 7-position with ethyl 2-bromoacetate was performed in DMF in presence of K_2CO_3 to obtain the ethyl ester **10** which was subsequently hydrolyzed using 10**%** sodium hydroxide to the corresponding acid intermediate **11**. Final compounds with general structures **3** and **4** were obtained by a coupling reaction of **11** with (substituted)aryl-, arylalkyl- or cycloalkyl-amines in presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 1-hydroxybenzotriazole hydrate (HOBt) in DMF, at room temperature. Final derivatives **5a–f** were synthesized via an analogous EDAC/HOBt-mediated condensation of **11** with 4-(substituted)phenyl-thiazol-2-yl amines which have been conveniently prepared from the appropriate commercially available (substituted)-acetophenones and thiourea.[30]

2.2. Evaluation of rat and human TRPA1 inhibition

Thanks to the identification of hTRPA1 antagonists which display very low or reverse (agonist) activity against the rat isoform of the channel, both the Amgen[25] and Abbott[31] groups, highlighted as TRPA1 is a problematic target as it is subject to a marked species-specific modulation. Although activity at the human receptor is, in fact, essential in view of future therapeutic perspectives, the ability to inhibit the rat/mouse receptor is strictly required because the vast majority of tests for inflammatory, neuropathic and cancer pain have been developed and validated in rodent species. In light of this, both human (fetal lung fibroblasts, IMR90) and rat (primary cultures of rat DRG neurons) cells that constitutively



Scheme 1. Reagents and conditions: (i) H_2SO_4/HNO_3, 0 °C, 45'; (ii) Morpholine, $HC(OCH_2CH_3)3$, 120 °C, 4h; (iii) Zn, CH_3CO_2H, 90 °C, 30'; (iv) $BrCH_2CO_2CH_2CH_3$, K_2CO_3, DMF, rt, 3h; (v) NaOH 10%, CH_3OH, rt, 2h; (vi) (substituted)aryl-, arylalkyl- or cycloalkyl- amines EDAC, HOBt, DMF, rt, 12–24 h for 3,4; 4-(substituted)phenyl-thiazol-2-yl amines, EDAC, HOBt, DMAP, DCE, rt, 8–12 h for 5.

expressed the TRPA1 channel, have been used in the present work for the in vitro functional screening. We based our research program on the evaluation of the modulation of native channels activity in cells which constitutively express the TRPA1 channel, thus overcoming problems not infrequently inherent with cloned receptors expressed in recombinant systems that may affect the precise estimate of potency measurement because of receptor over- or under-expression.

All the synthesized compounds were assayed to evaluate their ability to block acrolein-mediated activation of human (h) and rat (r) TRPA1 channels employing a fluorometric calcium imaging assay, and the results are shown in Table 1 (hTRPA1) and 2 (rTRPA1). HC-030031,[17] AP18,[26] and 4-(3-methoxy-phenyl)-2-thioxo-1,2,3,4-tetrahydro-indeno[1,2-*d*]pyrimidin-5-one[32] (compound **12** in tables and figures) were also tested as reference compounds (the chemical structures of reference compounds are depicted in Fig. 3 of the Supplementary data). The functional data are expressed as IC_{50} values and/or percentages of inhibition of channel activity at 3 and 30 μM concentration of antagonist. IC_{50} values at hTRPA1 have been calculated only for compounds with a percentage of inhibition higher than 50% at 30 μM.

Differently substituted-phenylacetamide chains have been introduced at the 7-position of the pyrrolo[3,2-*d*]pyrimidine-2, 4-dione nucleus (compounds **3a–t**, Table 1). The best results have been obtained in the case of monosubstitution at the 4′-position of the phenyl ring. Among the 4-halo derivatives **3a–d**, the 4-iodo-phenyl substitution appeared particularly effective (**3d**, IC_{50} = 1.8 μM). The introduction of a 4-fluoro function determined a slight but significant decrease of activity (**3a**, IC_{50} = 3.1 μM), while the 4-chloro substitution produced a marked loss of efficacy (compound **3c** showed a percentage of channel inhibition lower than 50% at a concentration of 30 μM). *meta*-Substitution with an iodine atom determined a similar decrease of activity if compared to *para*-substitution (see compound **3e** vs **3d**). Also bisubstitution of the phenyl ring appeared ineffective (compounds **3f** and **3g**). Analogous results have been achieved by the introduction of strong electron donating (**3i–k**) or withdrawing (**3l–m**) groups. The bisubstitution of the acetamide nitrogen determined a significant decrease of efficacy (see compound **3b** vs **3a**). This would indicate that the free N–H group could establish a favorable interaction with the receptor.

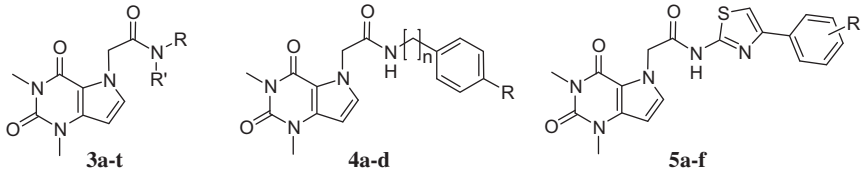
The efficacy of 4-iodo substitution suggested a possible steric control around 4-phenyl position, thus we tried to evaluate the effect of the introduction of hindered halogenated moieties, such as the $-CF_3$ group (**3h**), or bulky linear (**3p–q**), branched (**3n–o**) or cycloalkyl (**3r**) functions. Among these derivatives, the best results have been obtained with the introduction of a 4-trifluoromethyl group, which led us to the identification of compound **3h** as the most potent of the whole series (IC_{50} = 400 nM). This datum, along with the activity of the 4-*t*-butyl (**3o**, IC_{50} = 3.1 μM), the 4-*n*-butyl (**3p**, IC_{50} = 1.1 μM) and the 4-*n*-hexyl (**3q**, IC_{50} = 1.6) derivatives, confirmed that the presence of a bulky and lipophilic moiety at the 4-position of the phenyl ring may be of particular importance for ligand–receptor interaction. Nevertheless, it has to be noted that **3n**, the direct pyrrolopyrimidine analogue of HC030031, substituted with a 4-isopropyl moiety, proved to be inactive. This would suggest that the N^9 of the xanthine nucleus could be somewhat involved in a favorable interaction with the channel.

While branched and linear alkyl chains were shown to mostly enhance activity, the 4-cycloalkyl derivative **3r** showed a percentage of hTRPA1 inhibition lower than 50% at the highest concentration (30 μM). The introduction of heteroatoms in the 4-cycloalkyl function (see the 4-*N*-morpholinyl derivative **3s**), did not improve the activity (hTRPA1 inhibition <50% at 30 μM).

Compound **3t** is the only example of *N*′-cycloalkyl-acetamide functionalization of the series. In this derivative, indeed, a cycloalkyl moiety (1-adamantyl) has been directly linked to the nitrogen of the acetamide side chain. The lack of activity of **3t** would indicate the importance of a *N*-phenyl ring for receptor interaction. With the synthesis of the *N*-arylalkyl derivatives **4a–d** we intended to evaluate the effect on TRPA1 channel activity of a methylene (**4a**, **4d**), an ethylene (**4b**) or a propylene (**4c**) spacer between the acetamide nitrogen and the phenyl ring. Also in this case, the lack of activity testified the need of an *N*′-aryl-acetamide moiety on the N^7-side chain.

To further extend our research, the N^7-[4-(substituted-phenyl)-thiazol-2-yl]-acetamide derivatives **5a–f** have been synthesized as 9-deaza analogues of compounds with general structure **2** (see Fig. 1) previously reported by Hydra Biosciences.[33] As mentioned above, during the progress of our study, Glenmark Pharmaceuticals claimed an analogous series of fused pyrimidinedione derivatives as TRPA1 modulators.[28] In these derivatives, the thiazole ring of the lateral chains behaves as an aromatic spacer between the acetamide nitrogen and the terminal phenyl ring. Also in this small subset, the presence of fluorinated moieties at the 4-position of the terminal phenyl ring resulted effective to some extent for promoting antagonist activity (see compounds **5b** and **5d**), while the presence of a 4-iodine atom determined a substantial loss of activity (**5c**). The 3-CF_3,4-*F*-phenyl derivative **5e** is the only example of our series which has also been reported and characterized by Glenmark in its patent. The percentages of channel inhibition, obtained by Glenmark through an in vitro screening performed on a recombinant system (human TRPA1 CHO cells), were comparable to those we obtained with native channels and reported in Table 1. The pairwise comparison between the activities of analogously substituted phenylacetamide and (4-phenyl-thiazol-2-yl)-acetamide derivatives resulted in a variable and slight decrease of potency

Table 1
In vitro biological activity of the synthesized compounds in the human TRPA1 Ca^{2+} influx assay in IMR90 fibroblasts



	R	R′	*n*	hTRPA1[a]		
				% Inhibition[c]		
				IC_{50} (μM)[b]	R′	(30 μM)
HC-030031[17]				1.8 (1.1–2.8)	73.2 ± 6.4	97.5 ± 0.5
AP18[26]				2.2 (1.6–3.1)	69.1 ± 7.1	98.9 ± 0.6
12[d32]				1.5 (0.9–2.2)	83.1 ± 3.8	98.8 ± 0.9
3a	4-F-Ph	H		3.1 (1.9–4.9)	53.1 ± 7.9	96.7 ± 1.6
3b	4-F-Ph	Me				< 50
3c	4-Cl-Ph	H				< 50
3d	4-I-Ph	H		1.8 (0.7–3.9)	61.9 ± 16.4	82.2 ± 6.2
3e	3-I-Ph	H				< 50
3f	2,4-F-Ph	H				< 50
3g	3,4-F-Ph	H				< 50
3h	4-CF_3-Ph	H		0.4 (0.3–0.5)	99.0 ± 3.0	99.1 ± 2.0
3i	4-OCF_3-Ph	H		3.7 (2.3–6.2)	40.5 ± 9.0	95.6 ± 2.2
3j	4-SCF_3-Ph	H				<50
3k	4-OCH_3-Ph	H				<50
3l	4-(SO_2NEt_2)-Ph	H				<50
3m	4-(SO_2NHBn)-Ph	H				<50
3n	4-isopropyl-Ph	H				<50
3o	4-$C(CH_3)_3$-Ph	H		3.1 (1.6–6.0)	46.0 ± 7.8	89.6 ± 3.1
3p	4-*n*-Butyl-Ph	H		1.1 (0.8–1.4)	93.5 ± 4.2	99.0 ± 0.7
3q	4-*n*-Hexyl-Ph	H		1.6 (0.9–2.8)	74.5 ± 6.2	99.0 ± 1.0
3r	4-Cyclohexyl-Ph	H				<50
3s	*N*-Morpholinyl-Ph	H				<50
3t	1-Adamantyl	H				<50
4a	H		1			<50
4b	H		2			<50
4c	H		3			<50
4d	Me		1			<50
5a	H					<50
5b	4-F			4.1 (1.2–8.2)	47.9 ± 8.1	82.8 ± 4.7
5c	4-I					<50
5d	4-CF_3			2.1 (0.8–6.4)	66.8 ± 10.6	99.0 ± 1.0
5e	3-CF_3,4-F			1.3 (0.6–2.8)	76.1 ± 9.6	99.3 ± 1.2
5f	4-*n*-Hexyl			2.6 (1.2–5.6)	50.9 ± 11.5	99.0 ± 1.0

[a] Human TRPA1 receptor activated by acrolein (30 μM).
[b] Inhibitory potency is expressed as the molar concentration of antagonist producing 50% of the maximum effect (IC_{50}) with confidence interval (CI).
[c] % of inhibition (mean ± SEM) at indicated concentrations in at least 25 independent cells and three different experiments.
[d] 4-(3-Methoxy-phenyl)-2-thioxo-1,2,3,4-tetrahydro-indeno[1,2-*d*]pyrimidin-5-one.

for the thiazole-spaced compounds (see IC_{50} values and% of inhibition of **3a**, **3d**, **3h** and **3q** vs those of **5b**, **5c**, **5d** and **5f**, respectively). This effect is particularly significant in the case of 4-iodo- (see **5c** vs **3d**) or 4-CF_3- (see **5d** vs **3h**) substitution.

The most potent compounds against human TRPA1 channel have been screened for their potential to inhibit the activity of the rat channel subtype (rat DRG neurons). The resulting potencies, expressed as percentage of inhibition at 3 and 30 μM, have been reported in Table 2. While the efficacies toward rat and human TRPA1 at the highest concentration resulted mostly comparable, a sharp decrease of activity in DRG neurons has been occasionally detected at a concentration of 3 μM (see compounds **3d**, **3o**, **5b**). Nevertheless, compound **3p**, **5e** and **5f** confirmed their efficacy against rTRPA1 with percentages of inhibition higher than 80% at the lowest considered concentration.

Figure 2 depicts the dose-dependent inhibition curves evoked by **3h**, **5e** (as representative pyrrolo[3,2-*d*]pyrimidines), HC-030031, AP18 and **12** (as reference compounds) in human (A) or rat (B) TRPA1 (the concentration-dependent inhibition curves of the remaining derivatives of the series **3** and **5** obtained in human TRPA1 expressing cells have been reported in Fig. 4 of the Supplementary data). IC_{50} values from rat DRG neurons have been calculated for selected compounds (i.e. **3h** and **5e**, Fig. 2B) and even though **3h** proved to maintain antagonist properties, it resulted 7-fold less potent against the rat isoform of the channel, while the thiazole derivative **5e** showed to be equipotent.

Consistent with our experimental conditions, HC-030031 and AP18 displayed activities (IC_{50} and % inhibition) comparable to those previously reported and measured in different assays,[9] whereas compound **12**[32] resulted sensibly less potent (hTRPA1 IC_{50} = 1.5 μM; rTRPA1 IC_{50} = 3.4 μM) with regard to data previously obtained with hTRPA1 transfected in CHO cells (IC_{50} = 128 nM). This discrepancy could be partly related to differences in receptor expression between recombinant and native systems together with the employment of acrolein as agonist instead of cinnamaldehyde (CA) or allyl isothiocyanate (AITC) used in most of the reported studies.[9] Acrolein is an exogenous irritant component of air pollution and cigarette smoke[6], which, unlike CA and AITC, is

Table 2
Inhibitory effect of different concentrations on the calcium response evoked by acrolein (30 μM) in rat DRG neurons

	rTRPA1[a] % of inhibition[b]	
	3 μM	30 μM
HC-030031[17]	80.7 ± 5.2	99.8 ± 0.6
AP18[26]	45.2 ± 15.1	90.1 ± 9.8
12[c30]	40.2 ± 15.3	99.5 ± 0.9
3a	53.1 ± 10.9	96.7 ± 1.6
3d	12.4 ± 9.3	85.4 ± 14.6
3h	58.8 ± 11.8	99.7 ± 0.8
3i	36.8 ± 11.2	99.5 ± 0.6
3o	10.7 ± 6.6	95.8 ± 4.2
3p	94.4 ± 3.5	97.1 ± 2.1
3q	35.5 ± 13.0	93.3 ± 4.1
5b	3.6 ± 5.2	99.2 ± 0.8
5d	58.3 ± 7.6	80.2 ± 12.6
5e	80.6 ± 6.9	99.6 ± 0.8
5f	92.4 ± 5.7	99.4 ± 3.7

[a] Rat TRPA1 receptor activated by acrolein (30 μM).
[b] % of inhibition (mean ± SEM) at indicated concentrations in at least 20 independent neurons and three different experiments.
[c] 4-(3-Methoxy-phenyl)-2-thioxo-1,2,3,4-tetrahydro-indeno[1,2-*d*]pyrimidin-5-one.

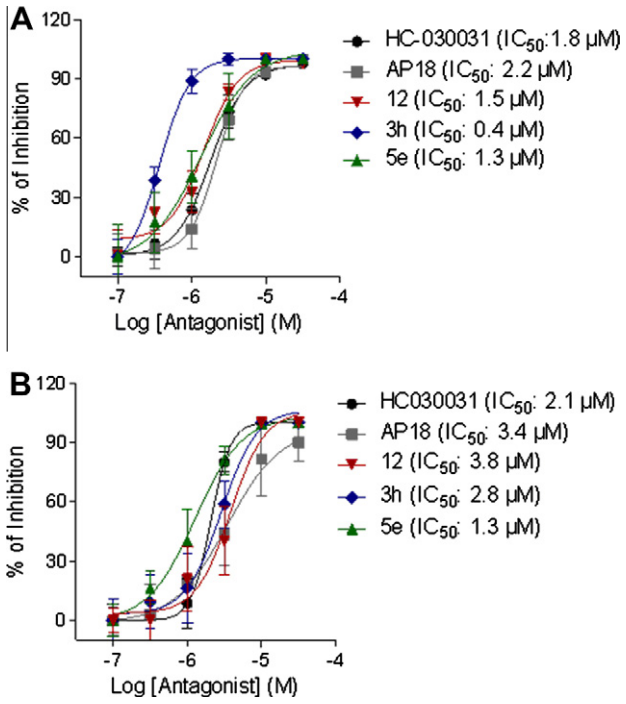


Figure 2. Concentration-dependent inhibition curves and IC_{50} values of the selective TRPA1 antagonists, HC-030031 and AP18, and the pyrrolo[3,2-*d*]pyrimidines derivatives obtained in human TRPA1 (A) or rat TRPA1 (B) constitutive expressing cells. Inhibitory potency on TRPA1 activation has been obtained testing the antagonists at different concentrations (all 0.1–30 μM) against a concentration of the selective endogenous TRPA1 agonist, acrolein (30 μM), which induces about 80% of the maximum effect. Each point represents the average of at least 25 independent cells; values are mean ± SEM.

generated endogenously during lipid peroxidation and contributes to oxidative stress-related pathophysiologic conditions in cells and tissues.[34] Thus, as a byproduct of oxidative/nitrative stress converging on TRPA1 to alert the sensory system of the presence of inflammation or tissue damage, acrolein has been chosen as TRPA1 agonist for in vitro screenings of potential antagonists. The advantage of using the present assay system (acrolein targeting the TRPA1 channel expressed by DRG neurons) is that this mimics real circumstances that may be encountered in vivo. The disadvantage is that acrolein, a very reactive byproduct of oxidative stress may act on the channel by still undetermined mechanisms and therefore its action at the native TRPA1 may result in a larger variability in the calcium response.

Compounds showing activity at the h/rTRPA1 have been also screened in rat DRG neurons, against capsaicin (0.1 μM) to verify the selectivity of the antagonism versus TRPV1. In no case was observed a significant inhibition (higher than 10%) on the response evoked by capsaicin.

3. Conclusions

The treatment of chronic pain, and particularly neuropathic pain, remains unsatisfactory. This observation underscores the importance of considering, validating, and pursuing alternative therapeutic targets. One emerging opportunity in this therapeutic area is offered by selective blockade of TRPA1 channels. In this study we described the design, synthesis and functional evaluation of a new series of molecules capable of inhibiting TRPA1. Most of the few specific antagonists so far reported suffered from low potency, and the limited number of lead compounds displaying nanomolar potency have been screened in recombinant systems against exogenous agonists. Our study, based on the employment of both human and rat cells that constitutively expressed the investigated target, led us to the identification of compound **3h** (hTRPA1: IC_{50} = 400 nM, percentage of channel inhibition at 1 μM = 88.67 ± 6.31) as the most potent of the series at the human channel. This derivative showed a considerably improved potency (sub-micromolar) with regard to some of the most representative antagonists previously reported (AP18, HC-030031 and **12**), and included in our screening program as reference compounds. In addition, **3h** proved to be selective versus TRPV1channel and to maintain a satisfactory rank of efficacy toward rTRPA1, which designates it as a possible candidate for future optimization in view of potential evaluation of in vivo efficacy in rodent animal models. It must be highlighted that the potency of **3h** has been assessed against acrolein as an endogenous mediator of inflammation. This contributes to the extension of the potential of such molecules as valuable tools for the pharmacological characterization of the TRPA1 channel, and its validation as a target in the therapeutic area of pain, inflammation, and airways diseases.

4. Experimental section

4.1. Chemistry

4.1.1. Materials and methods

AP18 was purchased from Tocris Bioscience, while HC-030031[6] and 4-(3-methoxy-phenyl)-2-thioxo-1,2,3,4-tetrahydro-indeno [1,2-*d*]pyrimidin-5-one[32] were synthesized as previously described. Reactions progress and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F254 Merck plates) and visualized by UV lamp (254 nm light source) or with aqueous potassium permanganate. Light petroleum refers to the fractions boiling at 40–60 °C. Chromatography was performed on Merck 230–400 mesh silica gel. After extraction from aqueous phases, the organic solutions were dried over anhydrous sodium sulfate. Melting points for purified products were determined in a glass capillary on a Stuart Scientific electro thermal apparatus SMP3 and are uncorrected. ^{1}H NMR data were determined in $CDCl_3$ or DMSO-d_6 solutions with a Varian VXR 200 spectrometer. Peak positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, and *J* values are given in Hertz. Splitting patterns are designed as s, singlet; d, doublet; t, triplet; q, quartet; m,

multiplet; b, broad. All the detected signals were in accordance with the proposed structures. Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Ferrara, and were within ±0.4% of the theoretical values for C, H, and N. All final compounds revealed a purity of not less than 95%. The mass spectra were obtained on a ESI Micromass ZMD 2000 mass spectrometer.

4.1.2. (1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetic acid ethyl ester (10)

1,3-Dimethyl-1,5-dihydro-pyrrolo[3,2-*d*]pyrimidine-2,4-dione **9** (2 g, 11.2 mmol) was dissolved in DMF (15 mL), K_2CO_3 (3.1 g, 22.4 mmol) was added and the mixture was stirred at room temperature for 10 min. After cooling at 0 °C, a solution of ethyl bromoacetate (2.05 g, 12.32 mmol) in DMF (5 mL) was added dropwise and the reaction was stirred at room temperature for a further 3 h. The solvent was evaporated and the residue partitioned between EtOAc (15 mL) and water (20 mL). The aqueous phase was further extracted with EtOAc (2 × 15 mL) and the organic layers were dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a solid residue which was filtered after suspension with a 1:1 mixture of Et_2O/Petroleum ether (15 mL). White solid; 80% yield; mp 171–172 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 1.29 (t, 3H, J = 7.4), 3.37 (s, 3H), 3.46 (s, 3H), 4.23 (q, 2H, J = 7), 5.10 (s, 2H), 5.99 (d, 1H, J = 3), 6.93 (d, 1H, J = 3). MS (ESI): $[MH]^+$ = 265.3.

4.1.3. (1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetic acid (11)

The ester derivative **10** (2 g, 7.55 mmol) was hydrolyzed by treatment with a mixture of NaOH 10% (20 mL) and MeOH (20 mL) for 2 h at room temperature under magnetic stirring. The solution was concentrated to half volume and the product was precipitated by acidification with HCl 10%, filtered, washed with water and dried in vacuum over phosphorus pentoxide. White solid; 84% yield; mp 264–265 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.19 (s, 3H), 3.37 (s, 3H), 5.04 (s, 2H), 6.17 (d, 1H, J = 3), 7.30 (d, 1H, J = 3), 13.00 (br s, 1H). MS (ESI): $[MH]^+$ = 337.1.

4.1.4. General procedure for the synthesis of pyrrolo[3,2-*d*]pyrimidin derivatives 3a–t and 4a–d

The acetic acid derivative **11** (0.4 mmol) was dissolved in DMF (3 mL) then EDAC (0.4 mmol) and HOBt (0.4 mmol) were added. The mixture was stirred at room temperature for 10 min, then the appropriate (substituted)aryl/arylalkyl/cycloalkyl amine (0.4 mmol) was added, and the reaction stirred at room temperature until disappearance of the starting reagents (12–24 h). The solvent was removed under vacuum to give a residue which was partitioned between EtOAc and water. The water layer was further extracted with EtOAc (2 × 10 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude residue which was purified by crystallization with the appropriate solvents.

4.1.4.1. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-fluoro-phenyl)-acetamide (3a).

The product was purified by crystallization with EtOH; white solid; 75% yield; mp 217–218 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.16 (s, 2H), 6.19 (d, 1H, J = 3), 7.10–7.19 (m, 2H), 7.33 (d, 1H, J = 3), 7.55–7.62 (m, 2H), 10.33 (s, 1H). MS (ESI): $[MH]^+$ = 331.4. Anal. ($C_{16}H_{15}FN_4O_3$) C, H, N.

4.1.4.2. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-fluoro-phenyl)-*N*-methyl-acetamide (3b).

The product was purified by crystallization with EtOH; white solid; 55% yield; mp 222–223 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.15 (s, 3H), 3.19 (s, 3H), 3.36 (s, 3H), 4.84 (s, 2H), 6.14 (d, 1H, J = 2.8), 7.19 (d, 1H, J = 3), 7.38–7.54 (m, 4H). MS (ESI): $[MH]^+$ = 345.2. Anal. ($C_{17}H_{17}FN_4O_3$) C, H, N.

4.1.4.3. *N*-(4-Chloro-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3c).

The product was purified by crystallization with MeOH; white solid; 60% yield; mp 262–263 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.18 (s, 2H), 7.60 (d, 2H, J = 9), 10.43 (br s, 1H). MS (ESI): $[MH]^+$ = 347.6. Anal. ($C_{16}H_{15}ClN_4O_3$) C, H, N.

4.1.4.4. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-iodo-phenyl)-acetamide (3d).

The product was purified by crystallization with DMF/Et_2O; white solid; 52% yield; mp 286–287 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.17 (s, 2H), 6.19 (s, 1H), 7.33 (s, 1H), 7.40 (d, 2H, J = 7.4), 7.64 (d, 2H, J = 6.8), 12.00 (br s, 1H). MS (ESI): $[MH]^+$ = 438.9. Anal. ($C_{16}H_{15}IN_4O_3$) C, H, N.

4.1.4.5. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(3-iodo-phenyl)-acetamide (3e).

The product was purified by crystallization with 1,4-dioxane; white solid; 42% yield; mp 286–287 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.17 (s, 2H), 6.20 (d, 1H, J = 2.8), 7.14–7.16 (m, 3H), 7.32 (d, 1H, J = 2.6), 8.09 (s, 1H), 10.40 (br s, 1H). MS (ESI): $[MH]^+$ = 439.1. Anal. ($C_{16}H_{15}IN_4O_3$) C, H, N.

4.1.4.6. *N*-(2,4-Difluoro-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3f).

The product was purified by crystallization with EtOH; white solid; 62% yield; mp 196–198 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.19 (s, 3H), 3.38 (s, 3H), 5.23 (s, 2H), 6.20 (d, 1H, J = 2.8), 6.99–7.16 (m, 1H), 7.22–7.41 (m, 2H), 7.80–8.00 (m, 1H), 10.09 (br s, 1H). MS (ESI): $[MH]^+$ = 349.1. Anal. ($C_{16}H_{14}F_2N_4O_3$) C, H, N.

4.1.4.7. *N*-(3,4-Difluoro-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3g).

The product was purified by crystallization with EtOH; white solid; 67% yield; mp 238–239 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.18 (s, 2H), 6.21 (d, 1H, J = 2.9), 7.20–7.42 (m, 3H), 7.60–7.80 (m, 1H), 10.54 (br s, 1H). MS (ESI): $[MH]^+$ = 349.3. Anal. ($C_{16}H_{14}F_2N_4O_3$) C, H, N.

4.1.4.8. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-trifluoromethyl-phenyl)-acetamide (3h).

The product was purified by crystallization with EtOH; white solid; 56% yield; mp 266–267 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.22 (s, 2H), 6.21 (d, 1H, J = 3), 7.34 (d, 1H, J = 3), 7.66–7.80 (m, 4H), 10.69 (br s, 1H). MS (ESI): $[MH]^+$ = 381.3. Anal. ($C_{17}H_{15}F_3N_4O_3$) C, H, N.

4.1.4.9. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-trifluoromethoxy-phenyl)-acetamide (3i).

The product was purified by crystallization with EtOH; white solid; 81% yield; mp 281–282 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.19 (s, 2H), 6.20 (d, 1H, J = 2.9), 7.30–7.34 (m, 3H), 7.66 (d, 2H, J = 9), 10.54 (br s, 1H). MS (ESI): $[MH]^+$ = 397.3. Anal. ($C_{17}H_{15}F_3N_4O_4$) C, H, N.

4.1.4.10. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-trifluoromethylsulfanyl-phenyl)-acetamide (3j).

The product was purified by crystallization with EtOH; white solid; 44% yield; mp 298–299 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.39 (s, 3H), 5.21 (s, 2H), 6.20 (d, 1H, J = 2.9), 7.33 (d, 1H, J = 2.8), 7.64–7.75 (m, 4H), 10.63 (br s, 1H). MS (ESI): $[MH]^+$ = 413.1. Anal. ($C_{17}H_{15}F_3N_4O_3S$) C, H, N.

4.1.4.11. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-methoxy-phenyl)-acetamide (3k). The product was purified by crystallization with MeOH; white solid; 60% yield; mp 238–239 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.38 (s, 3H), 3.71 (s, 3H), 5.14 (s, 2H), 6.18 (d, 1H, J = 3), 6.86 (d, 2H, J = 8.2), 7.32 (d, 1H, J = 3), 7.46 (d, 2H, J = 8.2), 10.11 (br s, 1H). MS (ESI): $[MH]^+$ = 343.3. Anal. ($C_{17}H_{18}N_4O_4$) C, H, N.

4.1.4.12. *N*-(4-Diethylsulfamoyl-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3l). The product was purified by column chromatography eluting with EtOAc; white solid; 35% yield; mp 217–218 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.02 (t, 6H, J = 7.2), 3.07–3.18 (m, 7H), 3.39 (s, 3H), 5.21 (s, 2H), 6.21 (d, 1H, J = 2.8), 7.33 (d, 1H, J = 3), 7.70–7.80 (m, 4H), 10.71 (br s, 1H). MS (ESI): $[MH]^+$ = 448.2. Anal. ($C_{20}H_{25}N_5O_5S$) C, H, N.

4.1.4.13. *N*-(4-Benzylsulfamoyl-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3m). The product was purified by column chromatography eluting with EtOAc; white solid; 45% yield; mp 273–274 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.40 (s, 3H), 3.95 (s, 2H), 5.22 (s, 2H), 6.21 (d, 1H, J = 2.8), 7.18–7.35 (m, 6H), 7.70–7.80 (m, 4H), 8.10 (br s, 1H), 10.8 (br s, 1H). MS (ESI): $[MH]^+$ = 482.2. Anal. ($C_{23}H_{23}N_5O_5S$) C, H, N.

4.1.4.14. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-isopropyl-phenyl)-acetamide (3n). The product was purified by crystallization with MeOH; white solid; 74% yield; mp 252–253 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.15 (s, 3H), 1.18 (s, 3H), 2.70–2.90 (m, 1H), 3.18 (s, 3H), 3.39 (s, 3H), 5.16 (s, 2H), 6.19 (d, 1H, J = 2.9), 7.16 (d, 2H, J = 8.6), 7.33 (d, 1H, J = 3), 7.47 (d, 2H, J = 8.2), 10.19 (br s, 1H). MS (ESI): $[MH]^+$ = 355.7. Anal. ($C_{19}H_{22}N_4O_3$) C, H, N.

4.1.4.15. *N*-(4-tert-Butyl-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3o). The product was purified by crystallization with MeOH; white solid; 65% yield; mp 238 °C dec; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.25 (s, 9H), 3.19 (s, 3H), 3.39 (s, 3H), 5.16 (s, 2H), 6.19 (d, 1H, J = 3), 7.29–7.33 (m, 3H), 7.48 (d, 2H, J = 8.8), 10.18 (br s, 1H). MS (ESI): $[MH]^+$ = 369.3. Anal. ($C_{20}H_{24}N_4O_3$) C, H, N.

4.1.4.16. *N*-(4-Butyl-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3p). The product was purified by crystallization with MeOH; white solid; 60% yield; mp 233–234 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 0.87 (t, 3H, J = 7.2), 1.20–1.40 (m, 2H), 1.40–1.60 (m, 2H), 2.48–2.55 (m, 2H), 3.18 (s, 3H), 3.39 (s, 3H), 5.16 (s, 2H), 6.19 (d, 1H, J = 2.8), 7.11 (d, 2H, J = 8.6), 7.31 (d, 1H, J = 2.8), 7.46 (d, 2H, J = 8.4), 10.18 (br s, 1H). MS (ESI): $[MH]^+$ = 369.5. Anal. ($C_{20}H_{24}N_4O_3$) C, H, N.

4.1.4.17. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-hexyl-phenyl)-acetamide (3q). The product was purified by crystallization with EtOH; white solid; 80% yield; mp 217 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 0.84 (t, 3H, J = 7.1), 1.10–1.40 (m, 6H), 1.40–1.60 (m, 2H), 2.48–2.55 (m, 2H), 3.18 (s, 3H), 3.38 (s, 3H), 5.15 (s, 2H), 6.17 (d, 1H, J = 2.6), 7.10 (d, 2H, J = 8.2), 7.31 (d, 1H, J = 2.6), 7.46 (d, 2H, J = 8.2), 10.17 (br s, 1H). MS (ESI): $[MH]^+$ = 397.0. Anal. ($C_{22}H_{28}N_4O_3$) C, H, N.

4.1.4.18. *N*-(4-Cyclohexyl-phenyl)-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3r). The product was purified by crystallization with EtOH; white solid; 82% yield; mp 276–277 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.10–1.50 (m, 6H), 1.60–1.80 (m, 4H), 2.40–2.49 (m, 1H), 3.18 (s, 3H), 3.39 (s, 3H), 5.15 (s, 2H), 6.18 (d, 1H, J = 3), 7.13 (d, 2H, J = 8.6), 7.32 (d, 1H, J = 2.8), 7.46 (d, 2H, J = 8.4), 10.20 (br s, 1H). MS (ESI): $[MH]^+$ = 395.0. Anal. ($C_{22}H_{26}N_4O_3$) C, H, N.

4.1.4.19. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-d]pyrimidin-5-yl)-*N*-(4-morpholin-4-yl-phenyl)-acetamide (3s). The product was purified by crystallization with 1,4-dioxane; white solid; 50% yield; mp 265–266 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.00–3.05 (m, 4H), 3.19 (s, 3H), 3.38 (s, 3H), 3.69–3.74 (m, 4H), 5.14 (s, 2H), 6.18 (d, 1H, J = 2.8), 6.88 (d, 2H, J = 9.2), 7.32 (d, 1H, J = 2.8), 7.43 (d, 2H, J = 9), 10.05 (br s, 1H). MS (ESI): $[MH]^+$ = 398.4. Anal. ($C_{20}H_{23}N_5O_4$) C, H, N.

4.1.4.20. *N*-Adamantan-1-yl-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (3t). The product was purified by column chromatography eluting with a mixture of EtOAc/Petroleum ether 7:3; white solid; 80% yield; mp 277–278 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.60 (br s, 6H), 1.91 (br s, 6H), 1.99 (br s, 3H), 3.19 (s, 3H), 3.37 (s, 3H), 4.87 (s, 2H), 6.12 (d, 1H, J = 2.8), 7.22 (d, 1H, J = 3), 7.60 (br s, 1H). MS (ESI): $[MH]^+$ = 371.0. Anal. ($C_{22}H_{26}N_4O_3$) C, H, N.

4.1.4.21. *N*-Benzyl-2-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-acetamide (4a). The product was purified by crystallization with MeOH; white solid; 61% yield; mp 213–214 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.21 (s, 3H), 3.38 (s, 3H), 4.28 (d, 2H, J = 5.8), 5.03 (s, 2H), 6.17 (d, 1H, J = 3), 7.20–7.40 (m, 6H), 8.51 (br t, 1H). MS (ESI): $[MH]^+$ = 327.6. Anal. ($C_{17}H_{18}N_4O_3$) C, H, N.

4.1.4.22. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-phenethyl-acetamide (4b). The product was purified by crystallization with EtOH; white solid; 30% yield; mp 192–193 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 2.70 (t, 2H, J = 6.8), 3.20–3.40 (m, 8H), 4.93 (s, 2H), 6.16 (d, 1H, J = 2.8), 7.17–7.27 (m, 6H), 8.04 (bt, 1H). MS (ESI): $[MH]^+$ = 341.6. Anal. ($C_{18}H_{20}N_4O_3$) C, H, N.

4.1.4.23. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(3-phenyl-propyl)-acetamide (4c). The product was purified by crystallization with EtOH; white solid; 44% yield; mp 190–191 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 1.65–1.74 (m, 2H), 2.49–2.59 (m, 2H), 3.00–3.10 (m, 2H), 3.19 (s, 3H), 3.37 (s, 3H), 4.95 (s, 2H), 6.16 (d, 1H, J = 3), 7.16–7.30 (m, 6H), 8.04 (bt, 1H). MS (ESI): $[MH]^+$ = 355.7. Anal. ($C_{19}H_{22}N_4O_3$) C, H, N.

4.1.4.24. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-methyl-benzyl)-acetamide (4d). The product was purified by crystallization with EtOH; white solid; 60% yield; 236–237 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 2.27 (s, 3H), 3.21 (s, 3H), 3.38 (s, 3H), 4.23 (d, 2H, J = 5.6), 5.01 (s, 2H), 6.17 (d, 1H, J = 3), 7.10–7.20 (m, 4H), 7.28 (d, 1H, J = 2.6), 8.45 (bt, 1H). MS (ESI): $[MH]^+$ = 341.6. Anal. ($C_{18}H_{20}N_4O_3$) C, H, N.

4.1.5. General procedure for the synthesis of pyrrolo[3,2-*d*]pyrimidin derivatives 5a–f

The acetic acid derivative **11** (0.4 mmol) was dissolved in DCE (3 mL), then EDAC (0.48 mmol), HOBt (0.12 mmol) and DMAP (0.04 mmol) were added. The mixture was stirred at room temperature for 15 min, then the appropriate 4-(substituted)phenyl-thiazol-2-yl amine (0.4 mmol) was added and the reaction stirred at room temperature until disappearance of the starting reagents (8–12 h). The solvent was removed under vacuum to give a residue which was partitioned between EtOAc and water. The water layer was further extracted with EtOAc (2 × 10 mL) and the combined

organic layers were dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude residue which was purified by crystallization with the opportune solvents.

4.1.5.1. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-(4-phenyl-thiazol-2-yl)-acetamide (5a). The product was purified by crystallization with DMF/H_2O; white solid; 30% yield; mp >300 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.17 (s, 3H), 3.40 (s, 3H), 5.32 (s, 2H), 6.23 (d, 1H, J = 3), 7.20–7.44 (m, 4H), 7.64 (d, 1H, J = 2.8), 7.89–7.94 (m, 2H), 12.5 (br s, 1H). MS (ESI): $[MH]^+$ = 396.3. Anal. ($C_{19}H_{17}N_5O_3S$) C, H, N.

4.1.5.2. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-[4-(4-fluoro-phenyl)-thiazol-2-yl]-acetamide (5b). The product was purified by crystallization with DMF/H_2O; white solid; 35% yield; mp >300 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.40 (s, 3H), 5.32 (s, 2H), 6.23 (d, 1H, J = 3), 7.23–7.36 (m, 3H), 7.63 (s, 1H), 7.91–7.98 (m, 2H), 12.60 (br s, 1H). MS (ESI): $[MH]^+$ = 414.3. Anal. ($C_{19}H_{16}FN_5O_3S$) C, H, N.

4.1.5.3. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-[4-(4-iodo-phenyl)-thiazol-2-yl]-acetamide (5c). The product was purified by crystallization with DMF/H_2O; white solid; 35% yield; mp >300 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.17 (s, 3H), 3.40 (s, 3H), 5.31 (s, 2H), 6.23 (d, 1H, J = 3), 7.35 (d, 1H, J = 3), 7.70–7.83 (m, 5H), 12.67 (br s, 1H). MS (ESI): $[MH]^+$ = 522.3. Anal. ($C_{19}H_{16}IN_5O_3S$) C, H, N.

4.1.5.4. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-[4-(4-trifluoromethyl-phenyl)-thiazol-2-yl]-acetamide (5d). The product was purified with DMF/H_2O; white solid; 32% yield; mp >300 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.18 (s, 3H), 3.40 (s, 3H), 5.33 (s, 2H), 6.23 (d, 1H, J = 3), 7.35 (d, 1H, J = 3), 7.79–7.88 (m, 3H), 8.12 (d, 2H, J = 8.2) 12.70 (br s, 1H). MS (ESI): $[MH]^+$ = 464.0. Anal. ($C_{20}H_{16}F_3N_5O_3S$) C, H, N.

4.1.5.5. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-[4-(4-fluoro-3-trifluoromethyl-phenyl)-thiazol-2-yl]-acetamide (5e). The product was purified by crystallization with DMF/H_2O; white solid; 22% yield; mp >300 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 3.16 (s, 3H), 3.41 (s, 3H), 5.31 (s, 2H), 6.22 (d, 1H, J = 3), 7.34 (d, 1H, J = 3), 7.40–7.70 (m, 1H), 7.75–7.90 (m, 1H), 8.10–8.30 (m, 2H), 12.80 (br s, 1H). MS (ESI): $[MH]^+$ = 482.0. Anal. ($C_{20}H_{15}F_4N_5O_3S$) C, H, N.

4.1.5.6. 2-(1,3-Dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-pyrrolo[3,2-*d*]pyrimidin-5-yl)-*N*-[4-(4-hexyl-phenyl)-thiazol-2-yl]-acetamide (5f). The product was purified by crystallization with DMF/H_2O; white solid; 42% yield; mp 270–272 °C; ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) 0.85 (t, 3H, J = 7.2), 1.20–1.40 (m, 6H), 1.42–1.70 (m, 2H), 2.50–2.70 (m, 2H), 3.18 (s, 3H), 3.40 (s, 3H), 5.31 (s, 2H), 6.22 (d, 1H, J = 3), 7.25 (d, 2H, J = 8.4), 7.35 (d, 1H, J = 3), 7.54 (s, 1H), 7.80 (d, 2H, J = 8.4), 12.60 (br s, 1H). MS (ESI): $[MH]^+$ = 480.1. Anal. ($C_{25}H_{29}F_4N_5O_3S$) C, H, N.

4.2. Functional studies

4.2.1. Animals

We used Sprague–Dawley rats (male, 100 g) (Harlan Laboratories; Milan, Italy) in compliance with the experimental procedures approved by the Animal Care Committee of the University of Florence. Animals were housed in a temperature- and humidity-controlled vivarium (12 h dark/light cycle, free access to food and water) for at least 48 h before test. Animals were euthanized with a high dose of sodium pentobarbital (200 mg/kg, intraperitoneal).

4.2.2. Cell culture and isolation of primary sensory neurons

We used fetal human lung fibroblasts (IMR90; American Type Culture Collection; Manassas, VA, USA), that constitutively express the human TRPA1 channel[2], and primary culture of rat sensitive neurons taken from dorsal root ganglion. Fibroblasts were grown in DMEM added with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in 95% air, 5% CO_2 at 37 °C in a humidified atmosphere. For calcium imaging measurement, cells were plated on glass coverslips and grown to a 60% of confluence. For isolation of rat primary sensitive neurons, ganglia were removed, placed in cold HBSS, and transferred to HBSS containing collagenase type 1A (2 mg/ml), trypsin (1 mg/ml), for 35 min at 37 °C. Ganglia, placed in warmed DMEM complete medium containing 10% FBS, 10% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, were dissociated in 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 resuspended in DMEM complete medium containing mouse-NGF (100 ng/ml) and cytosine-b-D-arabino-furanoside free base (2.5 μM). Neurons were plated on glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM) cultured for 2–3 days before calcium experiment.

4.2.3. Calcium imaging measurement

Intracellular calcium $[Ca^{2+}]_i$ fluorescence has been measured in single cells as previously reported.[6] Plated cells were loaded with Fura-2, AM-ester (5 μM) (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37 °C) containing the following: $CaCl_2$, 1.6 mM; KCl, 5.4 mM; $MgSO_4$, 0.4 mM; NaCl, 135 mM; D-glucose, 5 mM; HEPES, 10 mM; and BSA, 0.1%, at pH 7.4. After 40 min, cells were washed and transferred to a chamber on the stage of a Nikon Eclipse TE2000U microscope for recording. Cells were excited at 340 and 380 nM to indicate relative $[Ca^{2+}]_i$ changes by the F340/F380 ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCSoftware; Florence, Italy). Cells or neurons were exposed to a selective TRPA1 agonist, acrolein[13] in the presence of selective TRPA1 antagonists, HC-030031 or AP18 (both, 0.1–30 μM), and pyrrolo[3,2-*d*]pyrimidines analogues (all 0.1–30 μM), or their maximum vehicle (3% DMSO). In rat DRG neurons, the addition of capsaicin (0.1 μM) has been used to identify capsaicin-sensitive neurons and to verify the selectivity of all compounds. Inhibition lower than ~10% has been considered not effective. Calcium response has been normalized to the maximum response induced by ionomycin (5 μM) added at the end of each experiment. Results are expressed as % of inhibition of the effect induced by a concentration of acrolein (30 μM) which represents 80% of the maximum effect (EC_{80}) induced by the agonist (See the concentration–dependent response curves and EC_{50} values of acrolein in human fetal lung fibroblasts and primary rat DRG neurons in Fig. 5 of the Supplementary data). Antagonist potency is expressed as IC_{50}, that is, the molar concentration of antagonist producing 50% of the inhibitory measured effect with confidence interval (CI), calculated fitting data by a sigmoidal regression with variable slope (GraphPad, San Diego, CA, USA).

Acknowledgment

We acknowledge the Italian Institute of Technology (IIT) SEED and Regione Toscana for financial support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.020.

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